University of Alberta

Characterization of virulence factors expressed by Helicobacter pylori

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of *Master of Science*

in

Bacteriology

Department of *Medical Microbiology and Immunology*

Edmonton, Alberta

Spring 2006

Your file Votre référence
ISBN: 0-494-13889-0
Our file Notre référence
ISBN: 0-494-13889-0

Dedication

For P.G.S.

Abstract

The gastric pathogen *Helicobacter pylori* is associated with gastritis, duodenal ulcers and gastric adenocarcinoma. Several virulence factors have been identified, but are inconsistently correlated with clinical outcome. CagA, a virulence factor correlated with severe clinical manifestations in Caucasian populations, is translocated into host cells by a type four secretion system. To determine the translocation domain of CagA, adenylate cyclase was fused to portions of CagA. This showed that a central 371 bp region of *cagA* is not required for translocation. In addition, *H. pylori* isolates from symptomatic children were typed for virulence factors and Lewis antigen expression. A significantly greater frequency of pediatric isolates expressed both Lewis X and Lewis Y than asymptomatic adult isolates ($p<0.05$). Furthermore, a higher frequency of pediatric isolates were non-typable than symptomatic adult isolates ($p<0.001$). Expression of Lewis X was significantly correlated ($p<0.05$) with the virulence determinants CagA, and *iceA1*.

Acknowledgments

I would like to thank my supervisors Drs. Markus Stein and Diane Taylor for providing invaluable support and mentorship and allowing me to change directions midstream. I would also like to thank my committee members Drs. Michele Barry and Tracy Raivio for their helpful suggestions. This work was funded by an NSERC PGAS scholarship and laboratory funding was provided by Alberta Heritage and CIHR.

All of the members of the Stein and Taylor labs have been wonderful, but there are a few people I have to mention by name. I thoroughly enjoyed my scientific discussions with Marc Couturier (even though I don't think we have ever had the same opinion), and he and I have had fun navigating the often murky world of *H. pylori* research together. Sarah Keen taught me the laboratory skills I needed to know to be successful, and Dr. Monika Keelan provided insight into the world of clinical science. Finally, this project could not have been completed without the help of Dr. Joanne Simala-Grant. Thank you for helping me to assemble the pediatric strain collection; always being available to discuss results, ideas and methodologies; and editing my thesis.

I would also like to thank my support team outside of the university. My parents, Peter and Colleen Skipper, who always knew I could do it even when I wasn't quite as sure, and my sisters, Elysha and Vanya, who make sure I don't act like too much of a "grandma". I would also like to thank Rod and Laureen Wolansky for their support and lots of chocolate, which is known to help people keep writing. My partner, Mark Wolansky, has been wonderfully loving and supportive and I could not have finished without him. Finally, and this may be strange, but I would also like to thank my dog

Harley. He never lets me take myself too seriously and knows that the solution to any problem lies in a good long walk.

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List of Abbreviations

α(1,3) FucT	fucosyltransferase with α(1,3) activity
α(1,4) FucT	fucosyltransferase with α(1,4) activity
α(1,3/4) FucT	fucosyltransferase with α(1,3) activity and α(1,4) activity
μl	microliter
AC	adenylate cyclase
AGS	gastric adenocarcinoma cell line
ATP	adenosine triphosphate
BabA	blood group antigen binding adhesin
BB	Brucella broth
BG	blood group
bp	base pairs
c-Met	hepatocyte growth factor
Ca^{2+}	calcium ion
CaM	calmodulin
cAMP	cyclic adenosine monophosphate
CagA	cytotoxin associated gene A
*cag*PAI	cytotoxicity associated gene pathogenicity island
Csk	C-terminal Src kinase
CT	cholera toxin
ddH_2O	double distilled water
DC-SIGN	dendritic cell specifc ICAM-3 grabbing non-integrin

