University of Alberta

Using Phage Display to Identify Peptides that Bind to the Surface of

Helicobacter pylori

by

Praveen Raj Seetharaman Srinivasan

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Abstract

Recent studies have reported high rates of *Helicobacter pylori* (*H. pylori*) infection and gastric disease in the remote community of Aklavik (NWT) as compared with urban centers in Alberta. Current therapies fail to eradicate 20-25% of *H. pylori* infections, which may be related to antimicrobial resistance, poor compliance and, perhaps, inadequate drug delivery strategies. Proteins and unknown outer surface molecules (OSM) found on the bacterial surface of *H. pylori* play a major role in colonization and pathogen-host interaction. A novel therapeutic that will specifically target the OSM of bacteria and kill them may provide an effective alternative for the eradication of *H. pylori* infections. To this end, it is essential to identify peptides that bind specifically to the OSM of *H. pylori.* Phage display library is a tool that can interact with OSM of bacteria by exposing a library of a specific phage, each displaying one kind of the PIII minor coat proteins on its surface. This thesis Identified 20 peptides that bind to the OSM of four isolates of *H. pylori*, which is a first step towards the development of a targeted treatment for *H. pylori* infection.

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AA	amino acids
ATCC	American Type Culture Collection
ATP	Adenosine 5'-Triphosphate
BabA	Blood group antigen binding adhesin A
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
CFU	Colony Forming Unit
CWI	Clean Water Index
dupA	Duodenal ulcer promoting gene A
ds	Double-stranded
GERD	Gastroesophageal Reflux Disease
IDT	Integrated DNA Technologies
IPTG	Isopropylthio-β-D-galactoside
LB	Luria-Bertani
LPS	Lipopolysaccharide
MALT	Mucosal-Associated Lymphoid Tissue
MIC	Minimal Inhibitory Concentration
MW	Molecular Weight
oipA	Outer inflammatory protein A gene
OMP	Outer membrane protein
OSM	Outer surface molecule

PAI	Pathogenicity island
PCR	Polymerase chain reaction
PBS	Phosphate Buffered saline
PFU	Plaque forming unit
PF3	Filamentous phage 3
PUD	Peptic ulcer disease
UBT	Urea breath test
RF	Replicative form
sabA	Sialic acid binding adhesin A gene
SCVF	Single chain variable fragment
SD	Standard Deviation
ss	Single stranded
TET	Tetracycline
TBS	Tris-Buffered Saline
TFSS	Type IV secretion system
UV	Ultraviolet
vacA	Vacuolating cytotoxin A gene

Chapter 1: Introduction

1.1 Characteristics of *Helicobacter pylori*

Helicobacter pylori is a Gram negative bacterium that was first discovered by Warren and Marshall in 1982.(1) This bacterium is a pathogen that colonizes the stomachs of approximately half of the world's population.(2) *Helicobacter pylori* was initially named *Campylobacter pylori*, but later on, data from 16S ribosomal RNA sequencing revealed that the bacterium did not belong to the Campylobacter genus.(3) Hence, it was placed under the genus Helicobacter due to the helical shape of the bacterium, which is thought to have evolved in order to penetrate the mucoid lining of the stomach.(4,5) Most individuals infected by *H. pylori* are asymptomatic but approximately 10% may develop peptic ulcers, 1-3% may develop gastric adenocarcinoma and less than 0.1% may develop mucosal-associated lymphoid tissue (MALT) lymphoma (2). The infection is more prevalent in developing countries, while the rate of incidence is decreasing in developed countries.(6) *H. pylori* is considered a class 1 carcinogen by the International Association of Research on Cancer.(7) It is one of the most common causative agents of infection-related cancer that represents 5.5% of global gastric cancer cases.(8) Some studies indicate that *H. pylori* have been colonizing the human stomach for at least 58,000 years.(9) The genomes of *H. pylori* strains vary widely among geographic locations and cultural groups. An Important feature of *H. pylori* is that it is able to survive in the harsh stomach

environment by producing the enzyme urease that neutralizes gastric acid. (4,10-12)

Outer membrane proteins and unknown surface molecules found on the cell membranes of *H. pylori* play an important role in the initial colonization of the host.(13) The initial colonization of *H. pylori* to host gastric epithelial cells facilitates its access to nutrients and delivery of virulence factors, which leads to the development of gastric diseases.(14) The *H. pylori* genome encodes for five major outer membrane protein (OMP) families: adhesins, porins, iron transporters, flagellum-associated proteins and proteins of unknown function.(13) *H. pylori* is the only bacteria where the O antigen of lipopolysaccharide (LPS) expressed on its outer membrane can be fucosylated to form Lewis antigens like those found on the surface of human cells. This mimicry of Lewis blood group antigens found on the gastric epithelium aids *H. pylori* to avoid the human immune response.(1,13) The outer membrane of *H. pylori* contains the enzymes catalase and superoxide dismutase to protect against the toxic oxygen species produced by the human gastric epithelial cells' acute inflammatory immune response.(15) Catalase is present in the cytosol and periplasmic space of *H. pylori* and may also be expressed on its surface.(16) Mapping the epitope of OMPs expressed on the surface of *H. pylori* would provide a strategy to disrupt the initial colonization of the host and prevent *H. pylori* infection.

Recent studies have identified high rates of *H. pylori* infection and gastric disease in the remote community of Aklavik, NWT, as compared to the infection rates in urban centers in Alberta.(17) Current therapies fail to eradicate 20-25% of *H. pylori* infections.(18) This failure may be attributed to re-infection among patients,(19,20) poor patient compliance,(21) and the presence of antimicrobial resistance.(22) The features of the stomach, such as gastric emptying time and the ruggae surface of the stomach lining, may also hinder access of the drug to the site of bacterial infection.

1.1.1.1 Epidemiology

Numerous studies assessed prevalence, risk factors, transmission and the incidence of *H. pylori* infection from developed and developing countries with different socioeconomic, racial and cultural groups.(6) The majority of these studies were performed on defined subgroups such as pregnant women(23), teachers(24,25) and students(26). The urea breath test (UBT), serologic tests or histologic tests were usually used to detect the presence of *H. pylori* infections but a very few studies used stool antigen tests or polymerase chain reaction to detect the presence of *H. pylori* genes.(6)

1.1.2 Prevalence

Epidemiological studies on the prevalence of *H. pylori* infection described developed countries such as Canada and Western Europe as low prevalence, and developing countries like India and China as high prevalence (Figure 1.1).(27) In

Lebanon, recent studies reported that the prevalence of infection was higher in children from low-income families than children from high-income families.(6). Recent studies in Canada (Ontario) by Naja *et al.* 2007 (28) demonstrated that the *H. pylori* prevalence was lower among individuals with a higher level of education. Seroprevalence was 23.1% overall and was found to be greater among adults in low income families because of crowded households and also among people who are born in developed countries.(28)

1.1.3 Risk factors

Risk factors, such as the low socioeconomic status, low education level, poor sanitation, and birth in a developing country, are often associated with a high prevalence of *H. pylori* infection. In developing countries, the occurrence of reinfection is reported to be 50% each year due to overcrowded populations.(29,30) Prevalence of *H. pylori* infection in developed countries is mostly in children due to poor sanitation and low socioeconomic status.(6)

Recurrence of infection after treatment occurs more frequently in developing countries as compared to developed countries. Recurrence of infection in developed countries appears to occur via getting re-infected with the same strain.(29,31) Recrudescence means reappearance of the original strain of *H. pylori* in infected patients as would occur if the infection was temporarily suppressed rather than completely eradicated.(32) In developed countries such as the United Kingdom, following successful eradication, only 0.4% of patients

become re-infected each year,(29) whereas in developing countries, treated Individuals are re-infected with a new strain.(29)

Water quality plays a vital role in the transmission and acquisition of infection.(33) Recent studies demonstrated that the prevalence of *H. pylori* is associated with hygiene and sanitary water conditions.(33) In South India, prevalence studies reported that well water drinkers had a higher *H. pylori* prevalence (92%) relative to tap water drinkers (75%). A low clean water index (CWI), is defined as drinking water before boiling, taking bath just once a week and using water multiple times.(6,33) A recent study on medical students from Saudi Arabia found that prevalence of *H. pylori* infection was higher among people with a low CWI when compared to people with high CWI.(34)

1.2 Transmission

Transmission of *H. pylori* infection is likely through person-to-person contact.(35) The possible transmission routes are discussed as follows.(6)

1.2.1 Iatrogenic transmission

Iatrogenic transmission occurs when using an unsterile endoscope for diagnosing gastric diseases.(36) This transmission happens when an endoscope that was in contact with the stomach mucosa of an infected patient is reused for another patient without proper sterilization.(37)

1.2.2 Faecal-oral

Faecal-oral transmission occurs via oral ingestion of food or water contaminated with faeces containing *H. pylori* arising from hand-to-mouth transmission when hands are not washed following use of bathroom facilities. The faeces of *H. pylori*-infected young children are known to contain the bacterium, but studies have shown that the isolation of the bacteria from adult faeces is rare.(4,38) The failure to recover the bacterium from adult faeces may be due to inadequate recovery methods.(6,39) Water contaminated with faeces is one of the main sources of faecal-oral transmission.(38,39) Increased risk of infection was found in children who swam in the rivers, streams and swimming pools.(4) Recent studies conducted in South America suggest that the faecal-oral transmission of *H. pylori* occurred mainly through faeces-contaminated food or water.(36) These studies suggest that transmission may occur via food or water in developing countries.(4,38)

1.2.3 Vector- borne transmission

The human stomach is the major reservoir of *H. pylori* bacteria, however, non-human reservoirs are known to exist. Transmission of *H. pylori* via non-human contact is known as vector-borne transmission. Recent studies demonstrate that *H. pylori* infection may also be isolated from non-human primates such as macaques,(40) and some evidence suggests that insects such as house flies, could transmit the infection.(41) House flies may harbour *H. pylori* in their intestine and body hair, with high levels in the fly excreta.(41) Exposure of

flies to the bacteria may partially explain the high levels of *H. pylori* infected flies found in regions with poor sanitation, as well as how they serve as a potential vectors.(4)

1.2.4 Consequences of eradication of infection

Eradication of *H. pylori* with current antibiotic treatments will lead to the destruction of normal flora, which may be beneficial to the human digestive tract.(11)

1.3 Clinical manifestations

1.3.1 Gastritis

H. pylori colonization of gastric epithelial cells may lead to the development of chronic gastritis, an inflammatory condition of the stomach, and other gastro-duodenal diseases. (42) *H. pylori* can cause inflammation in the lining of the stomach and occasionally causes pain in the upper central abdomen. (37) Many people with gastritis do not have any symptoms. If present, symptoms of gastritis include loss of appetite, nausea, and vomiting.

1.3.2 Peptic ulcer disease (PUD)

The inflammation caused by *H. pylori* colonization of gastric epithelial cells sometimes results in erosion of the mucosal layer of the stomach or duodenum, known as PUD. Erosion of the mucosal lining of the stomach allows the secreted acid to irritate the lining of the gastric and duodenum leading to

formation of ulcers. (43) *H. pylori* is one of the major causes of PUD. The mechanism of the infection causing PUD is not clearly understood, but most likely involves genetic and virulence factors of *H. pylori*. (44). Several non-steroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenase-1 (COX-1), an enzyme responsible for the biosynthesis of eicosanoids in the stomach, which regulates secretion of various protective factors, and thereby increases the possibility of forming peptic ulcers.(43)

1.3.3 Gastroesophageal reflux disease (GERD)

Recently, much attention has been focussed on the possible relationship between *H. pylori* infection and GERD In western countries; 40% of GERD patients tested were positive for *H. pylori*.(45) GERD results when acid from the stomach contacts the esophagus to cause mucosal damage.(46)

In contrast, the treatment of the *H. pylori* infections has also been proposed to lead to increased risk of GERD in many patients. This point has proved to be controversial with several studies reporting conflicting results on the relationship between *H. pylori* and GERD.(13)

1.3.4 Gastric cancer

Gastric adenocarcinoma is the most common form of stomach cancer found worldwide.(2) Infection with *Helicobacter pylori* is believed to promote the development of gastric cancers due to long term inflammation of the stomach.(2) It is believed that the risk for developing gastric cancers is positively

correlated with duration of *H. pylori* infection. Stomach cancer may spread throughout the stomach and to other organs in the body.(47) Recent studies in Japan by Umera *et al*. reported that of 1526 patients, gastric cancer developed among approximately 3% of *H. pylori* infected patients compared to uninfected patients. (2,48)

1.4 Pathogenesis

1.4.1 Mechanism of infection

The *H. pylori* genome codes for about 1500 bacterial proteins, many of which are required for the colonization of the gastric mucosa.(9) Flagella, structures which also facilitate the transport of the organism to the surface of the mucosa, contain key proteins encoded by the genes *flaA* and *flaB*.(32) Virulence factors play a vital role in the infection of *H. pylori,* causing effects such as transient hypochlorhydria via unknown mechanisms. *H. pylori* produces urease which neutralizes acid and allows it to survive the harsh gastric environment.(14) Adherence of *H. pylori* to the stomach mucosa takes place by the interaction between the adhesins and peptidoglycan on the cell wall of the bacterium and by blood group antigens found on the stomach epithelial cells.(33) Once attached to the gastric mucosa, *H. pylori to* releases additional virulence genes and proteins into the human gastric mucosa that causes epithelial cell injury.(5) Approximately 4% of the *H. pylori* genome codes for outer membrane proteins (OMPS) which includes 21 Hop (Helicobacter outer

membrane porins) and, 12 Hor (Hop-related proteins)(10). In most cases, gastroduodenal diseases are often associated with expression of OMPs and adhesins.

1.4.2 Virulence factors

Most pathogenic bacteria express or secrete molecules called virulence factors, which are responsible for causing disease in the host.(49,50) Virulence factors produced by the pathogen allow the bacteria to initiate colonization of the host, the host immune response, and finally suppress the host immune response.(51) Virulence genes and proteins produced by *H. pylori* play a large role in the clinical outcomes of *H. pylori* infections. There is a high level of genetic diversity of the *H. pylori* genome and variations in regions coding for virulence factors.(50,51)

The cytotoxin-associated gene (*cag)* pathogenicity island (*cag PAI*) is a 40-kb DNA insertion element consisting of 27 to 31 genes, which encodes components of the Type IV secretion system (TFSS) that mediates the translocation of DNA and virulence factors to the host.(52) *Cag* PAI also encodes for one of the most important *H. pylori* virulence proteins, *CagA,* which triggers the inflammatory response in the host.(47)

CagA protein is the product of the 120-140 kb of the *cagA gene*. It is translocated to the cytoplasm of the gastric epithelial cells via a channel formed by the TFSS encoded by *cag*PAI of *H. pylori*.(52) Phosphorylation of *CagA* inside

the gastric epithelial cells activates a eukaryotic phosphatase (SHP-2) leading to cellular morphological changes such as cell elongation, increased cell proliferation and Induction of inflammation.(37)

The TFSS secretion system delivers peptidoglycan from the cell wall of *H. pylori* along with CagA protein into the host cell.(2) Intracellular recognition molecule, nucleotide-binding oligomerization domain (NOD1)-containing protein of the host cell acts as a sensor, and interacts with the entering *H. pylori* peptidoglycan leading to activation of the nuclear factor-kappaB (NF-kB)-dependant proinflammatory response.(13)

The vacuolating cytotoxin A (*vacA)* gene encodes for the toxic VacA protein that induces intracellular vacuolation of gastric epithelial cells.(2) The activation of host T-cells, which regulate the immune response to *H. pylori,* are suppressed b*y* VacA. VacA may also induce mast cell chemotaxis and stimulate mast cell expression of multiple proinflammatory cytokines.(53)

Several adhesins and OMPs, (including BabA, SabA, OipA, DupA and FlaA), have been identified to mediate the adherence of *H. pylori* to gastric epithelial cells.(14,54) These in turn facilitate the colonization and delivery of virulent genes to the host epithelial cells. One of the best-known adhesins is blood group antigen binding adhesin (BabA).(13) It is encoded by the *babA2* gene, and binds to the fucosylated Lewis^{b} (Le^{b}) antigen found on the surface of the gastric epithelial cells and increases the pathogenic potential for *H. pylori*.(13,14) *H.*

pylori strains isolated from countries such as Germany and Turkey expressing the *babA2 gene* are associated with gastric disease.(2,5)

Sialic acid binding adhesin (SabA) is encoded by the *sabA* gene and SabA binds to the carbohydrate structure of sialyl Lewis^x antigen expressed on gastric epithelium.(54) The expression of sialyl Lewis^x is induced during chronic gastric inflammation to regulate the host cell glycosylation patterns to increase the efficiency of *H. pylori* attachment and colonization (13). Recent studies reported that *H. pylori* strains expressing SabA protein are often associated with the increased risk of developing gastric cancer and reduced risk for duodenal ulceration.(2,55)

Outer inflammatory protein gene (*oipA*) encodes for the inflammation-related outer membrane protein OipA. The *oipA* gene is present inside *H. pylori* in a functional and a non-functional form. The functional *oipA* gene is often associated with duodenal ulcers, gastric cancer, and increased neutrophil infiltration, whereas the non-functional form is not associated with any gastric diseases.(2)

Duodenal ulcer promoting gene (*dupA)* is present in the plasticity zone (variable region) of the *H. pylori* genome. Analysis of the presence of *dupA* positive strains in persons among the 500 *H. pylori* strains obtained from the countries such as Columbia, South Korea and Japan revealed an increased risk of duodenal ulcer, and decreased risk of gastric cancer.(2)

1.5 Diagnosis

Patients with gastric or duodenal ulcers should be investigated for *H. pylori* infection. There are several standard methods that test for the presence of *H. pylori* infection but each test has its own limitations due to cost and availability of equipment, as well as method sensitivity and specificity.(13) Choice of diagnostic methods should reflect clinical presentations, population rates of infection, differences in test sensitivity and specificity, and the impact of current medications such as proton pump inhibitors and antibiotics.(13)

1.5.1 Urea breath test

This test detects the product of the urease enzyme produced by *H. pylori*.(56) [^{13}C]-urea drinks are given to patients with suspected infection. If present in the patient, *H. pylori* splits urea into ammonia and [^{13}C]-carbon dioxide. The [^{13}C]-carbon dioxide is detected in the exhaled air from the lungs within 1-2 hours of ingestion.(57) However, while this test provides a high degree of both specificity and sensitivity, it cannot be used if antibiotic or proton pump inhibitors have been administered in the previous two and four weeks, respectively. Since the effects of the medications remain in the body for up to four weeks after the last dose, testing too early would result in a false negative test.

1.5.2 Serologic test

As the human immune system produces specific antibodies against the *H. pylori* specific antigens, an ELISA (Enzyme-Linked ImmunoSorbent Assay) test can detect these antibodies in patient serum. In a population with a low prevalence of infection, this test should be used as a second line diagnostic method, as it frequently produces false positive results.(58) This test is simple, accurate and measures specific *H. pylori* IgG antibodies; however, these antibodies may persist long after the active infection has been eliminated, making it unable to be used to assess treatment progress. (59)

1.5.3 Stool antigen test

This method uses enzyme immunoassay to detect the presence of *H. pylori* antigens in stool specimens usually for young children.(58) Otherwise, it is not commonly used for testing despite its high sensitivity and specificity for *H. pylori* infections during and before treatment, as stool collection is not the sample of choice for most patients. (60,61)

1.5.4 Histology

Histological testing is the microscopic study of the diseased tissue, which is enhanced through the use of histological stains to highlight specific cells and cell components in biopsied tissues.(62,63) This approach is one of the best methods for the diagnosis of *H. pylori* infection due to its high sensitivity and

specificity, but there are certain disadvantages, such as the invasive nature and high expense of biopsy collection. (63)

1.5.5 Polymerase chain reaction (PCR)

PCR is used to detect specific DNA segments of *H. pylori* strains from a mixture of bacterial strains in gastric biopsies. However, PCR is not yet in routine use to detect *H. pylori* infection. In the future, PCR may play a larger role in diagnosis once tests are standardized and costs are reduced.(58) PCR may also be used to detect the presence of *H. pylori* infections in less invasive samples such as saliva and dental plaque from infected individuals. One of the major disadvantages is the false interpretation of other bacterial DNA bands as *H. pylori* DNA bands in gel electrophoresis.(64) PCR may also be used to detect genome variations in different strains of *H. pylori* for pathogenic and epidemiologic studies.

1.5.6 Rapid urease testing

One of the biopsies taken during gastroscopy can be introduced into urea-containing medium to screen for the presence of *H. pylori*. The presence of urease in the sample breaks down urea into ammonia and carbon dioxide, with a resultant increase in the pH of the medium that results in a colour change from yellow to red. It is a fast, inexpensive and widely used on-site screening test in endoscopy clinics; however, its post-treatment sensitivity is reduced because other bacteria in the human stomach may also produce urease. (56)

1.6 Current antimicrobial treatment therapies

Eradication of *H. pylori* using a multidrug regimen consisting of a proton pump inhibitor, along with the two antibiotics is generally believed to be safe and effective by many consensus groups. The proton pump inhibitor drastically reduces gastric acid secretion, which increases the effectiveness of the antibiotics.

1.6.1 First-line treatment

The standard first line therapy for *H. pylori* eradication consists of a proton pump inhibitor + amoxicillin + clarithromycin or metronidazole twice daily for 7 to 14 days.(18,65) Amoxicillin is a β-lactam antibiotic that inhibits peptidoglycan cross-linkage and prevents the formation of the bacterial cell wall.(66) Clarithromycin binds to the 50S subunit of the bacterial ribosome, thereby inhibiting protein synthesis.(67) Metronidazole is a prodrug that is reduced in bacterial cytoplasm; it damages DNA by oxidation and inhibits DNA synthesis, ultimately killing the bacteria.{{401 Lofmark,S. 2010}} However, antibiotic resistance is reducing rates of successful eradication.(68) Prevalence studies in Europe, North America, and Asia-Pacific region reports that the eradication rate of *H. pylori* with standard triple therapy reduced from 90% to <70%-80% over a 10 year time period.(65)

1.6.2 Second-line treatment

When the first line treatment for *H. pylori* fails, a second line treatment is offered that includes bismuth and levofloxacin.(69,70) Bismuth hydrolyzes to bismuth oxychloride and salicylic acid, which prevent the growth of bacteria by inhibiting the synthesis of proteins, cell wall and ATP.(69,71). Levofloxcin prevents the growth of bacteria by inhibiting DNA gyrase, which stops DNA replication and cell division.(71) Collective analysis of bismuth-based therapy reported that it achieves a 76% eradication rate.(70) A recent 10 day clinical study among 300 patients in Spain demonstrated that levofloxacin-based therapies achieved 81% eradication rate.(65,72) A mutation in the *gyrA* gene prevents levofloxacin from binding to DNA gyrase, and leads to levofloxacin resistance.(18,73)

1.6.3 Third-line and fourth line rescue therapy

When the second line treatment for *H. pylori* fails, then the patient is offered a third-line rescue treatment.(18) The rescue therapy for *H. pylori* infection is largely based on experimental observation.(18) Furazolidone, a synthetic nitrofuran derivative, a good antibacterial against many gram–negative organisms, eradicated 83% of *H. pylori* infections(73,74); however, in the fourth-line therapy, this was reduced to 57% due to increased resistance of *H. pylori*.(18,65)

1.6.4 Antimicrobial resistance rates

The success of *Helicobacter pylori* eradication treatment is falling.(18) *H. pylori* develop resistance to antibiotics through point mutations.(75) Resistance to antibiotics does not seem to involve drug efflux proteins but drug efflux may affect the bacterium's sensitivity to antibiotics.(76,77) An efflux system also contributes to the homeostatic maintenance of metal ion concentrations in the cell (78), which is important for the bacterium's adaption to gastric conditions. Recent studies suggest that in most European countries the resistance towards clarithromycin-based triple therapy has gradually reached 20% over the last 20 years [Megraud et al. 2011], while in the USA and Europe clarithromycin resistance has been reported to range from 4% to 18. Up to 72% resistance to metronidazole was reported in Europe, whereas resistance to amoxicillin was rarely detected.(79,80) *H. pylori* resistance to metronidazole and clarithromycin for 120 isolates from the remote northern community of Aklavik, NWT was 28% and 8%, respectively, with no amoxicillin resistance.(81).

1.7 Vaccines

A vaccine is a chemical substance usually prepared from a causative agent similar to the disease-causing microorganism, toxins or one of its surface proteins.(82,83) In order to enhance the body's immune system, antibodies related to that particular disease start to be generated and are sustained many years after. Vaccines to prevent and treat *H. pylori* infections would be particularly useful in regions where the prevalence of *H. pylori* infection is high.

Currently there are no vaccines available for the prevention or treatment of *H. pylori* infections. Vaccines contain molecules that often resemble a surface protein of the micro-organism to serve as an antigen to stimulate the immune response to clear the infection.

The best line of defense against micro-organisms for the body is the generation of antibodies by the immune system, which recognize a specific antigen on the micro-organism's surface. An epitope is the part of an antigen that is recognized by antibodies, B cells, or T cells. The part of an antibody that recognizes the epitope is called the paratope. The epitopes of protein antigens are divided into conformational and linear epitopes.(84) A conformational epitope is composed of a discontinuous sequence of amino acids. These epitopes interact with the paratope based on the formation of a stable tertiary structure with the antigen. Most epitopes are conformational. Linear epitopes are composed of a continuous sequence of amino acids that interact with the paratope based on their primary structure.(84) The identification of precise epitopes that stimulate the production of effective neutralizing antibodies would lead to the production of epitope-based vaccines.

1.8 Novel therapeutics

An effective vaccine to prevent or treat *H. pylori* infection is not currently available. The emergence of antibiotic-resistant strains of *H. pylori* highlights the need for effective alternative treatments for infection. Novel therapeutics for

treating pathogenic infections focus on significantly enhancing drug penetration and specificity to increase the therapeutic efficiency, while reducing the side effects, and improving patient compliance.(85)

1.9 Phage therapy

A virus that infects only bacteria is commonly called a bacteriophage or phage.(86) Phage therapy is a target-specific approach that has been used to treat infections caused by pathogenic *E. coli*.(87,88) Smith *et al.* demonstrated that a single dose of anti-k1 phage was more effective than intramuscular doses of antibiotics such as tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulfafurazole.(88,89). An important application of phage therapy is its use in the control of foodborne diseases, which are caused by pathogens such as *C. jejuni, Salmonella species* and *Listeria monocytes*.(87) Using genome informatics, Liu *et al.* developed a procedure to identify and select DNA sequences for expressing peptides on the phage surface that have antimicrobial properties.(90) The identified sequences reduced the growth of *Staphylococcus aureus* by inhibiting the DNA or RNA synthesis of the bacteria.(87) Other phages (such as ϕX174 which have single-stranded DNA or RNA) do not have genes that code for proteins such as lysins and holins. Instead these phages have lysis gene *E* that encodes for proteins that inhibit the synthesis of the bacterial cell wall.(87) When the phage (phiX174) carrying lysis gene *E* was introduced into *H. pylori* strains through a plasmid vector, they completely destroyed *H. pylori* cells by

causing a transmembrane tunnel through the cytoplasm, forming what was then termed “ghost cells” for they lacked cytoplasm and DNA.(91)

Phage therapy has several advantages because it is effective against multidrug-resistant pathogenic bacteria, highly target specific, cost effective, and does not affect the common microflora present in the environment.(87) A serious drawback to using phage is the immune response developed against them can be severe so that only one dose can usually be administered.(87)

A modified filamentous M13 phage, which has a protein coat expressing peptide sequences that are antibody binding specific to molecules expressed on the cell surface, was used to eliminate *H. pylori* infection.(92) However, the endotoxin released by the modified filamentous phages following bacterial lysis can cause undesirable side effects, such as decreased oxygen levels in patient tissues (circulatory shock proteins). Hagens *et al*. constructed a *Pseudomonas aeruginosa* filamentous phage (Pf3) with a defective endolysin gene incorporated into its genome, so that as soon as the Pf3 genome inserts into *P. aeruginosa,* it digests the DNA and kills the bacteria with a minimum release of endotoxins.(93)

1.9.1 Phage

All phages contain a head structure of varying size and shape, and also an outer protein coat called a capsid, which covers the genetic material.(94,95) Most phages are smaller in comparison to bacteria and range in size from 24-200

nm in length. One of the largest phages, T4, is approximately 200 nm long and 80-100 nm wide.(96,97) Phages are found in all locations especially in the places that have large bacterial populations such as seawater, soil and intestines of animals.(98)

1.9.1.1 Classification of phage

About 5100 phages that lyse the *Micrococcus* (*Staphylococcus*) and dysentery bacilli have been identified at the late end of the 20th century.(97) These phages are divided into 13 families depending upon the type of nucleic acid, and by the presence or absence of a protein coat (Table 1.1). Most of the identified phages are composed of an icosahedral head and tail and contain double-stranded DNA (dsDNA), single-stranded DNA (ssDNA) or ssRNA.(86) The tailed phages are further classified into three groups based on the different structures: *Myoviridae* have a contractile tail (e.g. T-even phages), *Siphoviridae* have a long non-contractile tail (e.g. λ phage), and *Podoviridae* have a non-contractile tail (e.g.T7 phage). The other 4% of identified phages are filamentous, such as M13 and Pf3, belong to the *Inoviridae* family and contain either the dsDNA or ssDNA or ssRNA as the genome. M13 filamentous phages express peptides attached to the PIII coat protein on the phage surface, which interact with unknown surface molecules of the bacterium. (86,87,99,100) The pIII gene of the M13 filamentous phage genome is responsible for displaying peptides on its surface.

1.9.2 Life cycles of phage

1.9.2.1 Lytic cycle

In this cycle, lytic or virulent phages lyse the bacterial host immediately after their replication. In the lytic cycle, after the phage genome is inserted into the host, the phage DNA replicates separately from the host bacterial DNA using the host enzymes. The bacterial enzymes convert the injected ssDNA into the dsDNA replicative form, (RF DNA). This RF DNA encodes for proteins that aid phage assembly, and also for lytic proteins, holins and endolysins, that lyse the bacterial cell wall for the release of matured phages. After assembly, the matured virions are ready to infect other bacterial cells present in the environment (Figure 1.2).(101)

1.9.2.2 Lysogenic cycle

In this cycle, the phages do not cause lysis after immediate replication. In the lysogenic cycle, the phage genome is incorporated into the bacterial host DNA, and starts replicating along with the bacterial genome. The replicated phages remain dormant until the host is depleted of nutrients. Once the nutrients are depleted, the phages become active and cause lysis of the host cells.(102) Occasionally the lysogenic phage incorporates toxic genes into the bacterial host genome. Incorporation of the toxic genes aids the bacterial host to develop resistance to infection by these phages and closely related previously lysogenized phage.(87)

1.9.3 Mechanism of phage infection

1.9.3.1 Attachment

To infect the bacterial host, the phage has to attach to outer surface molecules of the bacterial cell. Tail fibers mediate the attachment of the phage to the surface of the bacteria.(95) The receptors are proteins on the outer surface of the bacterium, LPS, pili, and lipoprotein. The base plate of the bacteriophage assists in permanent attachment of phage to bacteria.(103)

1.9.3.2 Penetration

Following permanent attachment of the phage to the outer surface of the bacterium, the tail sheath (for those phages which have a sheath) and tail fiber contract. The contracting tail fiber pierces through the cell wall of bacteria and uses a hypodermic syringe-like motion to inject the phage DNA into the host cell by using the ATP present in the tail.(103)

1.9.3.3 Synthesis of proteins and nucleic acids

As soon as phage DNA enters the bacterial host cell, bacterial enzymes convert the injected ssDNA into the dsDNA replicative form, (RF DNA). This RF DNA replicates along with the host DNA and acts as a template for the production of new phages and proteins, such as holins and endolysins.(103) Holins assists in forming the pore in the bacterial cell membrane (95,102) Endolysins are a peptidoglycan-degrading enzymes which cause lysis of the cell by degrading the bacterial cell wall.(87)

1.9.3.4 Virion assembly and release

Virions are non-replicable carriers of the viral genome. They contain components specific to the virus such DNA and proteins.(95) Once newly formed, phage virions need assistance from helper proteins pI-pXII, which are expressed by the phage genome inside the host to aid in phage assembly. Assembly of tail with the head containing the genomic material follows. The virions are then assembled and ready for release.(87,95) The matured phages are extruded from the bacterial cell membrane by using the proteins (holins and endolysins), which are synthesized from their own DNA. The released phages may bind to the bacterial host of interest and continuously replicate within the host to produce new virions until they have lysed all viable bacteria.(87)

1.9.4 Phage display of foreign peptides

Phage vectors used for recombinant DNA research are the phages that infect *E. coli*, a frequently used host.(88,89,104) These phage vectors can accommodate the foreign DNA sequence of interest within the pIII gene and replicate the phage genome by infecting the host bacteria. Accordingly, the foreign peptide of interest is expressed fused to the PIII coat proteins on the phage surface. This is known as phage display, as a foreign peptide is 'displayed' on the coat protein of the phage.