DNA	deoxyribonucleic acid
ECL	enhanced chemiluminesence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
ENA-78	epithelial cell-derived neutrophil activating protein 78
EPIYA	glutamate proline isoleucine tyrosine alanine
ERK	extracellular related kinase
FBS	fetal bovine serum
fmol	femptomolar
Fuc	fucose
fucT	fucosyltransferase gene
FucT	fucosyltransferase enzyme
g	gram
Gal	galactose
Glc	glucose
GlcNAc	*N*-acetylglucosamine
GlcNAc-MurNAc	*N*-acetylglucosamine-*N*-acetylmuramic acid
glmM	phosphoglucosamine mutase
GM-CSF	granulocyte-monocyte colony stimulating factor
HP-NAP	*H. pylori* neutrophil activiating protein
HRP	horse radish peroxidase
HS	horse serum
IDA	iron deficiency anemia

iceA	induced by contact with epithelium A
IL-	interleukin
JAM	junctional adhesion molecule
kB	kilobases
kDa	kilo-Dalton
l	liter
LB	Luria broth
Le^a	Lewis A
Le^b	Lewis B
Le^x	Lewis X
$Le^{x/y}$	Lewis X and Lewis Y
Le^y	Lewis Y
LPS	lipopolysaccharide
M	molar
MAb	monoclonal antibody
MALT	mucosa associated lymphoid tissue
MCS	multiple cloning site
MHC	major histocompatibility
mins	minutes
ml	milliliter
MOI	multiplicity of infection
NaCl	sodium chloride
NF-κB	nuclear factor kappa B

nm	nanometer
NtHP	non-typable *H. pylori*
OD	optical density
ORF	open reading frame
p	probability
PAI	pathogenicity island
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMN	polymorphonuclear leukocyte
NHL	Non-Hodgkins lymphoma
NTHp	nontypable *Helicobacter pylori*
NSAIDS	non-steroidal anti-inflammatory drugs
RAP	recurrent abdominal pain
rpm	revolutions per minute
RTK	receptor tyrosine kinase
RUT	rapid urease test
SabA	sialic acid binding adhesin
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLe^x	sialyl-Lewis X
T3SS	type three secretion system
T4SS	type four secretion system
TCP	toxin-coregulated pili

TNF-α	tumor necrosis factor alpha
TSB	trypticase soy broth
ure	urease
V	volts
VacA	vacuolating cytotoxin A

Chapter One

Introduction

1.1 General Introduction

Helicobacter pylori was first isolated in 1983 by Drs. Marshall and Warren (Warren and Marshall 1983). It is a gram negative, microaerophilic, flagellated curved rod shaped organism (Owen 1995, Lee and O'Rourke 1993). It belongs to the family *Helicobacteraceae*, order *Campylobacterales*, and is included in the ε- subdivision of Proteobacteria. It is believed to be the second most common bacterial infection in the world (Telford *et al.* 1997), and affects greater than 50% of the world's population (Ge and Taylor 1999, Gomez-Duarte *et al.* 1999). In the developing world infection rates are higher than in developed countries (Rothenbacher and Brenner 2003). Although *H. pylori* is a model system for chronic bacterial infections (Monack *et al.* 2004) and is associated with severe clinical manifestations, little is known about the infection process including the mode of transmission. Nonetheless, several recent advances have furthered our understanding of pathogenicity mechanisms.

The majority of individuals infected by *H. pylori* are asymptomatic carriers, but approximately 10% progress to more serious pathologies (Dunn *et al.* 1997). Clinical manifestations include chronic gastritis (Kuipers 1997), recurrent duodenal ulcers (Blaser 1992, Buck 1990, Marshall *et al.* 1988), gastric ulcers (Graham *et al.* 1992), peptic ulcer disease (PUD) (Kuipers 1997, Cover and Blaser 1996), gastric adenocarcinoma (Kato *et al.* 2000, Dubois 1995, Parsonnet *et al.* 1991) and mucosa-associated lymphoid tissue (MALT) lymphoma (Forman *et al.* 1993, Parsonnet *et al.* 1991, Wotherspoon *et al.* 1991). Gastric adenocarcinoma is one of the most common human cancers (Dubois 1995), and in 1994 *H. pylori* was the first bacterium to be designated as a class I carcinogen by the World Health Organization (WHO) (IARC 1994, Logan 1994).