1.9.4.1 Phage display libraries

Using the phage display method, biologists can create a library of phages, displaying unique peptides by manipulating the DNA encoding for a selected peptide.(104) This method allows the ability to express different kinds of peptides, which include the selection of peptides with desired properties, such as specific binding ability. The phage display method enables us to distinguish unique peptides in a pool of peptides with these properties, such as stability and folding. The concept of phage display was proven to be successful against *E. coli*.(94,104) It led to the development of many other alternative phage display systems including other *E.coli*-specific phage (i.e λ-phage(105), T4 phage(86)), and also against eukaryotic viruses.(86,106)

1.9.4.2 Targeted construction of phage display libraries

A phage display library is a collection of phages, each phage containing one kind of peptide expressed on the PIII coat protein on its surface.(104) Construction begins by inserting containing random nucleotide sequences inserted into the pIII gene coding for amino acid peptides of certain lengths, which are later selected depending upon the binding affinity of the peptides (fitness). The fitness is selected by the affinity of the peptides towards the target receptors.(107) The fittest sequence identified in the initial library might be far inferior to the best global sequence. The phages expressing random DNA and RNA libraries are capable of heritable mutation, evolving the parent DNA sequences present in the initial phage library. This strategy of mutating and

identifying the peptides of closely related mutants is called a “greedy strategy”.(104) In this strategy, the first step is to select the mixture of sequences in order of fitness by incubating the phage library with the target receptors. The second step is to mutagenize all the eluates by inserting the selected sequences that have the highest binding affinity from the first step. Step three is to then select the mutagenized sequence that has good binding affinity to the receptor compared to the first identified best sequence. Finally, steps two and three are repeated until only the highest binding affinity peptides are selected. Peptide selection based upon fitness affinity for a target receptor molecule is called the “affinity maturation program”.(104) Affinity maturation begins with the different rounds of affinity selection and mutagenesis, while the selection of peptide with good fitness is kept at very low concentration during the selection process. The phages selected from these alternating rounds will bind to specific receptors, and then again, these clones are subjected to additional rounds of affinity selection without mutagenesis to find the highest affinity clones, which are then sequenced and analyzed.(104,107)

1.9.4.3 Structure, assembly, replication, and release of M13 filamentous phage used in phage display libraries

The filamentous M13 bacteriophage particle is 65 Å in diameter and 9300 Å in length.(94) It consists of a ssDNA genome of 6400 bases which is coated with approximately 2700 copies of the major coat protein PVIII.(95,108) One end of the protein is capped with five copies of each of the minor coat proteins (PIII

and PVI) and the other end is capped with five copies each of the minor coat proteins PVII and pIX) (

Figure 1.). The coat proteins are usually small in size except for PIII, which is a 42 kDa polypeptide made up of three separate structural domains (N1, N2 and CT).(104) N1 and N2 polypeptides together form a protein structure, which is necessary for infection. The C-terminal (CT) is involved in creating contacts between the coat of the phage and the bacterium and for the incorporation of PIII protein into phage particles.(107) Concurrently, the domain N2 binds to the F-pilus of the *E. coli,* which causes the pilus to retract the phage to the membrane surface, and the N1 domain to bind to TolA, an outer membrane protein. The retraction of the host pilus causes the phage tail to be compressed, providing potential energy, which leads to the injection of the phage genome into the host cell. The ssDNA phage genome is then converted to dsDNA by host proteins. This dsDNA acts as a template for the synthesis of new ssDNA genomes as well as phage proteins, to provide all materials necessary for the assembly of new phage particles.(95) Assembly begins when a sufficient concentration of phage structural proteins are inserted into the host inner membrane. Simultaneously, the ssDNA-binding protein pV is expressed and reaches a similar, critical concentration to coat newly synthesized ssDNA genomes, preventing them from converting to the double-stranded form. pV-associated DNA has a hairpin structure which is recognized by a complex of three assembly proteins (pIV, pXI, pI) which forms a pore that spans both inner and outer membranes.

ssDNA is extruded through this pore at the site of assembly and is stripped of from pV, being replaced by phage structural proteins.(104) PVII and pIX proteins cap the initiating end of the assembling phage, PVIII proteins cover the entire length, and the termination end is composed of cap proteins PIII and PVI. The PIII protein dissociates from the membrane at the termination end and the fully assembled phage is released from the host cell.(87,104)

1.9.4.4 Types of phage display systems

Phage display systems are classified according to the expression of peptides by coat protein genes on the phage surface. In the type 3 phage display system, the PIII gene incorporates the DNA of interest that encodes for the peptide expressed in PIII coat protein.(94) The type 8 and 6 phage display systems, the PVIII gene and PVI gene, respectively, incorporate the foreign DNA that results in the peptide displayed in coat proteins PVIII and PVI.(104) The type 88 system is unique as it has two types of the PVIII gene: one is a recombinant DNA and the other is wild-type DNA.(94,104) Similarly, the type 33 system bears two different types of the PIII gene.(94) The type 8+8 system is totally different from the type 88 system. In the 8+8 system, the two different molecules of PVIII are on different phages. The wild type PVIII is on the helper phage and the recombinant PVIII is on a special kind of plasmid called a phagemid.(94) Unlike the other recombinant DNA plasmids, a phagemid has the origin of replication and the antibiotic resistance gene transferred to the host, which can be used to select for the plasmid-bearing host cells. The helper phage initiates replication

and the production of progeny virions. The type 3+3 and 6+6 systems are similar to type 8+8 system, except the recombinant gene is PIII and PVI, respectively.(87,107)

1.9.4.5 Recombinant phage display system

In this system, monoclonal antibodies specific to antigens presented on the outer surface of host cells can be selected through an enzyme-linked immunosorbent assay (ELISA). Monoclonal antibodies are introduced into M13 phage through a recombinant phage display system.(101,109) The single chain variable fragment (ScFv) is digested with restriction enzymes such as *NotI* and *SfiI*, and then ligated into a phagemid vector such aspCANTAB5 or pAK300.(88) This genetically engineered phagemid vector is introduced into the host bacterium through electroporation. Then helper phage, like M13K07 that aids initiation of replication of phagemid vectors containing the ScFv, result in production of antibodies specific to bacterial host and kill the bacterium.(107)

1.9.4.6 Ff-tet: the parent of the vectors in the phage display system

The tetracycline resistant filamentous (fd-tet) phage has a 2775 base pair of *Bg*/II fragment in which the transposon Tn10 genome is inserted into the *BamHI* site of the phage genome. The transposon Tn10 genome transfers the *tetA* gene, which codes for the tetracycline resistance protein in a bacterial host, and is replicated along with the phage genome. The insertion of the Tn10 genome into the fd-tet phages disrupts the minus-strand, which is the origin of

replication.(95,104) This greatly reduces the number of RF DNA without affecting the yield of virions. If the same Tn10 gene were inserted in some other loci of the fd genome, it would result in “cell killing” where the bacterial host is killed without yielding any phage particles. The disadvantage of this system is that it needs large cultures of infected bacteria for the phage purification step to isolate sufficient amounts of phage DNA.(107)

1.9.4.7 Titering fd-tet phage Infectivity

Phage infectivity is determined by counting the number of plaque-forming units (PFU). Serially-diluted phage particles are incubated with infectible host cells and then transferred to solid agar media containing tetracycline.(95) During the infection, the fd-tet phage confers tetracycline resistance gene to the host cells resulting in survival of the host on the tetracycline medium. After an overnight incubation, a count of the number of tetracycline-resistant colonies reflects the number of phage particles in PFU/mL. Thus phage infectivity is determined by measuring the number of tetracycline-resistant infected host cells.(107)

1.9.5 Applications of phage display

1.9.5.1 Identifying new target receptors and natural ligands

A list of identified target receptors has been used to select specific target peptides from phage display libraries.(110) There are diverse targets available for receptor binding. Although most of the receptors recognize

proteins, there remains a chance that they may recognize non-protein ligands, which are totally irrelevant to natural ligands. Ligands can be used as probes to identify unknown receptors such as antibodies or hormone receptors by identifying the ligand that binds again and again to that specific receptor.(104) Researchers use this technique to identify novel proteins that are critical for cell signalling pathways. Specific ligands are affinity-selected from random peptide libraries.(94,94)

1.9.5.2 Epitope discovery: a new route to vaccines

An epitope is the small portion of the ligand or antigen that is responsible for binding to a specific receptor, known as the antigenic determinant.(111) The receptors used to select the antigenic determinant are antibodies that identify the antigenic mimics from random peptide libraries. On a protein antigen, continuous epitopes contact 3 to 4 amino acids of specific antibody receptor over a sequence of 6 amino acids. However, there are some discontinuous epitopes that consist of critical binding residues that are far from the primary sequence but are close to the folded native conformation. The receptor may bind to a specific random peptide of the phage library, which facilitates mapping of the epitope of the receptor molecule. The sequence motif of the identified peptide is compared with the amino acid sequence of the natural ligand. If the motif matches the sequence of the amino acid found in the natural protein ligand, then the epitope can easily be mapped.(107) Since this phage display method is replicable and widely available, it is considered to be a cheap and easy

method for epitope mapping. If the selected peptides bind to the antibody, they are called 'immunogenic mimcs' and are used to immunize naïve animals. (104,107) This method of identifying immunogenic mimics may be a new strategy to diagnose diseases and develop vaccines. Peptides selected through epitope mapping may act as specific probes for antibodies used to treat diseases and to identify the antigen responsible for unknown diseases.(94,104)

1.9.5.3 Drug discovery

Receptors that select specific protein ligands from a phage display library are the targets of drug discovery programs such as antibiotics.(91,101) This approach of affinity selection is a traditional method of identifying new drugs by screening libraries for synthetic or natural products that can bind to the target receptors and act as inhibitory antagonist molecules.(112) Affinity selection has the ability to select different compounds at the same time, whereas chemical libraries have to screen one compound at a time. Phage display makes important contributions in the field of drug discovery where the selected peptides themselves have therapeutic properties.(94,107)

1.9.5.4 Selection of DNA binding proteins

Phage display may be used to design proteins that specifically bind to target DNA sequences.(113) A tetracycline-resistant peptide library is constructed within the parent coat protein sequence displayed on the phage, which then will be affinity-selected depending upon binding capacity and

specificity. (104) This display method identifies the nucleotide sequence that encodes the peptide selected by affinity binding, and is very easy, cheap and familiar to all molecular biologists. The fascinating thing is that a 1.5 mL microcentrifuge tube can contain a few hundred trillion phage particles, displaying billions of different random peptides, which can be used to select clones for different purposes.(94,104)

1.10 Rationale of proposed research

Recent studies have identified high rates of *H. pylori* infections and gastric disease in the remote community of Aklavik, NWT, as compared to the infection rates in urban centers in Alberta.(17) Current standard first line therapies fail to eradicate 20-25% of *H. pylori* infections.(18) This failure may be attributed to reinfection among patients(19,20), patient non-compliance(21), and the presence of antimicrobial resistance.(22) Gastric emptying time and the ruggae surface of the stomach wall may also hinder the access of the drug to the site of bacterial infection thereby leading to low drug efficiency.

A novel therapeutic that specifically targets the outer surface molecules of *H. pylori* for drug delivery may provide an effective alternative for the treatment of *H. pylori* infection without killing other bacteria in the gastrointestinal tract. The problem lies in detecting the surface molecules on the bacterium that could be used as a potential target. Phage display is a method used to identify unknown outer surface molecules expressed on the surface of

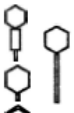
the bacterium.(94) This method has been used to successfully identify peptides that bind to the outer surface molecules of the *C. jejuni* bacterium, a close relative to *H. pylori*.(114). Each phage clone expresses a unique kind of peptide attached to the PIII coat proteins, one of which may bind to the outer surface molecules of the bacterium.(86) In my project, an M13 filamentous phage display library of peptides is used as a probe to identify the peptide that can specifically bind to the outer surface molecules of *H. pylori*.(92) Surface panning incubates a phage display library with an *H. pylori* isolate that is bound to a solid surface.(104) Bound phages are eluted after surface panning. Eluted phages are again used to surface pan the same *H. pylori* isolate; bound phages are eluted and once more used to surface pan the same *H. pylori* isolate. The phages in the final eluate are amplified in *E. coli*, which is then plated onto agar to identify phage-infected bacteria. Individual phage-infected bacteria are amplified to amplify the phage clone. Individual phage clones are extracted & purified. The pIII gene of the phage clone is sequenced and the nucleotide sequence is translated to amino acids. These peptides may specifically bind to the outer surface of *H. pylori* and have antimicrobial activity.

1.11 Research objectives

- To identify 7-mer peptides that bind specifically to the outer surface of *H. pylori* using M13 phage display at physiological conditions of the stomach.

- To determine the antimicrobial activity of the identified phage displayed 7-mer peptides against *H. pylori* isolates.

Table 1.1 Phage classification

Order	Family	Morphology	Nucleic acid
Caudovirales	*Myoviridae*		Double-stranded DNA
	Siphoviridae		
	Podoviridae		
	Tectiviridae[a]		
	Corticoviridae[a]		
	Lipothrixviridae[b]		
	Plasmaviridae[b]		
	Rudiviridae		
	Fuselloviridae		
	Inoviridae		Single-stranded DNA
	Microviridae		
	Leviviridae		Single-stranded RNA
	Cytoviridae[b]		Segmented, double-stranded RNA

[a] Lipid containing
[b] Enveloped

Reproduced with permission. (87)

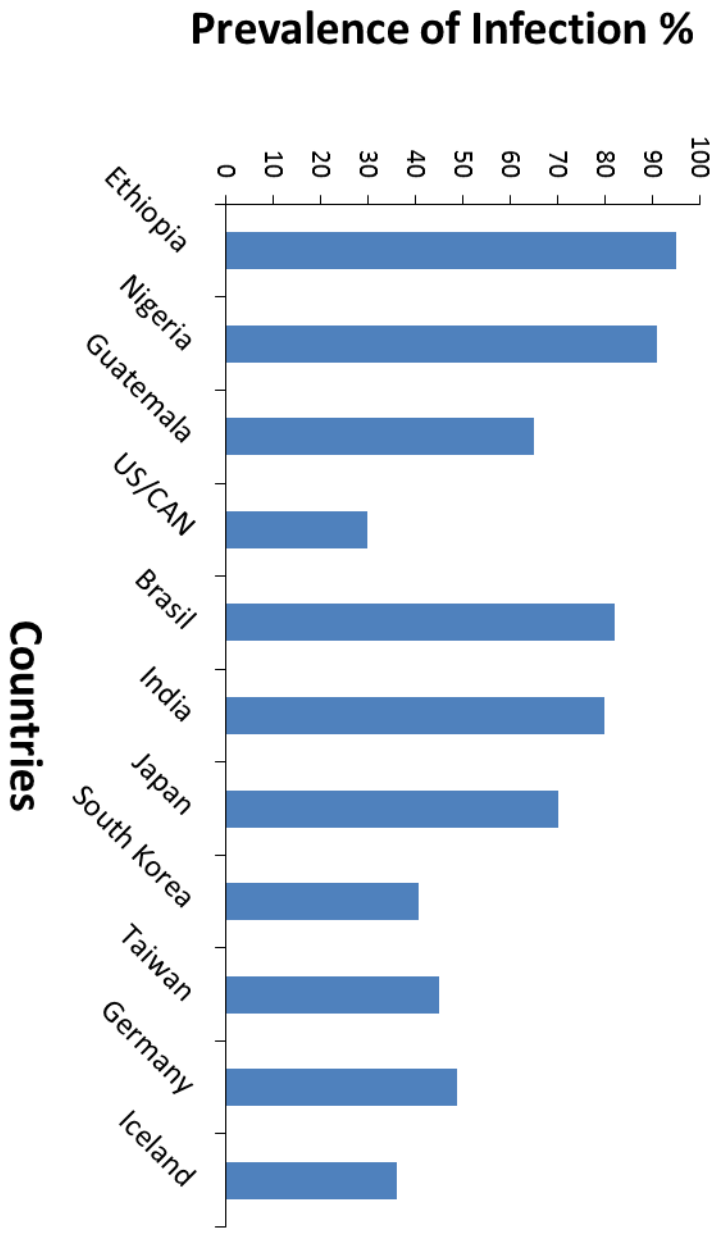


Figure 1.1 *Helicobacter pylori* infection globally

Prevlaence of *H. pylori* infection among adults from countries around the world as summarized by Thjodleifsson *et al.,* 2007. (27)

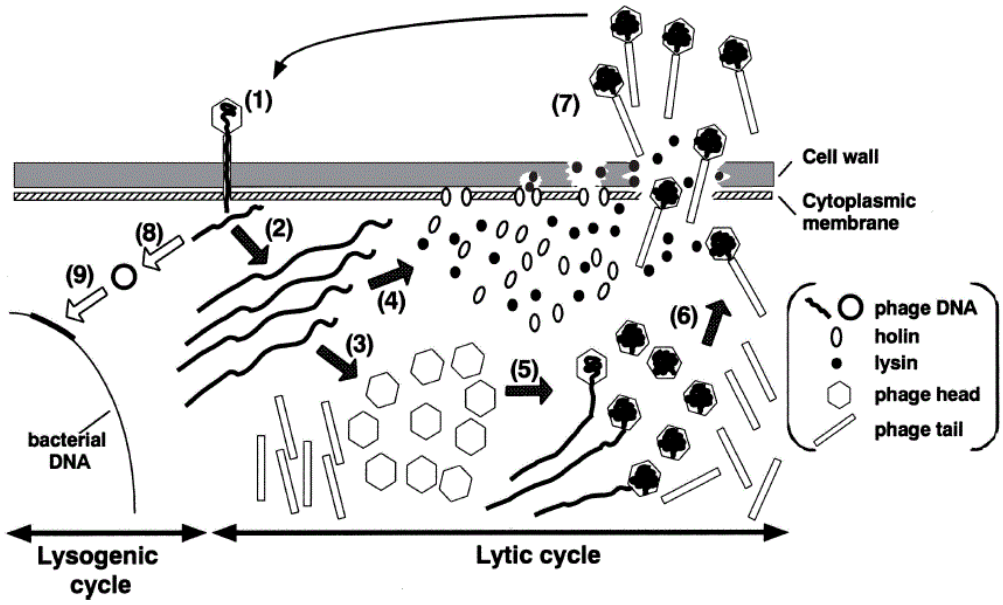


Figure 1.2 Phage life cycle

Schematic illustration of phage-induced bacteriolysis from Matsurzaki *et al*., 2005.(87) (1) Adsorption and DNA injection;(2) DNA replication; (3) production of head and tail; (4) synthesis of holin and lysin; (5) DNA packaging; (6) completion of phage particle; (7) disruption of the cell wall and release of the progeny; (8) circularization of phage DNA; (9) integration of the phage DNA into the host genome.

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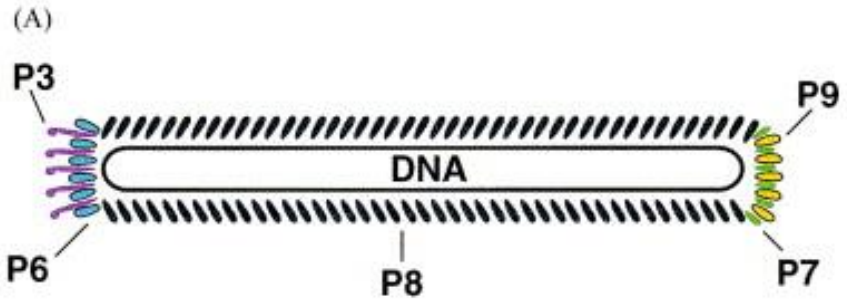

Figure 1.3 M13 filamentous phage

P3 (or PIII) coat protein is responsible forattachment of M13 filamentous phage to bacterial cells. (115)

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Chapter 2: Materials and Methods

2.1 Reagents and equipment

Reagents, chemicals and equipment were purchased from Fisher Scientific, Ottawa, ON, Canada unless otherwise stated.