The natural reservoir and mode of transmission are unknown, but has been hypothesized to be fecal-oral or oral-oral (Chelimsky *et al.* 2000). Infection occurs in childhood, but severe clinical manifestations are seen primarily in adults (Passaro *et al.* 2002). Inside the stomach, the pathogen uses multiple flagella to move towards the mucous layer of the gastric epithelium. The mucosa provides protection from low pH and clearance by peristalsis allowing colonization of the mucous layer and the luminal surface of the gastric epithelium in the antrum and the corpus (Figure 1.1) (Kirschner and Blaser 1995, Lee 1993). *H. pylori* can also persist in intracellular vacuoles (Amieva *et al.* 2002). Members of the genus *Helicobacter* are the only bacterial species that have been repeatedly cultured from the human stomach over an extended time period (McGowan *et al.* 1998).

H. pylori elicit a strong local and systemic immune response, but the pathogen cannot be cleared by the adult immune system (Rathbone *et al.* 1986, Wyatt *et al.* 1986). Production of superoxide dismutase and catalase also protect the bacteria from the immune response (Wirth *et al.* 1996). Additionally, host phagocytes, antibodies and complement are less effective in the gastric lumen (Wirth *et al.* 1996). Individuals are occasionally colonized by multiple variants (Blaser and Atherton 2004) and clinical strains have highly polymorphic genomes (Salama *et al.* 2000).

The acute phase of infection occurs post colonization. This phase includes hypochlorhydia, bacterial proliferation and gastric inflammation. This phase is followed by the asymptomatic chronic phase characterized by a return to normal gastric pH and a low level inflammatory response resulting in superficial gastritis. An individual can be infected for decades with no clinical symptoms. It is not known what initiates the shift

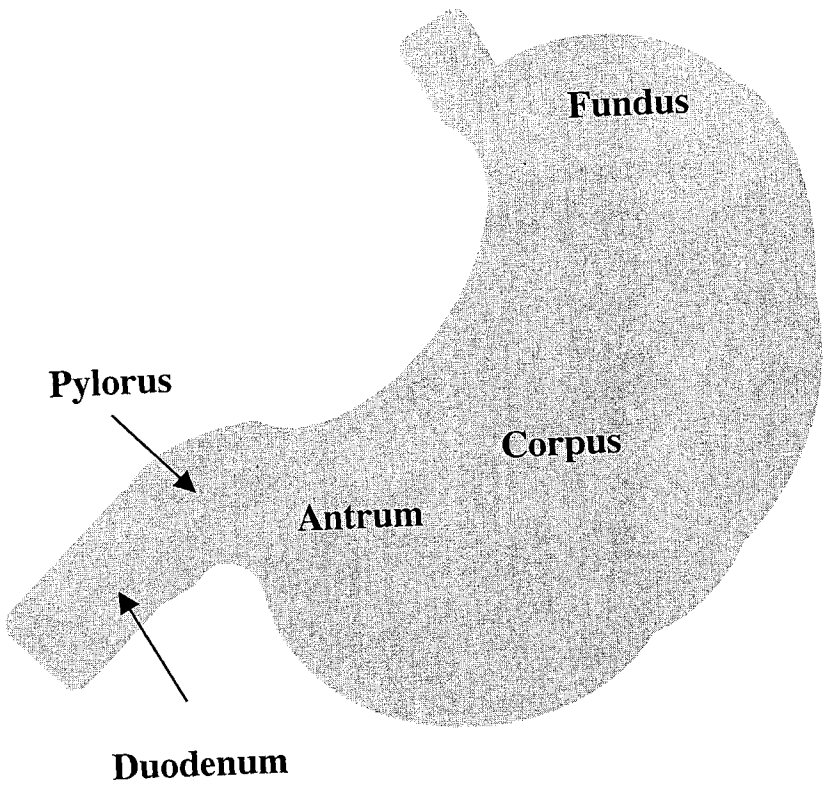


Figure 1.1- Anatomy of the human stomach. *H. pylori* colonizes the luminal surface of the gastric epithelium in the antrum and the corpus.