The following media were used in the study:

3.7 g% Brain Heart Infusion (BHI) (37 g/L) (Oxoid, Basingstoke Hampshire, United Kingdom), 5 g% Yeast Extract (YE) (Becton, Dickinson and Company, Sparks, San Jose, USA) and 1.5 g% Select Agar (Invitrogen, Carlsbad, CA) were dissolved in distilled deionized ultrafiltered water (MQ water) in an Erlenmeyer conical flask and autoclaved for 30 min at 121°C. After autoclaving, the conical flask containing the hot agar suspension was cooled in a 55°C water bath for at least 30 min. Then, 5% horse serum (Gibco® Invitrogen, St Louis, MO, USA) 1.5 mg% Amphotericin B (Sigma Aldrich, Oakville, Ontario, Canada) and 1.5 mg% Vancomycin (Abraxis Biosciences, Richmond Hill, ON, Canada) were added to agar suspension, swirled briefly and then 22-24 mL was poured into each individual agar plate. This media was abbreviated as BHI+YE+HS+amp+vanc. Broth was prepared in the same manner but without agar. All antibiotics were prepared in MQ water and filtered through a 0.22 μm filter before adding to media.

Luria-Bertani broth containing 10 g% Bacto-Tryptone, 5 g% YE, and 5 g% NaCl and 1.5 g% Select Agar were dissolved in MQ water in an Erlenmeyer conical flask and autoclaved for 30 min at 121°C. The conical flask containing the hot agar suspension was cooled in a 55°C water bath for at least 30 min. Then, 20 mg% Tetracycline (Sigma Aldrich & Co, St Louis,MO, USA) was added, swirled briefly and then 22-24 mL was poured into each individual agar plate. This media was abbreviated as LB+Tet. Broth was prepared in the same manner but without agar. Tetracycline was prepared in MQ water and filtered through a 0.22 μm pore filter before adding to media.

Top Agar containing containing 10 g% Bacto-Tryptone, 5 g% YE, and 5 g% NaCl, and 2.5 g% Select agar were dissolved in MQ water in an Erlenmeyer conical flask and autoclaved for 30 min at 121°C. The conical flask containing the hot agar suspension was cooled in a 55°C water bath for at least 30 min. The media was dispensed as 3 mL aliquots into sterile 15 mL conical tubes and stored @ 4°C.

X-gal agar plates were prepared as for LB agar (without tetracycline). After cooling the media to 55°C, 50 g% IPTG (isopropyl-β-D-thiogalactoside) and 40 g% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside)(Sigma Aldrich & Co, St Louis,MO, USA) were added. This media was abbreviated as LB+IPTG+X-gal.

All the reagents used for the experiments were sterilized by autoclaving for 30 min at 121°C and 15 psi. While working with phages, any phage spills were

decontaminated immediately by 70% ethanol. Extra care should be taken when using automatic pipettes by using the pipette tips only once to avoid cross contamination.

2.2 Bacterial strains and culture conditions

2.2.1 *H. pylori* isolates

H. pylori 26695 was originally obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA) and Aklavik isolates A43, A96, A101, A104, and A167 were previously cultured from gastric biopsies obtained from residents of the remote community of Aklavik, NWT of Canada. *H. pylori* was isolated and cultured on BHI+YE+HS+amp+vanc agar plates under microaerobic conditions at 37°C. *H. pylori* growth was suspended in 20% glycerol in BHI broth, placed into 2 mL cryovials, flash frozen in liquid nitrogen, and stored at -80°C.

H. pylori were cultured from frozen stock vials on BHI-YE-HS+amp+vanc agar under microaerobic conditions at 37°C for 48 hours. Microaerobic conditions were achieved by placing broth or agar cultures in an anaerobic jar, evacuating the jar to -25 psi using a vacuum pump, and then replacing the vacuum with a gas mixture containing 5% CO_2, 5% H_2, 90% N_2 (Gas cylinder from Praxair, Edmonton, AB).

2.2.2 *E. coli* K12 ER2738

E. coli K12 ER2738 (Ph.D.™-7 Phage Display peptide Library Kit, New England Biolabs, Canada) was used as a host strain to titer and amplify bound

phages.(116) *E. coli* K12 ER2738 contains F´ *proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ*(lacZ)M15 zzf::Tn10 (Tet*^{R}*)/fhuA2 glnV* Δ*(lac-proAB)* Δ*(hsdMS-mcrB)5* [$_{k-}$ m$_{k-}$ McrBC^{-}]. *E. coli* K12 ER2738 is a tough F+ strain with a high growth rate more suitable for M13 propagation. This strain carries the tetracycline resistance gene, *GlnV* gene in order to suppress amber (UAG) stop codons within the library with glutamine, and also the *lacZ*α-peptide cloning sequence which permits blue/white screening of phages which have been inserted in the vicinity of the (+) strand origin of replication.

E. coli K12 ER2738 was streaked onto LB+Tet agar and grown under aerobic conditions at 37°C for 12 h. *E. coli* stock cultures were prepared by suspending cultures in 50% glycerol in LB broth and stored at -80°C.

2.3 M13 phage library

Phage Display Peptide Library stock solution, containing 1 x 10^{13} PFU/mL (Ph.D. ™-7 Phage Display Library Kit, New England Biolabs, Canada) of M13KE filamentous phages was supplied in 100 µL Tris-buffered saline (TBS [50 mM Tris-HCl (pH 7.5), 150 mM NaCl] with 50% glycerol.(116) The M13KE filamentous phage contains a single-stranded genome that is approximately 7220 nucleotides in length. Approximately 5% of the phage display library is composed of parent vector M13KE phage that is derived from the common cloning vector M13mp19 carrying the *lacZα* gene, which allows the phage plaques to appear blue when plated on media containing X-gal and IPTG. Environmental filamentous phages

are considered to be contaminants when observed during surface panning experiments, and will typically yield white/colorless plaques when plated on the same media. These plaques are also larger and “fuzzier” than the library phage plaques. Phage Display Peptide Library working stock solution was prepared by diluting 10 µL of the Phage Display Peptide Library stock solution in 90 µL phosphate-buffered saline (PBS)[0.08 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.02 M KCl, 1.4 M NaCl, pH 7.4] to get a final concentration of 10^{10} PFU/10 µL, which is used for surface panning experiments.

2.4 Growth curve experiments

2.4.1 *H. pylori*

Each *H. pylori* isolate – 26695, A43, A96, A101, A104, and A167 – was cultured onto BHI-YE-HS+amp+vanc agar from frozen stock for 48 h, subcultured once and incubated for 48 h. Each 48 h agar subculture was suspended in 12 mL of BHI-YE-HS+amp+vanc in a sterile 50 mL conical tube. Each suspension's optical density was measured at 600 nm (OD_{600}) in a spectrophotometer (Ultrospec 2000, Pharmacia Biotech) and adjusted to 0.100 ± 0.005. From each suspension, 5 mL aliquots were dispensed into two separate 25 cm^2 cell culture flasks, with canted neck and vented cap with a 0.2 µm hydrophobic filter (Corning Incorporated, New York, USA) labelled "A" and "B". The vented culture flasks were placed in an anaerobic jar and incubated under microaerobic conditions in a temperature-controlled orbital shaker (Forma orbital shaker, Thermo Electron Corporation, Gormley, ON, Canada) at 120 rpm at 37°C for 56 h.

The study design for sampling *H. pylori* broth cultures was similar to that previously used by Bury-Mone and co-workers.(117) Aliquots (300 µL) of bacterial suspensions were removed at 4 h time intervals over 56 h, alternately from 'A' and 'B' flasks so that there would be sufficient volumes of *H. pylori* broth suspension to measure over the 56 h incubation period. The OD_{600} was measured on 120 µL of each aliquot and three 50 µL aliquots were serially diluted 1/10 from 10^{-1}-10^{-7}. Then, 50 µL of bacterial suspension from each dilution tube was spread onto fresh BHI-YE-HS+amp+vanc agar plates in triplicate

and incubated under microaerobic conditions for 72 h at 37°C. After the incubation, the number of live bacterial cells present on each plate was reported by counting the number of bacterial colony forming units. The number of colony forming units per mL (CFU/mL) was calculated using the following formula:

For each bacterial dilution:

CFU/mL = average number of colonies per 50 µL X 20 X dilution factor

Where: 20 = factor to convert 50 µL to 1 mL

dilution factor= 1/dilution

A growth curve was performed on three different days for each isolate (n=3).

2.4.2 *E.coli* K12 ER2738

A single colony of an overnight culture of *E. coli* K12 ER2738 on LB+Tet agar was inoculated into 10 mL of LB+Tet broth in a 250 mL Erlenmeyer flask and incubated under aerobic conditions in an orbital shaker at 250 rpm under aerobic condition at 37°C for 12 h.

Aliquots of bacterial suspensions (300 µL) were removed at 2 h intervals over a 12 h time period from each 250 mL Erlenmeyer flask. The OD_{600} was measured on each aliquot and three 50 µL aliquots were serially diluted 1/10 from 10^{-1}-10^{-9}. Then, 50 µL of bacterial suspension from each dilution tube was spread onto fresh LB+Tet agar plates in triplicate and incubated under aerobic conditions at 37°C for 12 h. After the incubation, the number of CFU/mL was

calculated using the formula in section 2.3.1. A growth curve was done on two different days (n=2).

2.5 Selection of phage displayed peptides

2.5.1 Preparation of *H. pylori* suspensions for surface panning

A 48 h *H. pylori* culture from BHI+YE+vanc+amp agar plates was suspended in 12 mL BHI+YE+amp+vanc broth, OD_{600} adjusted to 0.100, transferred 5 mL each to two T25 vented culture flasks and cultured for 22 h at 37°C under microaerobic conditions. After incubation, 3mL of the *H. pylori* broth suspension was transferred into two 1.5 mL microcentrifuge tubes. The tubes were centrifuged at 5000 X g (9000 rpm) in a GSA rotor in a SORVALL RC2-B refrigerated centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 10 min at 4°C. The supernatant was discarded and each of the two pellets was resuspended in 1.5 mL of phosphate-buffered saline with gelatin (PBSg) [0.08 M Na_2HPO_4, 0.01 M KH_2PO_4, 0.02 M KCl, 1.4 M NaCl, pH 7.4 with 0.1% {w/v} gelatine] for pH 7.4 condition experiments; or in phosphate citrate buffer (PCB)[0.1 M citric acid, 0.2 M Na_2HPO_4, pH 4.0] for pH 4.0 condition experiments. Then, 1.5 mL of pellet suspension was transferred from each tube to one sterile 60 mm X 15 mm polystyrene petri dish for a final volume of 3 mL of *H. pylori* suspension.

2.5.2 Surface panning

Surface panning was performed for *H. pylori* 26695 at three different conditions 37°C pH 4.0, 37°C pH 7.5, room temperature RT and pH 4.0. Aklavik

isolates A43, A104, & A167 were panned with the working stock solution of phage display library at 10^{10} PFU/10 µL at 37°C and pH 4.0. For all conditions, the experiment was performed in the following way: 10 µL of phage display library were pipetted into each *H. pylori* Petri dish to obtain a suspension containing 10^{10} PFU phage per 10^{9} CFU *H. pylori*. The number of phage particles should be 10 fold higher than number of CFU of *H. pylori* to capture all the potential phages with high binding affinity. The phage/*H. pylori* mixture was incubated for 45 min on a GX-1000 elliptical platform shaker (Labnet International Inc., Edison, NJ, USA). The phage-*H. pylori* mixture was transferred to two 1.5 mL microcentrifuge tubes and centrifuged at 3200 X g (7600 rpm) for 5 min at 4°C.

Unbound phages remained in the supernatants and were discarded. Loosely bound phages were removed from each pellet by a series of 10 washes: each wash consisted of resuspending one pellet in 1 mL of PBSg (pH 7.5) or PCB (pH 4.0) and centrifugation for 5 min at 4°C. The pellet was then resuspended in 200 µL of elution buffer (0.1 M Glycine ($H_2NCH_2CO_2H$)·HCl (pH 2.2), 250 mg/mL BSA) and mixed gently for 20 min at room temperature on a rocker. The phage suspension was then neutralized upon addition of 30 µL of Tris-HCl (1 M Tris-HCl, pH 9.1). The eluates contained tightly bound phages and were transferred to a new 1.5 ml microcentrifuge tube and stored at 4°C for 2-3 weeks for later phage amplifcation.

2.5.3 Phage Titering

Before phage amplification, three 100 µL aliquots of bound phages from each first round of panning were serially diluted 1/10 from 10^{-1}-10^{-3}. From additional rounds of panning, three 100 µL aliquots were serially diluted 1/10 from 10^{-1}-10^{-4}.

After phage amplification following the first round of panning, three 100 µL aliquots of amplified bound phages were serially diluted 1/10 from 10^{-1}-10^{-9}. After additional rounds of panning, three 100 µL aliquots were serially diluted 1/10 from 10^{-1}-10^{-11}.

Then for both before and after phage amplification procedure, 10 µL of each phage dilution was added to individual 200 µL aliquots of mid-log phase *E. coli* K12 ER2738 freshly cultured in LB+Tet broth for 5 h (until it reaches mid-log phase OD_{600}=0.6-0.8). The *E. coli*-phage mixture was vortexed and incubated at room temperature for 5 min, transferred to culture tubes containing 3 mL Top agar (previously melted and maintained at 45°C), vortexed briefly and immediately poured onto fresh LB+IPTG+X-gal agar plates, and gently swirled to uniformly distribute the top agar suspension across the plate. The plates were cooled 5 min and incubated under aerobic conditions at 37°C overnight. The next day, the number of blue colonies on each plate was counted to determine the number of bacteria infected with phages, where one colony represents one

plaque-forming unit (PFU). The number of PFU/mL was calculated using the following formula:

For each phage dilution:

PFU/mL= average number of PFU per 10 µL X 100 X dilution factor

Where: 100 = factor to convert 10 µL to 1 mL

dilution factor = 1/ dilution

2.5.4 Phage pool amplification

The remaining eluate aliquots (130 µL) containing bound phages from each round of panning — except the final round (see section 2.5.5) — were individually incubated with 20 mL of a 1:100 freshly diluted overnight *E. coli* culture in LB+Tet broth in a 250 mL Erlenmeyer flask. The phage-*E. coli* mixture was incubated under aerobic conditions with shaking at 250 rpm for 4.5 h at 37°C, and then centrifuged at 12,000 X g (14,500 rpm) for 10 min at 4°C. The pellet (containing *E. coli*) was discarded. Then 80% of each supernatant (containing phages) was transferred to a new tube containing 1/6 volume of 20% polyethylene glycol (PEG)/2.5 M NaCl and vortexed briefly. The phage-PEG precipitate was incubated overnight at 4°C and then centrifuged at 12,000 X g (14,500 rpm) for 15 min at 4°C. The supernatant was decanted and discarded. The tube was centrifuged again to remove the residual supernatant. The pellet was re-suspended in 1 mL TBS (to elute phages from PEG) and centrifuged at 12,000 X g (14000 rpm) for 5 minutes at 4°C (to pellet the PEG).

Again 80% of the supernatant was transferred to a new tube containing 1/6 volume of 20% PEG/2.5M NaCl and vortexed briefly. The pellet was discarded. The phage-PEG precipitate was incubated for 1 h on ice and then centrifuged at 14,500 rpm (12,000) for 10 min at 4°C. The supernatant was decanted and discarded. The tube was centrifuged again to remove the residual supernatant. The final pellet was suspended in 200 µL TBS and centrifuged for 1 min.

The supernatant was transferred to a new tube and 100 µL aliquot was used to determine the concentration of phage that was amplified (see section 2.5.3). The rest of the aliquot was used as a stock for the second round of panning. In order to determine number of phages bound to the *H. pylori*, the eluted phages from the second round of panning were titered and amplified. The amplified phages from the second round of panning were used as a stock for the third round of panning and titered.

2.5.5 Phage clone amplification

From the titer plates (< 1-3 days old) containing less than 100 plaques after the third final round of panning, 20 well-separated individual plaques were stabbed and transferred to 20 Individual 2 ml culture tubes containing 1 mL of 1:100 freshly diluted overnight culture of *E. coli* K12 ER2738 grown in LB+Tet broth. Each phage clone/*E. coli* mixture was incubated with shaking at 250 rpm for 4.5 h at 37°C, then transferred to 1.5 mL microcentrifuge tubes and

centrifuged at 12,000 X g for 30 sec. Each supernatant was transferred to a new tube and centrifuged again. Again, each supernatant (containing approximately 1.9 mL amplified phage clone stock) was transferred to a new tube and stored at 4°C for 2-3 weeks. If necessary, amplified phage clone stock can be diluted 1:1 with sterile glycerol for long term storage at -20°C.

2.6 DNA analysis of insert sequences

2.6.1 ssDNA extraction

Amplified individual phage clone stock (500 µL) was transferred to a fresh tube containing 200 µL of 20% PEG/2.5M NaCl, inverted several times to mix the phage-PEG precipitate, and Incubated for 10-20 min at room temperature. Then, the tubes were centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant was carefully removed by pipette leaving a small white phage pellet at the bottom of the tube (not always visible). The tube was centrifuged briefly and the residual supernatant was removed carefully. The pellet was re-suspended in 100 µL Iodide buffer (10 mM Tris-HCl, pH 8.0), 1 mM EDTA $C_{10}H_{16}N_2O_8$, 4 M NaI) by flicking the tube. Then, 250 µL ethanol was added to the pellet suspension and incubated for 20 min at room temperature to preferentially precipitate the phage ssDNA and leave most of the protein in solution. The phage precipitate was centrifuged at 14,000 rpm for 10 min at 4°C and the supernatant carefully removed by pipette and discarded. The pellet was washed by resuspension in 0.5 mL 70 % ethanol (stored at -20°C), centrifuged at

14,000 rpm for 10 min at 4°C and the residual supernatant was discarded. The pellet was briefly vacuum-dried and then suspended in 30 µL of sterile MQ water and stored at -20°C.

2.6.2 Amplification and purification of pIII gene

The random insert sequence (21 bp) of pIII gene, which encodes for peptide, is located between *Kpn*1 and *Eag*1 restriction sites (Figure 2.1). The pIII part of phage genome was amplified using the Phagef-F (Integrated DNA Technologies (IDT), Inc, Iowa, USA), which binds at position 1235, and 96 gIII primer (Phage Display Library Kit, New England Biolabs, Canada), which binds at position 1736 (Figure 2.1).

The PCR reaction mixture contains a final volume of 50 µL containing 1 µL of 20 ng/µL template phage ssDNA, 5 µL of 10 X Hi-fidelity PCR reaction buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl); 10 µL of 1 mM dNTPs (Invitrogen, Burlington, ON, Canada); 2 µl 50 mM $MgSO_4$; 0.5 µL of each 10 µM primer (Table 2.1) and 0.2 µL of 5 U/µL Hi-fidelity *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada). Thirty-five cycles of amplification consisted of 30 sec denaturation at 94°C, 45 sec annealing at 52°C, and 60 sec primer extension at 69°C. The PCR products (amplicons) were separated by 2% agarose gel electrophoresis dissolved in 1X TE buffer run at constant voltage (100 V) for 30 min, and the size (~ 450 bp) confirmed against a 1 kb DNA ladder (Invitrogen, Burlington, ON, Canada). The amplicons were purified using the GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, Inc, MO USA). The advantage of this kit is that it uses a

combination of silica binding with a spin column format, which does not need expensive resins or toxic organic compounds, such as phenol and chloroform, to remove the primers and other impurities. Each amplicon concentration was determined by measuring the absorbance at 260 nm using the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Accordingly, DNA concentration was calculated as follows:

dsDNA concentration (ng/μL) = A_{260} x 50 ng/μL x dilution factor

where: 50 ng/μL = DNA concentration when A_{260} = 1.0

dilution factor = 1/dilution

DNA purity was assessed from the ratio of absorbance measurements at 260 nm and 280 nm as follows:

$A_{260}/A_{280} \geq 1.8$ indicates "pure" DNA

where: A_{260} = absorbance of nucleic acids at 260 nm

A_{280} = absorbance of proteins and other impurities at 280 nm

2.6.3 DNA sequence analysis

The purified amplicons were diluted to 0.5 ng/μL and sent to The Applied Genomics Center (University of Alberta, Alberta, Canada) for DNA sequencing by the Sanger sequencing method.(118) Sanger sequencing uses selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during DNA replication. After the insert sequences were identified, they were translated to amino acids using DNAMAN software (Lynnonn Corporation, Quebec, Canada). The nucleotide sequences and 7-mer peptides were then

sorted using Microsoft Excel (Microsoft, Redmond,WA,) to assess sequence similarity.

2.6.4 *In vitro* antimicrobial assay

Individual amplified phage clones from the third round of panning of each of the four *H. pylori* isolates 26695, A43, A104 and A167 were incubated with each of the *H. pylori* isolates to assess their antimicrobial activity as described by Bishop-Hurley and co-workers.(114) *H. pylori* isolates were cultured under microaerobic conditions with shaking at 120 rpm for 4 hr at 37°C to obtain 1 X 10^7 CFU/mL, which was verified by measuring OD_{600} and culture of serial dilutions.

The antimicrobial assay was carried out in 96-well flat bottom microtiter plates (Nunclon™, Roskilde, Denmark). Twenty phage clones that bound to one *H. pylori* isolate were tested against the four *H. pylori* isolates per experiment, for a total of 80 clones over four experiments. For each *H. pylori* isolate, 135 µL BHI+YE+HS+amp+vanc broth suspension (1 X 10^7 CFU/mL) was added to 24 wells, and 40 µL of one of the following:

- TBS *H. pylori* suspension *with* one of the 20 phage clones (10^{12} PFU/mL)
- TBS *H. pylori* suspension *without* phage (*H. pylori* growth control in TBS)
- TBS only (blank for *H. pylori* growth in TBS)
- BHI-YE-HS+amp+vanc *H. pylori* suspension *without* phage (*H. pylori* growth control reference for *H. pylori* in TBS)
- BHI-YE-HS+amp+vanc broth only (blank for *H. pylori* growth in BHI)

The plates were then incubated under microaerobic conditions with shaking at 120 rpm for 18 h at 37°C. The OD was measured at 595 nm (OD_{595}) to assess inhibition of *H. pylori* growth as a measure of phage clone antimicrobial activity in a microplate reader spectrophotometer: The difference in OD_{595} in the presence and absence of phage represents the corrected *H. pylori* growth in the not inhibited by the presence of the phage clone. For each *H. pylori* isolate, the OD_{595} of the corrected *H. pylori* growth was plotted for one set of phage clones per graph; therefore, each *H. pylori* isolate has four graphs of antimicrobial activity for the four sets of phage clones.

Table 2.1 Primers used to amplify pIII gene of M13 phage genome

pIII Primers	Sequence (5'→3')
Phage f- F	TTT CGT TTT AGG TTG GTG CC
96 gIII- R	CCC TCA TAG TTA GCG TAA CG

From Ph.D.™-7 Phage Display Library Kit (116)

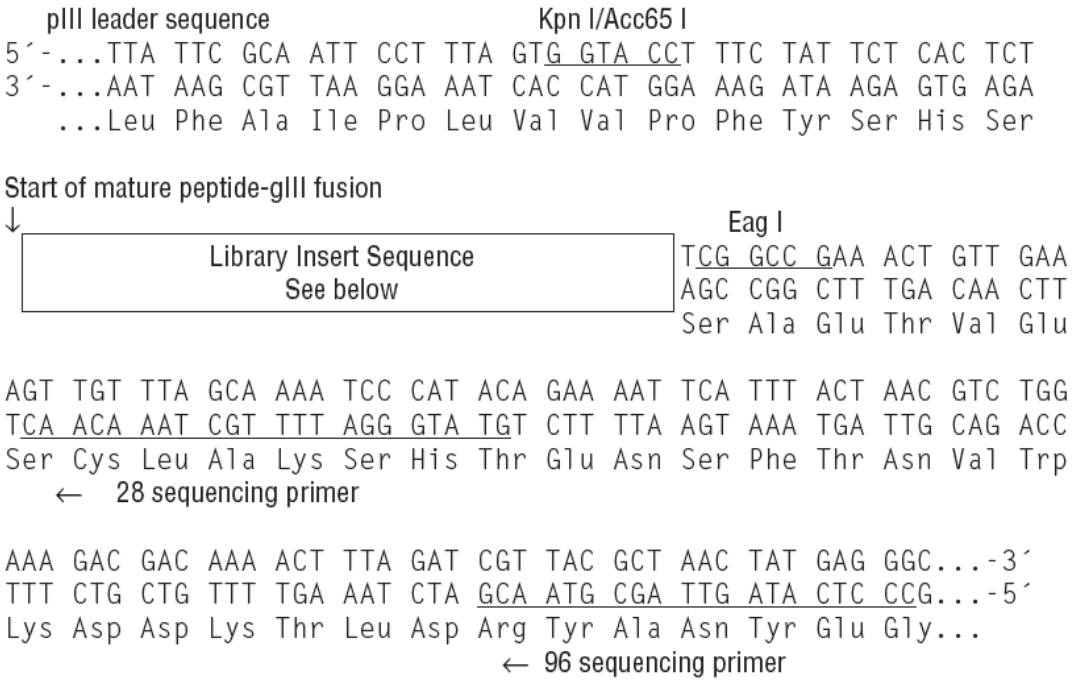


Figure 2.1 Sequence of M13KE phage pIII gene near insert sequence. (116)

Chapter 3: Results

3.1 Length of incubation required to achieve 10^9 CFU *H. pylori*/mL

The growth curves of *Helicobacter pylori* 26695, A167, A43, A96, A101, and A104 were determined over 56 h incubation. Aliquots were taken to measure the optical density and determine the CFU/ml at selected time points. The CFU/mL vs time growth curves were similar for 26695 and all Aklavik isolates with respect to the onset of lag, log and stationary phases (Figure 3.1**)**. The target initial bacterial concentration of 10^9 CFU/mL was achieved by *H. pylori* 26695 (3.4 x 10^9 CFU/mL) at 22 h (Table 3.1). However, the Aklavik strains only achieved 4.9- 6.5 X 10^8 CFU/mL at 24 h incubation (Figure 3.2, Table 3.1). The maximum CFU/mL achieved by *H. pylori* isolates varied from OD_{600}= 1.009-1.630 (Table 3.1).

3.2 Verification of mid-log phase of *E.coli* K12 ER2738

The mid-log phase was achieved at 5 h incubation where the CFU/mL was 7.0 X 10^8 CFU/mL (Figure 3.3).

3.3 Selection peptides bound *H. pylori*

Phage library displaying 7 amino acid peptides on minor coat protein PIII was used to affinity-select for peptides that bound to the cell surface of *H. pylori* 26695 at four different conditions. After three rounds of panning, similar amounts of phage bound to 26695 regardless of temperature and pH (Table 3.2). For the Aklavik isolates, all surface panning experiments were carried out at 37°C

and pH 4.0 to mimic conditions in the human stomach. After three rounds of panning 3.0-7.4 x 10^7 PFU/ml of enriched pool of phage clones were eluted from the surface of isolates A43, A104, and A167 (Table 3.3). A96 and A101 were not used for the surface panning studies due to time limitations.

3.3.1 pIII gene sequence analysis directly from amplified phage clone DNA

H. pylori 26695 were initially surface-panned at RT and pH 7.5. Twenty individual well-separated phage clones were randomly selected and amplified. DNA extraction yielded high 260/280 values (Table 3.4), - greater than 1.8, the value which indicates pure DNA, - yet sequence analyses yielded unreadable DNA sequences. The poor DNA sequence data suggested the presence of impurities following the DNA extraction procedure. In later experiments, the pIII gene was first amplified from the amplified phage clone DNA by PCR before doing sequence analysis to eliminate any impurities or contaminants that may interfere with the DNA sequencing method.

3.3.2 DNA sequence analysis on PCR amplified pIII gene

For each *H. pylori* isolate, 20 individual well-separated phage clones were randomly selected and amplified from the phage titering plates following the third round of surface panning. ssDNA was isolated for each of the 80 phage clones, and then the pIII genes containing the insert sequences were amplified by PCR. The expected amplicons (~450 bp) were confirmed by agarose electrophoresis, purified and quantified (

Table 3.5). The amplified pIII gene of the M13KE parent vector was used as the positive control. The DNA quality of the amplicons as assessed by A_{260}/A_{280} was within 1.8-2.0. Phage clones that bound to *H. pylori* were labelled “P” (bound to 26695), "Q" (bound to A43), "R" (bound to A104), and "S" (bound to A167).

3.3.3 Identification of pIII gene insert sequences

Of the 80 phage clones sequenced, only 44 clones contained an insert sequence (Figure 3.5). The remaining 36 clones had either no insert sequence or multiple insert sequences and were not analyzed further. Each of the insert DNA sequences of the 44 phage clones were translated to amino acids using DNAMAN software (Tables 3.6-3.9). Each of the insert sequences of the 44 identified phage clones that bound to the *H. pylori* isolates was unique. There were 13 phage clones that contained insert sequences, which bound to *H. pylori* A104 (Table 3.8). There were 12 phage clones that contained insert sequences, which bound to *H. pylori* A43 (Table 3.7). There were 11 phage clones that contained insert sequences, which bound to *H. pylori* 26695 (Table 3.6). In contrast, there were only 8 phage clones that contained insert sequences, which bound to *H. pylori* A167 (Table 3.9).

3.4 Antimicrobial activity of phage clones

Forty-four identified phage clones were further tested to see if they inhibit the growth of the bacteria. Only 3 phage clones - P7, P8, P17 - completely inhibited the growth of all the *H. pylori* isolates tested in this study, including

26695 (Table 3.10). Twenty of the 44 phage clones that contained insert sequences completely inhibited the growth of Aklavik *H. pylori* isolates. Phage clones that bound to the A167 had greater antimicrobial activity against the *H. pylori* isolates 26695, A43, A167 and A43 when compared to other bound phage clones (Figure 3.6, Figure 3.11). All but 2 phage clones (R15, S12) completely inhibited the growth of A104 (Figure 3.9, Figure 3.13). All but 6 phage clones (P5, P19, Q7, R8, S9, S12) completely inhibited the growth of A167 (Figure 3.10, Figure 3.14). In contrast, 21 phage clones completely inhibited the growth of A43 (Figure 3.8, Figure 3.12). Only 4 phage clones (P7, P8, P11, P17) completely inhibited the growth of 26695 (Figure 3.7, Figure 3.11).

Table 3.1 *H. pylori* growth at 24 h

H. pylori	**Maximum OD_{600}**	**CFU/mL**
26695	1.630	3.4×10^9
A43	1.184	5.2×10^8
A96	1.009	4.4×10^8
A101	1.076	6.2×10^8
A167	1.083	6.5×10^8

Table 3.2 Effect of temperature and pH on the number of phages bound

Round of Surface Panning	Number of phages bound to *H. pylori* 26695 (PFU/mL)			
	37°C		RT	
	pH 4.0	pH 7.5	pH 4.0	pH 7.5
First	2.97×10^5	2.47×10^5	3.60×10^5	3.77×10^5
Second	3.63×10^6	3.66×10^7	6.10×10^4	2.47×10^7
Third	1.29×10^7	1.50×10^7	8.23×10^6	5.73×10^6

Table 3.3 The number of phages bound to Aklavik *H. pylori*

Round of Surface Panning	Number of phages bound to Aklavik *H. pylori* (PFU/mL)		
	A167	**A104**	**A43**
First	3.03×10^5	6.77×10^5	1.32×10^6
Second	1.05×10^7	9.80×10^6	9.13×10^6
Third	8.30×10^6	7.37×10^7	3.03×10^7

Table 3.4 ssDNA quantitation of individual phage clones

Phage clone	DNA ng/µL	A_{260}/A_{280}
P1	63.0	5.2
P2	36.5	2.5
P3	22.0	2.9
P4	29.0	2.4
P5	38.5	3.2
P6	58.5	2.7
P7	58.5	2.3
P8	33.5	4.2
P9	47.5	3.0
P10	30.0	2.8
P11	27.5	3.0
P12	31.5	3.3
P13	32.0	2.6
P14	36.0	3.0
P15	33.0	1.6
P16	15.7	2.3
P17	28.0	3.8
P18	53.5	2.6
P19	34.5	2.9
P20	28.0	2.3

Table 3.5 PCR amplified pIII gene DNA quantitation and quality from individual phage clones

***H. pylori*: phage clones bound**	**DNA ng/µL**	**A_{260}/A_{280} ratio**
26695: P1-P20	3.0-5.2	1.8-2.0
A43: Q1-Q20	2.9-7.8	1.8-2.0
A104: R1-R20	2.4-6.3	1.8-2.1
A167: S1-S20	2.5-6.7	1.8-2.1

Table 3.6 Insert sequences for phage clones bound to *H. pylori* 26695

Phage clone	DNA Sequence	Peptide Sequence
P2	GTA ATA CTC AGC CGG GGT TTT	G N T Q P G F
P3	CTT AAT CCG GAT AAG GAT GAG	L N P D K D E
P4	ATG TCG ACG CAG TCG AAG GAT	M S T Q S K D
P5	AAT GCT TTG CCG TTG GAT TAT	N A L P L D Y
P6	ATG TTT GCG AGG TTT AAT GAG	M F A R F N E
P7	CTT TCT GAT GAT ACG AGT AAT	L S D D T S N
P8	TCT GAG GCG TTT GCG GAT ATT	S E A F A D I
P10	TAT ACG AGT AAG AAT AAT GCG	Y T S K N N A
P11	AAT ACT TAT GTT AAT AGG ATG	N T Y V N R M
P17	GGT AAG CCT TTG GGG AAT AAT	G K P L G N N
P19	CCG TCT CAT ATG TTT CGG ACG	P S H M F R T

Table 3.7 Insert sequences for phage clones bound to *H. pylori* A43

Phage clone	DNA Sequence	Peptide Sequence
Q2	GAG AAT TTG CCT GGT GCT TCG	E N L P G A S
Q3	AAT ATG TTT AGG CCT CCT GTG	N M F R P P V
Q4	GGT ACG *C*AG AGG TTT CTG TCG	G T Q R F L S
Q5	ACG ATG CAT AAG GGG AAG CAT	T M H K G K H
Q6	ACG AAG GTG GCT GGG ACT TGG	T K V A G T W
Q7	GTT GAT CCG TTT GCG TAT TAT	V D P F A Y Y
Q8	CTG GGG CAT GCT CTG ACG GTG	L G H A L T V
Q10	AAG GCT TCT TAT GCG CAG GGG	K A S Y A Q G
Q15	GTT GGG TTG TAT ACG CAG TCG	V G L Y T Q S
Q16	AGT TTG ACT TAT AAG GGG GTT	S L T Y K G V
Q18	ATG AAT TCN CAT GGT GTG ACT	M N S H G V T
Q19	CAG CTG CAG GAT GTG CTG GGT	Q L Q D V L G