from an asymptomatic to a symptomatic state, but the effects are due to chronic inflammation, cell proliferation and tissue damage (reviewed by Bourzac and Guillemin 2005). Severe, symptomatic gastritis is correlated with epithelial cell proliferation and apoptosis (Scotiniotis *et al.* 2000).

The most widely used method of detection is the ^{13}C urea breath test (UBT) (Kindermann *et al.* 2000, Sheu *et al.* 2000). Combinations of histology, serology and endoscopy are also used. *H. pylori* is usually eradicated by a triple therapy including a proton pump inhibitor and two antibiotics, typically clarithromycin-amoxicillin, amoxicillin-metronidazole, tetracycline-metronidazole or amoxicillin-rifabutin (Megraud and Lam 2003, Bock *et al.* 2000, Realdi *et al.* 1999). However, the occurrence of refractory infections is increasing. Quadruple therapy including a proton pump inhibitor, two antibiotics and bismuth salt is now recommended for refractory cases (Malfertheiner *et al.* 2002), but antibiotic resistance to metronidazole, clarithromycin and tetracycline continues to increase (reviewed by Megraud and Lam 2003, Trieber and Taylor 2002, Canton *et al.* 2001).

Sequencing and DNA fingerprinting have revealed that *H. pylori* is one of the most genetically diverse bacterial species (Akopyanz *et al.* 1992, Tee *et al.* 1992). The genomes of two strains, J99 (1 643 831 bp, 1495 ORFs) and 26695 (1 667 867 bp, 1552 ORFs), have been sequenced (Tomb,Alm *et al.* 1999). Orthologues from J99 and 26695 have from 21% to 91% identity (Eppinger *et al.* 2004). This information has advanced research and provided a basis for comparison between strains.

Research has been hampered by the lack of a good animal model. Most experiments have been done using tissue culture, which does not reflect the complexity of

the plethora of interactions *in vivo*. Utilization of a mouse model does not reflect the colonization and infection process in humans. The most important adhesin, BabA, cannot bind to the mouse epithelium and the most important virulence factor, CagA, is not tolerated in the mouse background (Guruge *et al.* 1998). Additionally, most of the severe pathologies associated with *H. pylori* are not manifested in an infected mouse. In an outbread Mongolian gerbil model, animals develop intestinal metaplasia and gastric adenocarcinoma (Honda *et al.* 1998, Watanabe *et al.* 1998). The drawback of this model is that little Mongolian gerbil DNA sequence is available hampering the utilization of new technology including microarrays.

To date, *H. pylori* research has focused on pathogenicity, but interest in this organism is increasing in other disciplines. *H. pylori* and humans have a long history of association. Falush and colleagues used this bacterium to track historical human migrations (Falush *et al.* 2003). Interestingly, it was recently hypothesized that *H. pylori* was originally a commensal organism and has recently evolved pathogenic function (Blaser and Atherton 2004). Research in this area could be important to the study of evolutionary ecology.

1.2 Important Virulence Factors

Progress has been made in identifying factors involved in the pathogenesis of *H. pylori*. Two major virulence factors, vacuolating cytotoxin, VacA (Papini *et al.* 1996), and cytotoxin associated gene A, CagA (Censini *et al.* 2001, Covacci *et al.* 1993), have been identified. VacA interferes with endosome trafficking (Papini *et al* 1997, Papini *et al* 1996) and vacuolar ATPase activity inhibiting phagosome/lysosome fusion (Zheng and Jones 2003). CagA is translocated into host cells by a type four secretion system (T4SS)

(Odenbreit *et al.* 2001, Asahi *et al.* 2000, Backert *et al.* 2000, Stein *et al.* 2000, Segal *et al.* 1999). The *H. pylori* T4SS belongs to a subset of contact dependent T4SS that share a functional convergence with type III secretion systems (T3SS) (Cascales and Christie 2003). T3SSs and T4SSs are both responsible for exporting macromolecules across membranes (Cascales and Christie 2003). The T4SS and CagA are chromosomally encoded by the *cag* pathogenicity island (*cag* PAI). Depending on the strain being studied, the *cag* PAI contains anywhere between 27 and 31 genes (Fischer *et al.* 2001, Stein *et al.* 2000). Inside host cells CagA causes cytoskeletal rearrangements resulting in an elongated cell phenotype (Segal *et al.* 1999) and disrupts epithelial tight junctions (Amieva *et al* 2003).

1.2.1 *cag* Pathogenicity Island (PAI)

The *cag* PAI is 37 kB long and encodes from 27 to 31 genes (Akopyants *et al.* 1998) (Figure 1.2). Based on GC content, it is believed to have been acquired by horizontal transfer and is inserted within the glutamate racemase gene (Censini *et al.* 1996). In some strains, including G27, the PAI is split into two segments, *cagI* and *cagII*, by insertion sequence (IS) 605 (Akopyants *et al.* 1998, Censini *et al.* 1996). In a minority of strains the two segments are interrupted by chromosomal DNA sequences.

A functional *cag* PAI was suggested to mediate bacterial attachment and is essential for induction of epithelial cell-derived neutrophil activating protein 78 (ENA-78) (Rieder *et al.* 2001), granulocyte-monocyte colony stimulating factor (GM-CSF), tumor necrosis factor alpha (TNF-α) (Foryst-Ludwig and Naumann 2000), interleukin 8 (IL-8) (Keates *et al.* 1999, Keates *et al.* 1997), and translocation of cytotoxin associated