Table 3.8 Insert sequences for phage clones bound to *H. pylori* A104

Phage clone	DNA Sequence	Peptide Sequence
R1	ATT CCT GTG AAT CGG GCT CTT	I P V N R A L
R2	AGT CCT CAG ACG ATG AGG ACG	S P Q T M R T
R4	TCT ACT GAG GCT GTG AAG TAT	S T E A V K Y
R5	ATT GCG ACG ACG AAG ACT TTG	I A T T K T L
R6	CAT TCG AGT TAT AGG ATT GTG	H S S Y R I V
R7	CTG GAG CGT ACT ACG CAT TCT	L E R T T H S
R8	GAT GCT AAT ATT TTG TCG AAG	D A N I L S K
R9	TTT CCT AAT TGG GCT TCG GGT	F P N W A S G
R13	TTG AAG AAT TTG GAG CGT GCG	L K N L E R A
R15	CTG CCT TCG AGT AAT CTG ACG	L P S S N L T
R16	ACT GAG CAT CAT AAG TTT TAT	T E H H K F Y
R18	TGG GAG GAT GGG GTT AAT ACT	W E D G V N T

Table 3.9 Insert sequences for phage clones bound to *H. pylori* A167

Phage clone	DNA Sequence	Peptide Sequence
S3	CCG GCT ATT GGT AGT AAT CAT	P A I G S N H
S5	CAG ACG GAT GTT CAT GCT AGT	Q T D V H A S
S7	TTT CAT ACG CCG TGG CAG GAG	F H T P W Q E
S9	TTT CGT ACT CTG CAT ACT AAT	F R T L H T N
S11	GTG AGT AAG CAT ATG GGG CTT	V S K H M G L
S12	ACG TCT TTG AAT AGT GTT CAG	V P M V G A V
S14	GTT CCT ATG GTG GGT GCG GTG	R M A N D A M

Table 3.10 Antimicrobial activity of 44 phage clones bound to *H. pylori* isolates

Phage clones	26695	A43	A104	A167
P 7, 8, 17	-	-	-	-
P 2, 4, 10 R 9 Q 2, 3, 5, 6, 8, 10, 15, 16, 18, 19 S 11, 14	+	-	-	-
P 3, 6 Q 4, R 1, 2, 4, 5, 7, 8, 13, 16, 18, 20 S 3, 5, 17	+	+	-	-
P 5, 19 Q 7	+	-	-	+
R 6, S 7, 9	+	+	-	+
P 11	-	+	-	-
R 15	+	+	+	-
S 12	+	+	+	+

(+) grow1th

(-) inhibited growth

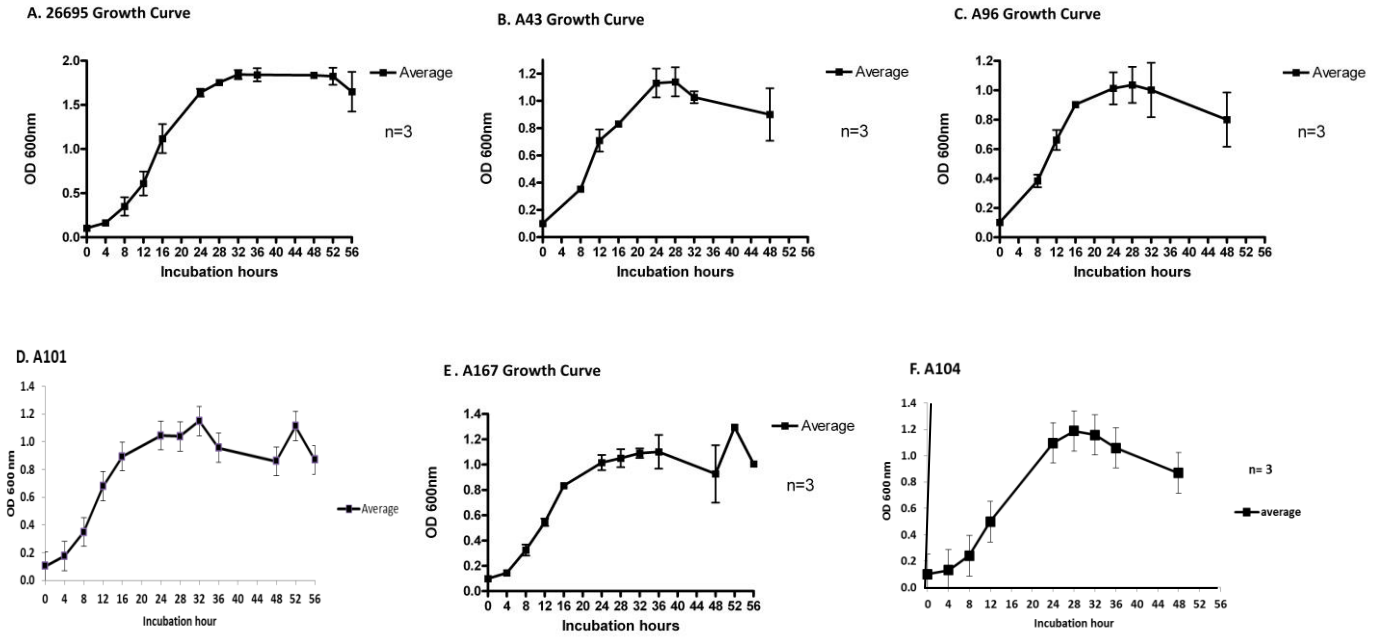


Figure 3.1. Growth curve of *H. pylori* isolates (OD_{600} vs time)

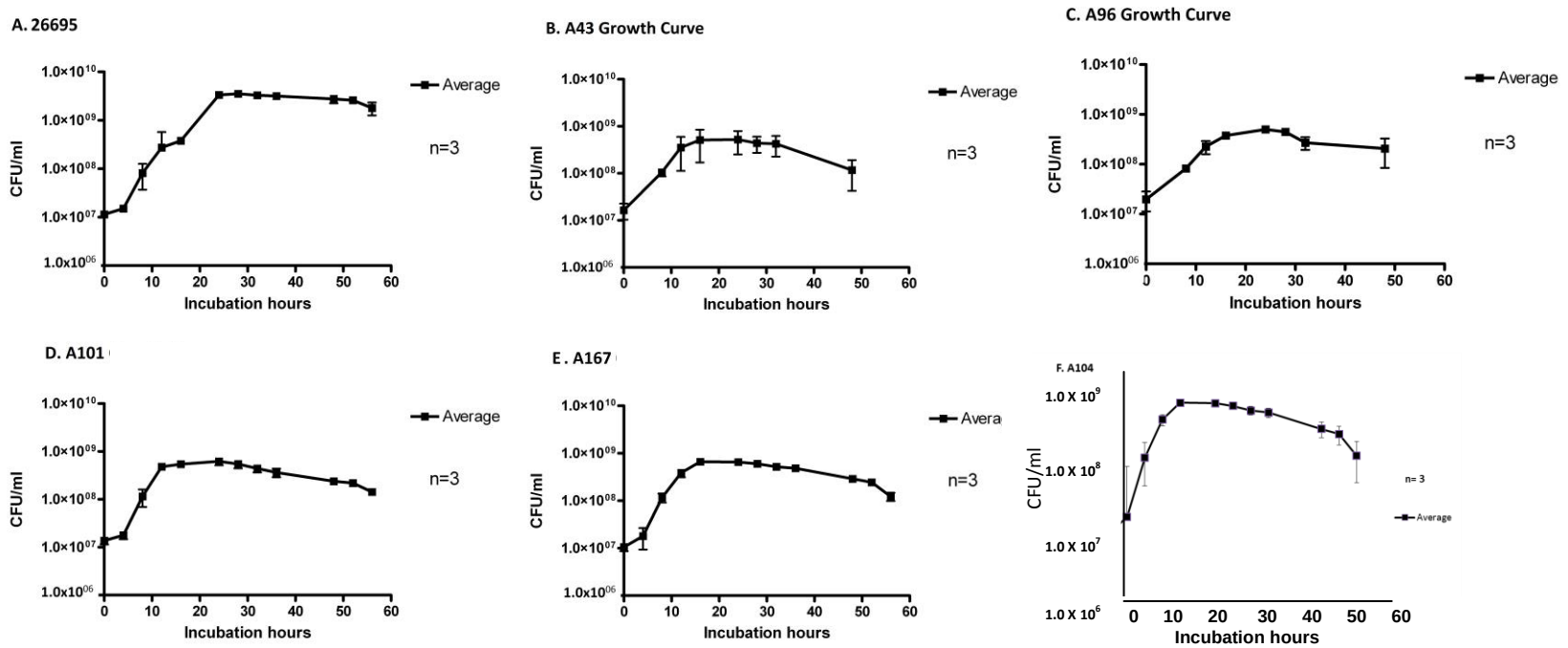
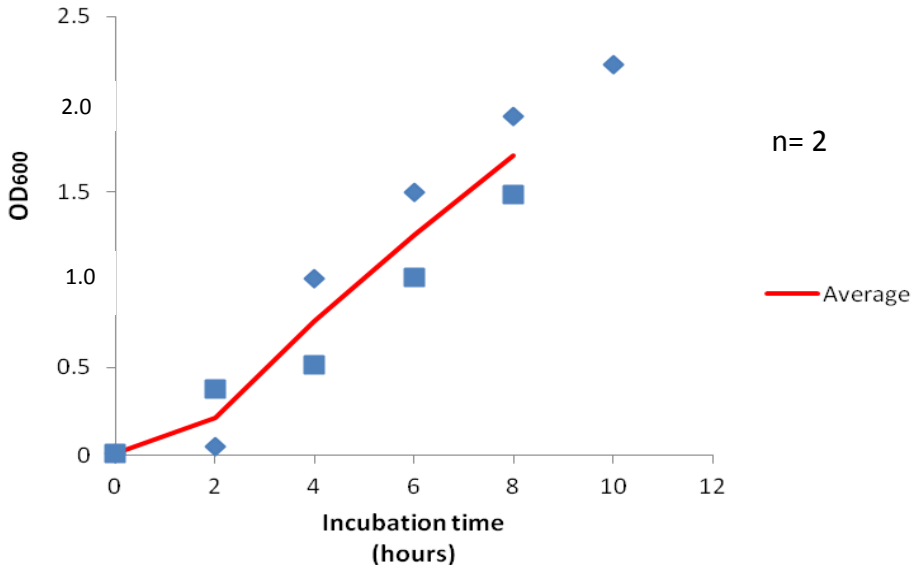


Figure 3.2. Growth curve of *H. pylori* isolates (CFU/mL vs time).

A



B

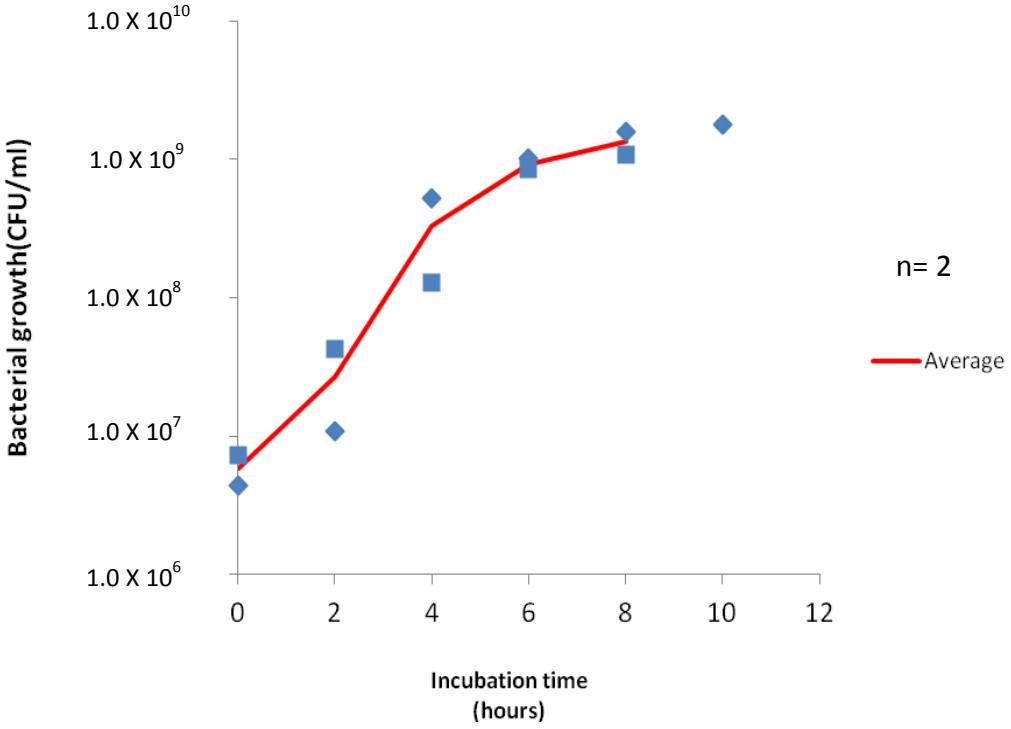


Figure 3.3 Growth curve of *E. coli* K12 ER2738

OD_{600} (A), and CFU/mL (B) of *E. coli* K12 ER2738 cultured from 0-10 h.

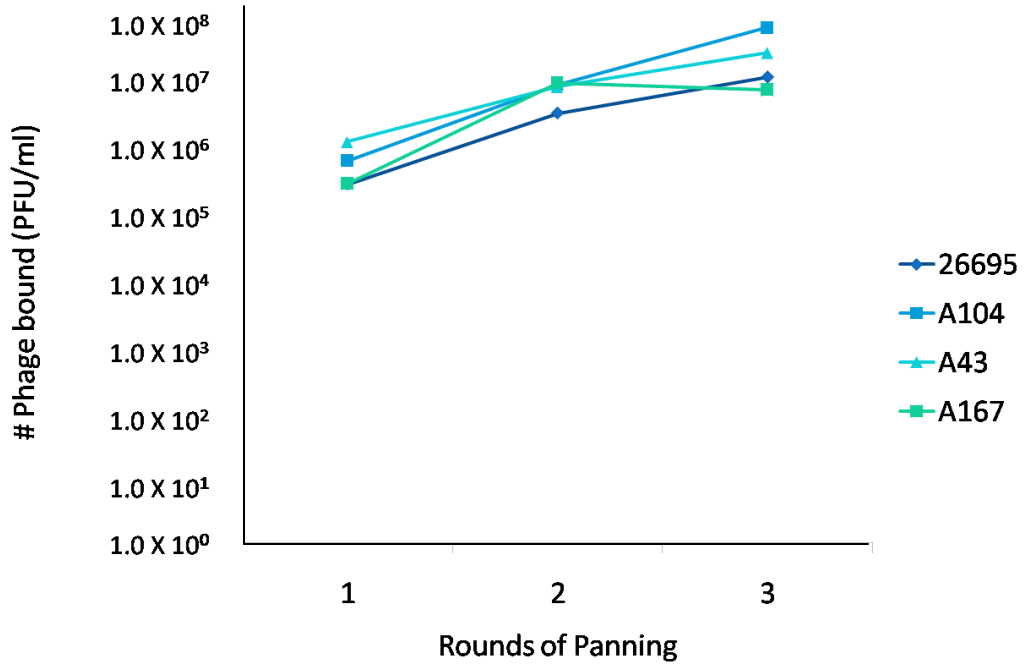


Figure 3.4 Number of phages bound to *H. pylori* isolates over three rounds of surface panning.

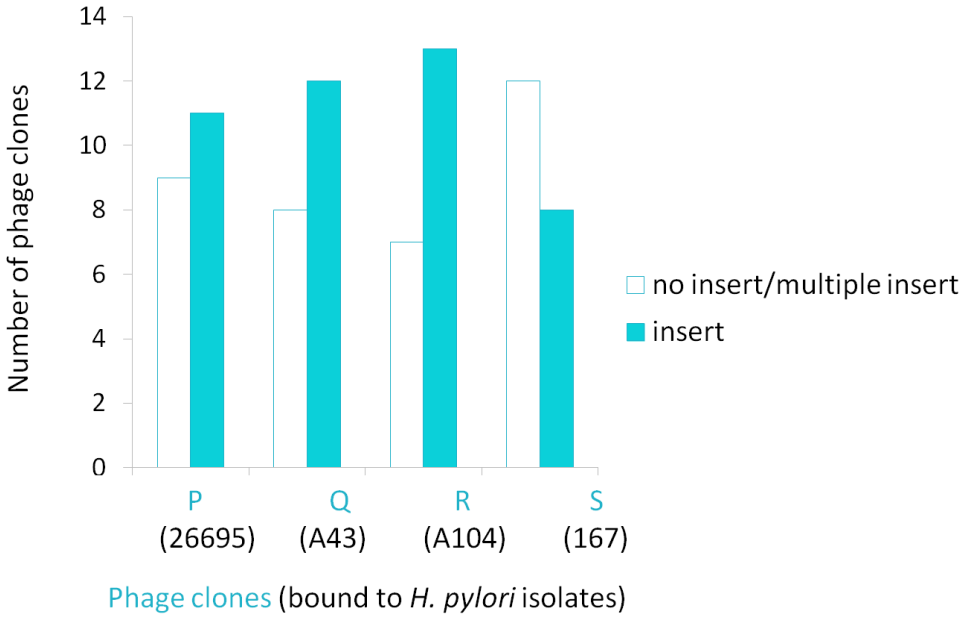


Figure 3.5 Number of phage clones bound to *H. pylori* isolates containing insert sequences

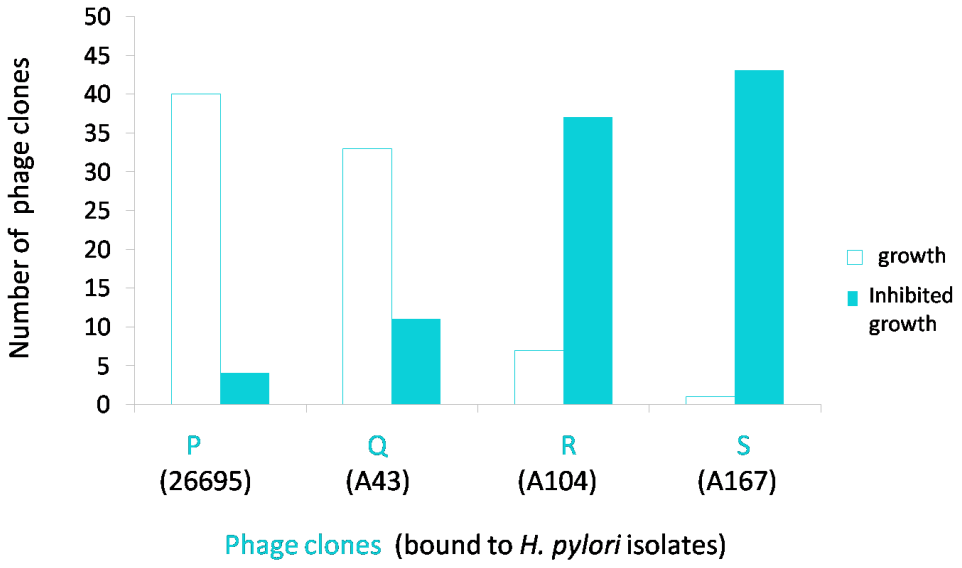


Figure 3.6 Number of bound phage clones that inhibited *H. pylori* growth

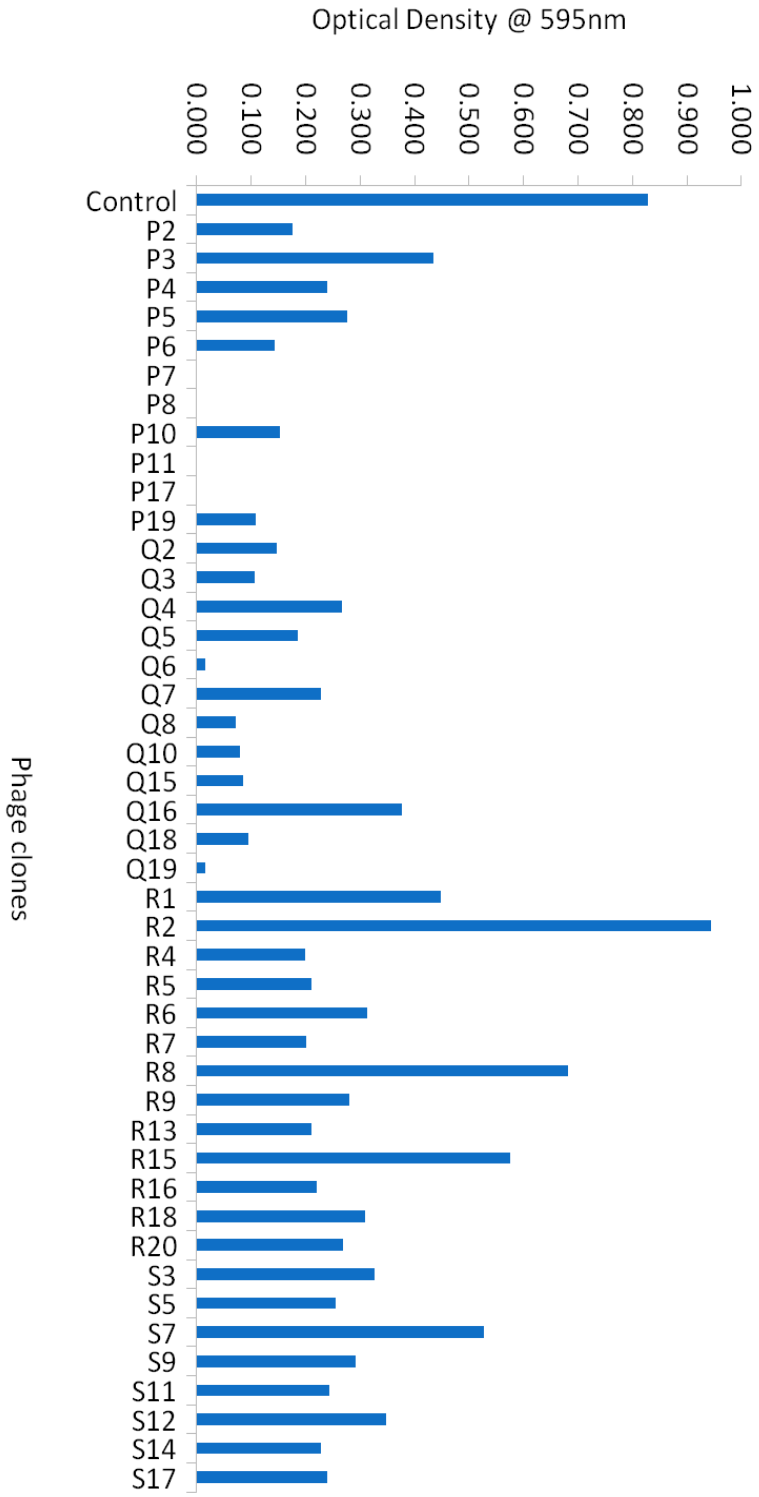


Figure 3.7 Antimicrobial activity of phage clones for *H. pylori* 26695
Decrease in optical density from control represents inhibition of growth.

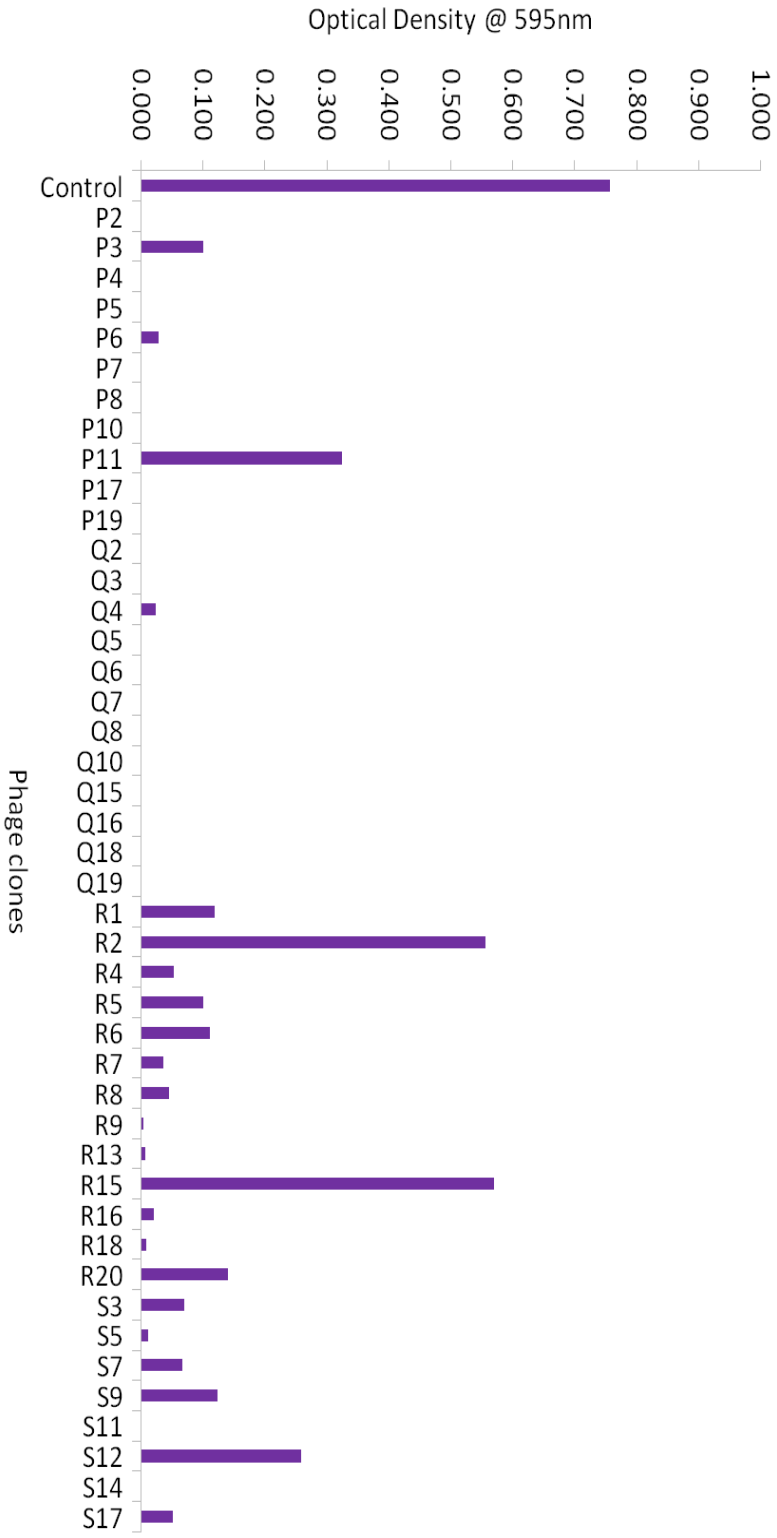


Figure 3.8. Antimicrobial activity of phage clones for *H. pylori* A43
Decrease in optical density from control represents inhibition of growth.

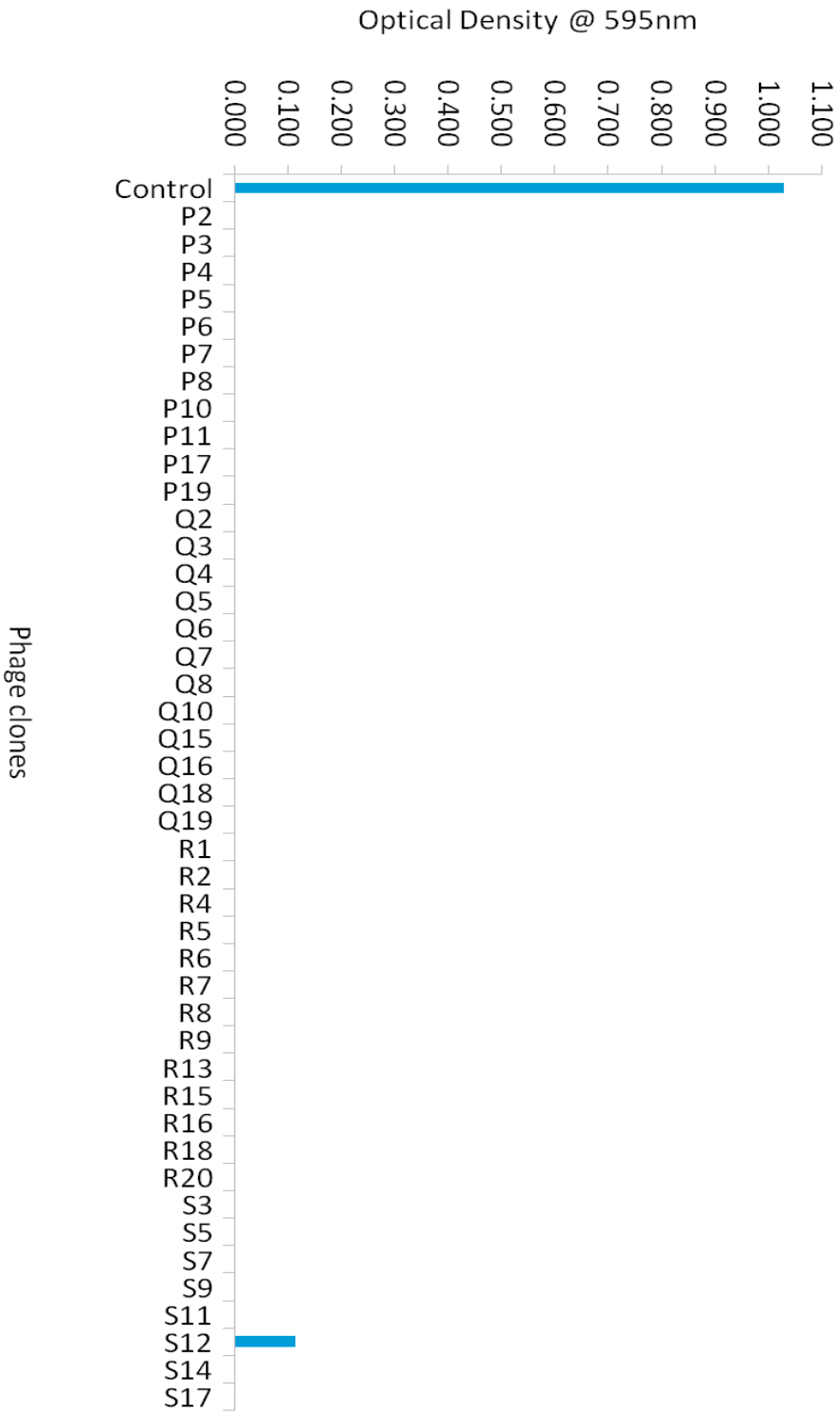


Figure 3.9 Antimicrobial activity of phage clones for *H. pylori* A104
Decrease in optical density from control represents inhibition of growth.

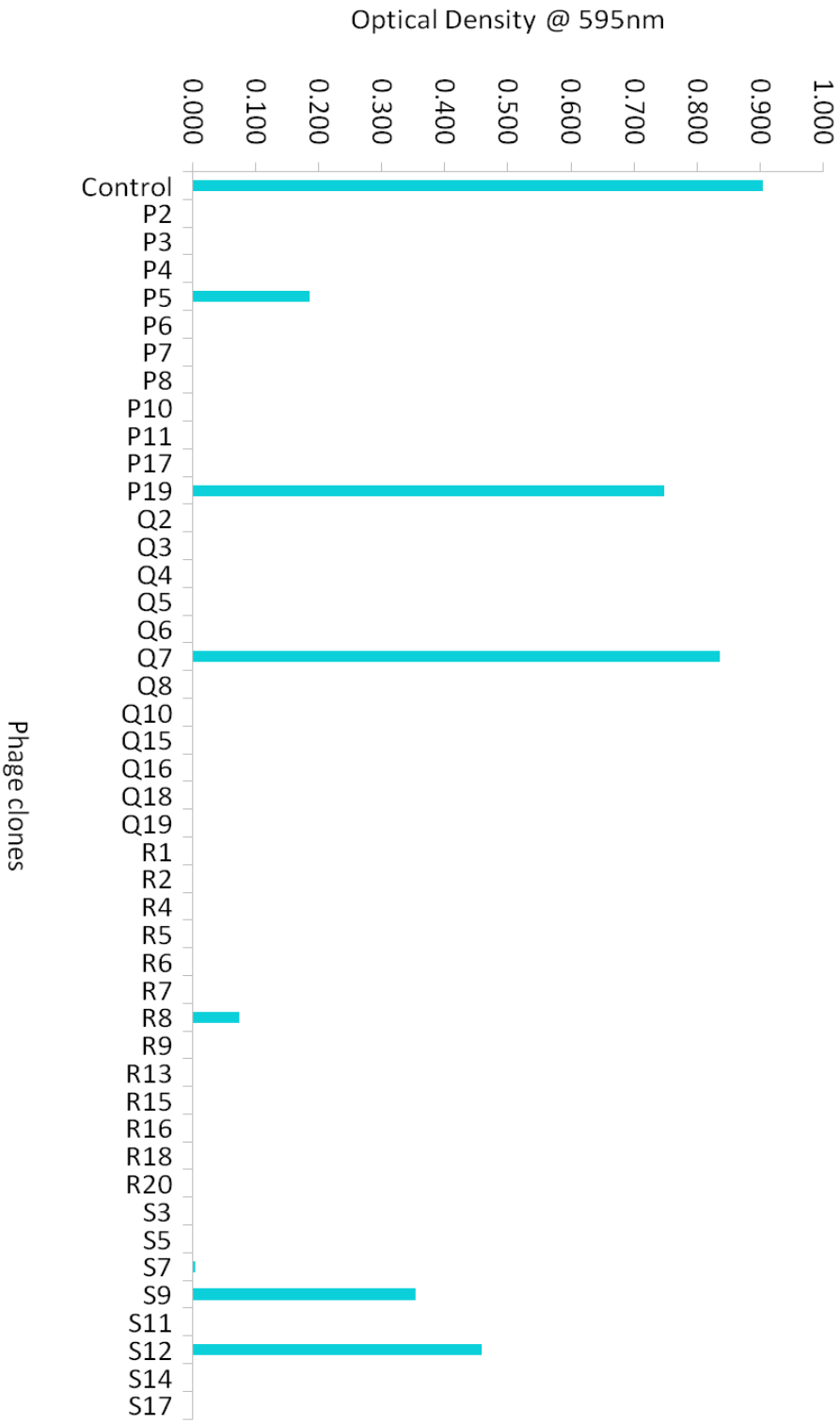


Figure 3.10 Antimicrobial activity of phage clones for *H. pylori* A167
Decrease in optical density from control represents inhibition of growth.

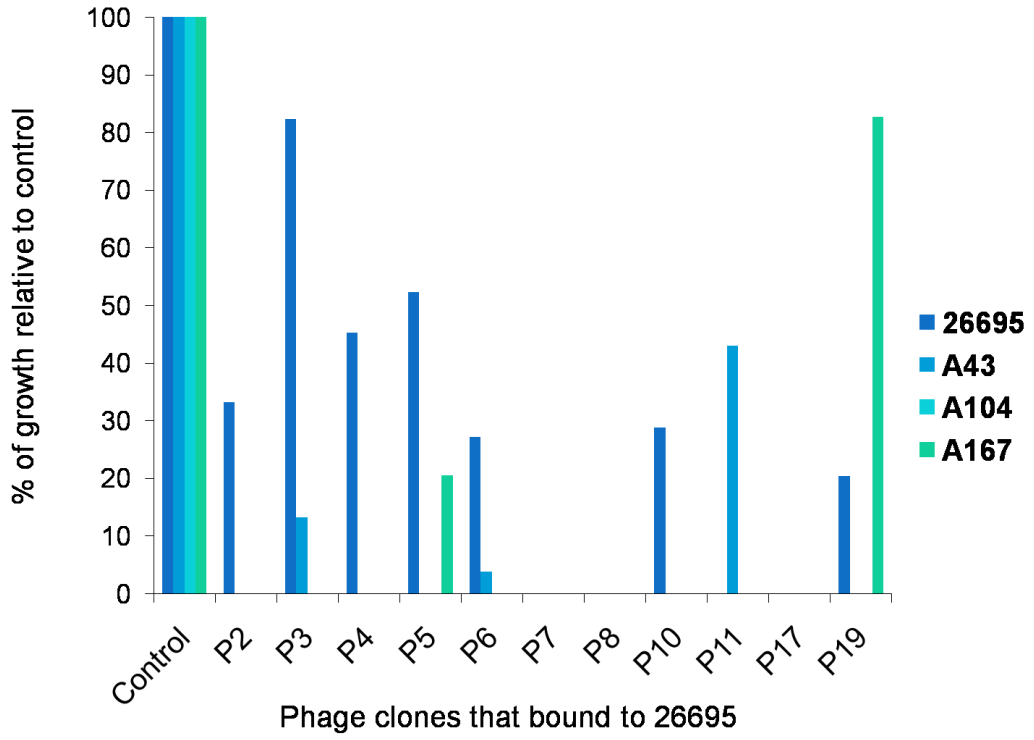


Figure 3.11 Antimicrobial activity by phage clones that bound to 26695

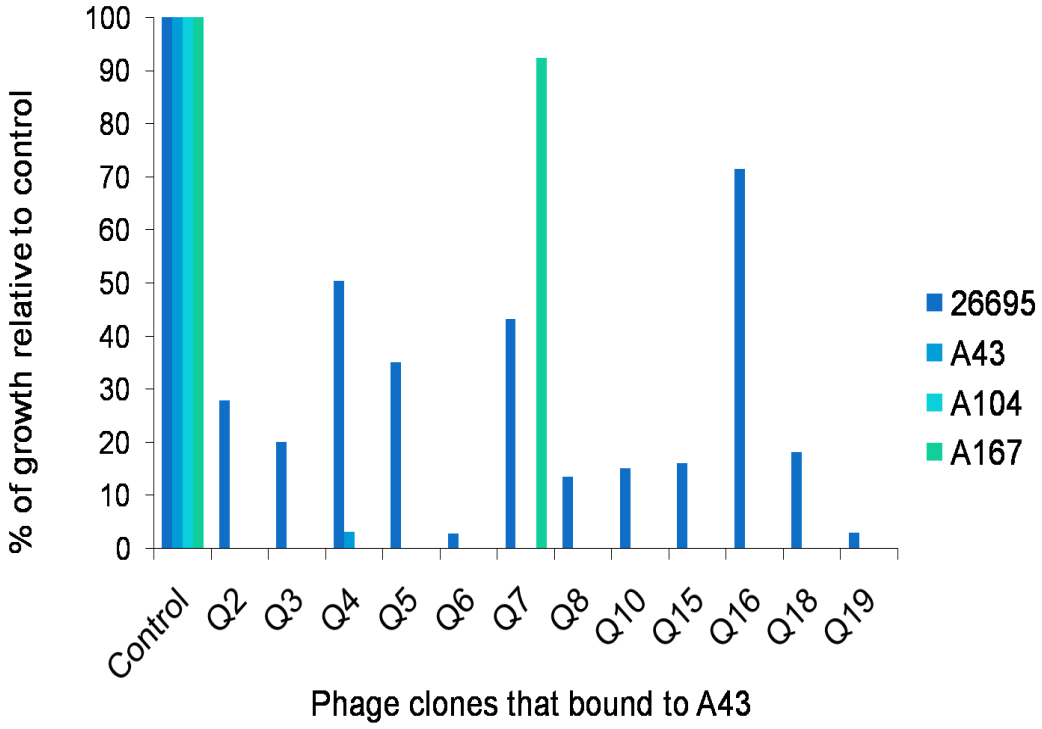


Figure 3.12 Antimicrobial activity by phage clones that bound to A43

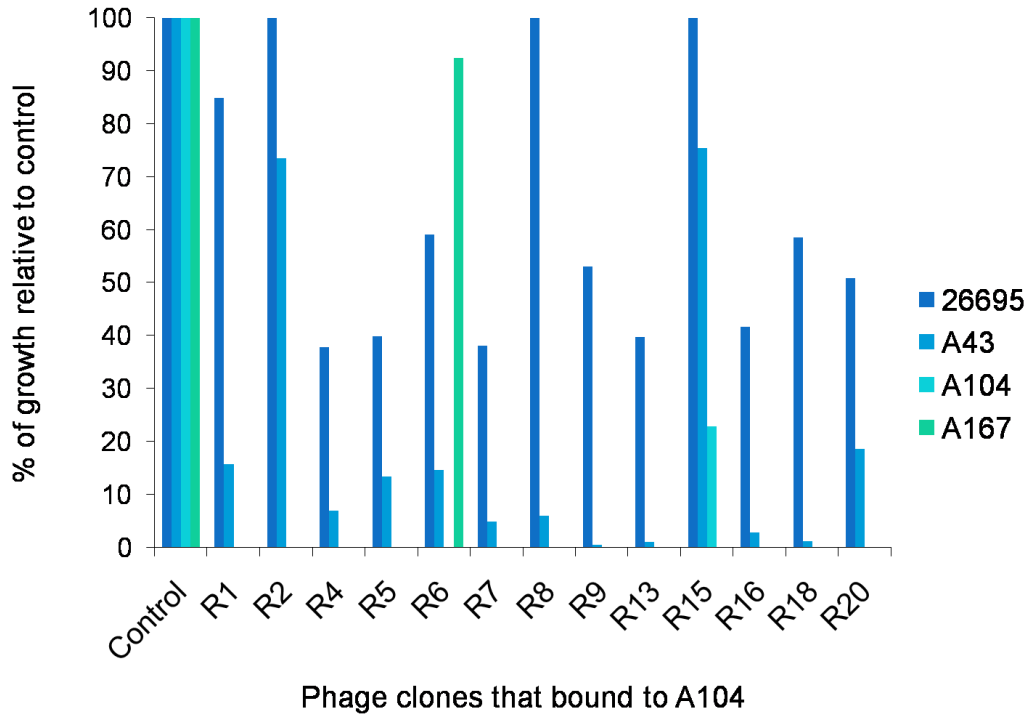


Figure 3.13 Antimicrobial activity by phage clones that bound to A104

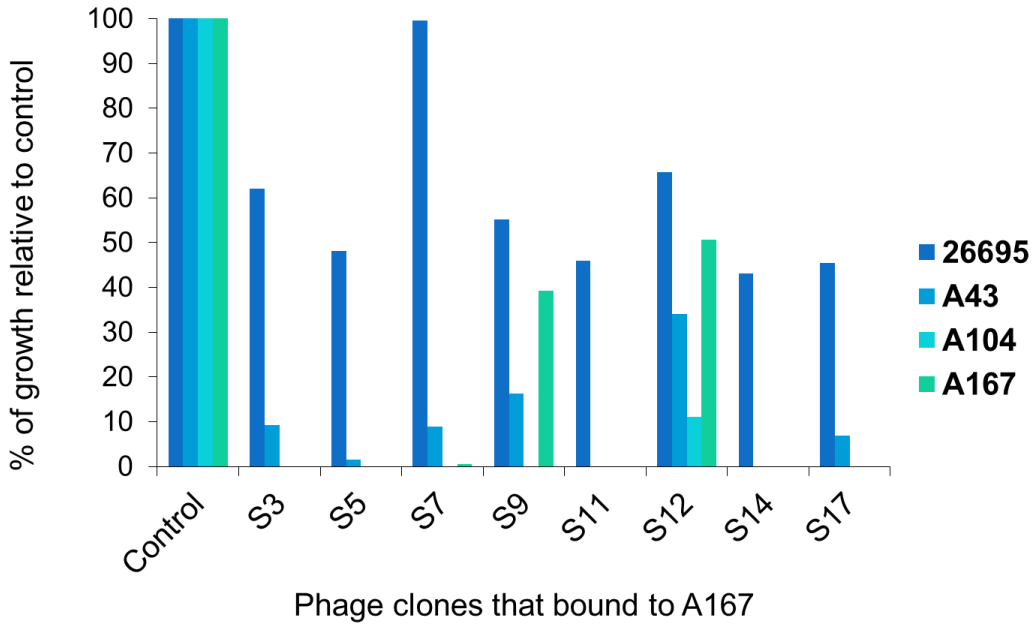


Figure 3.14. Antimicrobial activity by phage clones that bound to A167

Chapter 4: Discussion

Challenges in the treatment of *H. pylori* infections encourage researchers to develop new therapies. The challenges include a high prevalence of *H. pylori* infection among the northern communities of Canada, 20-25% of infected patients fail standard first line therapy, and finally an inability to specifically target *H. pylori.* This thesis is the first to report the use of a phage display to identify peptides that can bind to *H. pylori.* Other studies have focused on creating phage libraries displaying monoclonal antibodies to map the epitope of *H. pylori* antigens such as catalase, HopQ, or urease expressed on the outer surface.(87,101,109)

A phage display method using tetracycline-resistant filamentous phage was used to identify peptides that bind to the outer surface of *C. jejuni* (114), which is closely related to *H. pylori*. The Fd-tet filamentous phages are similar to the M13 phage used in this thesis. Shilpakala *et al.* used a phage-display library with random peptides that bound to the cell surface of *E. coli*.(119) The identified peptide (EC5) that bound to the cell surface of *E.coli* contained several arginine and lysine residues.(120,121) Antimicrobial assay of the identified peptide demonstrated that the peptide exhibited high antimicrobial activity against Gram-negative organisms such as *E. coli* and *P. aeruginosa.* Similar methods were used by Tsuyoshi Tanaka to identify six peptides, which exhibited antimicrobial activity against *Bacillus subtilis*, but not against *E. coli* and *Saccharomyces cerevisiae*. These identified peptides were highly cationic.(122)

Antimicrobial peptides bind to the outer membrane of the bacteria by interacting with the negatively charged phospholipids, leading to the permeability of the bacterial membrane. The peptide molecules outside the membrane enter the cytoplasm membrane, resulting in cell lysis.(121,122)

Bishop-Hurley *et al*. used a phage to *C. jejuni* ratio of 10:1 (10^{10} PFU/mL phage to 10^{9} CFU/mL bacteria) to successfully identify peptides that bind to *C. jejuni* and exhibit antimicrobial activity. Accordingly, similar experimental conditions were used for studies with *H. pylori* 26695. However, the Aklavik isolates (A43, A104, and A167) achieved a maximum concentration of only 10^{8} CFU/mL in broth culture. This study used a phage concentration of phage to 10^{09} PFU/mL to achieve the phage to bacteria ratio of 10:1 for the antimicrobial activity assays. The difference in growth in broth culture may be because *H. pylori* 26695 is a laboratory adapted strain that has been subcultured multiple times, while the Aklavik isolates were subcultured a maximum of three times from the original stock culture.

Phage particles can survive harsh conditions, such as low pH 2.2 and low temperature without losing their ability to infect bacteria.(123) Differences in temperature (RT and 37°C) and pH (7.5, 4.0) did not affect the number of phages bound to *H. pylori* 26695 (Table 3.2). The phage display library kit method uses RT and pH 7.5 as its standard conditions, whereas the surface panning for Aklavik *H. pylori* isolates were done at physiological conditions of the stomach, 37°C and

pH 4.0, to identify peptides that can specifically target the *H. pylori* in the stomach.

A problem with the phage display kit protocol was the use of the iodide buffer DNA extraction method. The extracted DNA represented amplified phage DNA but yielded poor sequencing results. To overcome this problem, a fragment of the pIII gene containing the insert sequence was amplified for each of the 80 phage clones using PCR. After the amplicons were purified (PCR reaction components removed), clear readable DNA sequences were obtained as observed by well- separated, individual peaks representing the nucleotide bases.

Among the 80 phage clones that were sequenced, 36 clones contained either no insert or multiple insert sequences (Figure 3.5). A known limitation of the phage display kit is that 5-10% of clones in the phage library are the parent vector (M13KE), which does not contain an insert. The number of insertless clones that bound to *H. pylori* in surface panning experiments varied for each isolate. It is possible that the insert-containing clones may have a lower infectivity than the wild type insertless parent vector. (116) Identification of insertless clones indicates the target bacteria have weak selection for the parent vector clones that represent non-selective background binding. Increasing the sample size of phage clones selected for sequencing would increase the probability of identifying more phage clones containing an insert sequence.

All phage clones containing an insert sequence, except S12, had some level of antimicrobial activity against *H. pylori* isolates (Figure 3.11-Figure 3.14). This may possibly be due to weak binding of S12 phage to the *H. pylori*. Interestingly, most of the phage clones did not inhibit the growth of *H. pylori* 26695 (Figure 3.14.), but the phage clones that bound to *H. pylori* 26695 did have antimicrobial activity against other *H. pylori* isolates.

Phage need long term incubation with bacteria to exhibit antimicrobial activity. In the surface panning experiments, phages were incubated with *H. pylori* for 45 min to achieve phage binding to the bacterial surface, whereas in the antimicrobial assay phage was incubated for 18 h to observe an inhibition in bacterial growth. *H. pylori* 26695 are a lab-adapted strain that no longer has the same growth characteristics as bacteria that are freshly isolated from gastric biopsies. Accordingly, it may not be necessary to demonstrate antimicrobial activity for this lab-adapted bacteria. The aim of this study is to inhibit the growth of Aklavik *H. pylori* isolates because of the association of Aklavik *H. pylori* with gastric diseases; two cases of gastric cancer have been reported since the Aklavik study began in 2008 (personal communication, Dr. Monika Keelan). The phage clones expressing the peptides P7, P8 and P17 inhibited the growth of all 4 *H. pylori* isolates. The phage clones expressing the peptides P2, 4, 10; Q2, 3, 5, 6, 8, 10, 15, 16, 18, 19; R9, 13, 18; and S11, S14 inhibited the growth of the Aklavik isolates A43, A104, A167 (Table 3.10). Although these phage clones inhibited the growth of *H. pylori* isolates, they may not be specific to *H. pylori*. These phage

clones must be tested for antimicrobial activity against other Gram negative and Gram positive bacteria that may be present in the human stomach.

4.1 Concluding Remarks

This thesis used phage display to identify 20 7-mer peptides that completely inhibited the growth of *H. pylori* associated with gastric diseases in the remote northern community of Aklavik, NWT. In this study, only three phage clones P7, P8 and P17 completely inhibited the growth of the three Aklavik *H. pylori* isolates and the lab-adapted isolate, 26695 (not related to the Aklavik *H. pylori*). The thesis is the first report of phage display as a useful tool to identify peptides that bind to the outer surface of *H. pylori* Aklavik isolates that can completely inhibit their growth. This is particularly important because *H. pylori* are difficult to treat in infected individuals that live in remote communities, like Aklavik, where the prevalence of infection and gastric disease is high. Accordingly, the findings of this thesis are the first step towards a new strategy to overcome the problem of treatment failure for *H. pylori* infections. This strategy could contribute to the design of novel therapeutics for the specific treatment of *H. pylori* Infections because it offers a targeted approach to treat *H. pylori* without affecting the other normally resident gastrointestinal microflora that play a role for human health.

4.2 Future Directions

Surface panning of the identified peptides with the other Gram negative and Gram positive bacteria that colonize the stomach — such as *lactobacillus, Bifidobacterium* and *E. coli* — will clarify whether the phage clones that bound to *H. pylori* 26695, A43, A104 and A167 are specific to *H. pylori*.

In vitro antimicrobial assays of identified phage clones that inhibited the growth of *H. pylori* 26695 and Aklavik isolates must be performed with other *H. pylori* isolates to determine if their growth can also be inhibited by the same 7-mer peptides. These peptides would be able to specifically target *H. pylori* isolates for drug delivery without affecting the other beneficial microflora in the gastrointestinal tract.

In order to assess the binding affinity of the selected peptides, a Phage ELISA Binding Assay with Direct Target Coating experiment has to be performed.(116) In this experiment, a microtiter plate is coated with the target, and then each individual identified amplified phage clone from the third round of panning are dispensed to the plate at various dilutions and incubated for 2 h. After the incubation, bound phages are then detected by using an anti-M13 antibody.

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