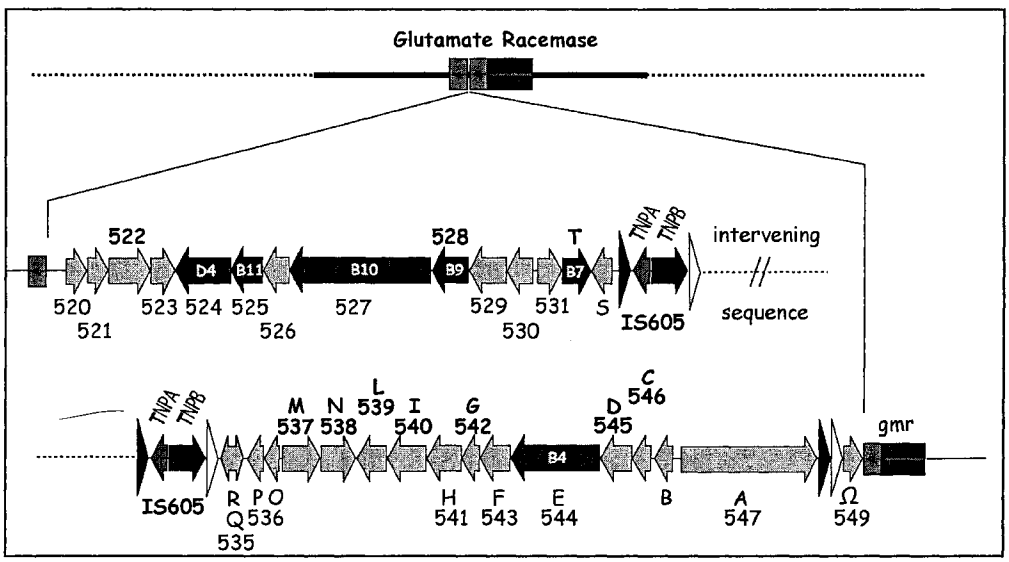


Figure 1.2 - The G27 *cag*PAI. Proteins with similarity to the VirB/D4 system of *A. tumefaciens* are indicated by the gene name in white inside a dark arrow. HP gene numbers (Tomb *et al.* 1997) are indicated by the numbers above or below the gene and named Cag proteins are indicated by a letter above or below the gene (eg. H/541 is CagH or HP541). (Figure courtesy of M. Stein)

gene A (CagA) into host cells (Odenbreit *et al.* 2001, Asahi *et al.* 2000, Beckert *et al* 2000, Stein *et al.* 2000, Segal *et al.* 1999).

The T4SS responsible for CagA translocation is encoded by the *cag* PAI. Several proteins of the T4SS show similarity to components of the *vir* T4SS of *Agrobacterium tumefaciens* (Stein *et al.* 2000). Gene content and arrangement is well conserved among strains (Akopyants *et al.* 1998), but some genes including *cagA* are polymorphic.

1.2.1.1 Type Four Secretion Systems (T4SS)

T4SS are the most versatile class of secretion systems (Cascales and Christie 2003). Whereas T3SS are evolutionarily related to the flagellar gene cluster and deliver proteins intracellularly (Macnab 1999), T4SS are derived from conjugation systems (Cascales and Christie 2003). T4SS can act as conjugation systems, DNA uptake and release systems or effector translocation systems (Christie and Vogel 2000, Winans *et al.* 1996). T4SSs can target bacteria, plants, fungi and animals and are employed by pathogenic and nonpathogenic bacteria (reviewed by Christie and Vogel 2000).

T4SSs were first identified as conjugation systems mediating DNA transfer in gram-negative and gram-positive bacteria (Cascales and Christie 2003). Later, the ability of T4SS to take up and release DNA independent of target cells was discovered. The *H. pylori* ComB system (Hofreuter *et al.* 2001) is an example of a DNA uptake system and *Neisseria gonorrhoeae* has a PAI that encodes a T4SS for DNA release (Dillard and Seifert 2001, Hamilton *et al.* 2001). Finally, T4SSs are effector translocation systems capable of translocating proteins, nucleo-protein complexes and possibly carbohydrates into host cells (Cascales and Christie 2003).

Although structural variations exist, T4SSs are composed of membrane associated and trans-membrane proteins that are believed to form a basal body spanning the periplasmic space and a pilus that may protrude into the extracellular milieu. Two models exist to explain how effector translocation systems could work (Cascales and Christie 2003). In the channel model the pilus acts as a tunnel from the bacterium into the host cell. The piston models suggests translocation is a more active process and the T4SS acts like a piston in a car and pushes substrates into host cells.

The *vir* system of *A. tumefaciens*, a plant pathogen and the causative agent of crown gall disease, is the best studied T4SS. This system is comprised of twelve essential components, VirB1-VirB11 and VirD4, located on the Ti plasmid (Zupan *et al.* 2000). The *cag* T4SS has fourteen essential genes some of which have similarity to *vir* genes (Figure 1.3). These include three inner membrane putative ATPases VirB4 (CagE/HP544), VirB11 (HP525) and VirD4 (HP524) (Bourzac and Guillemin 2005, Lai and Kado 2000). Although the major *A. tumefaciens* pilus subunit, VirB2, has no orthologues in *H. pylori*, VirB7 (HP523) and VirB9 (HP528) have been found to localize to the base of the *H. pylori* pilus (Tanaka *et al.* 2003). Rohde *et al.* also observed VirB7 in a ring structure associated with the pilus and VirB10 (CagY/HP527) in the core of the pilus (2003). Three other putative homologs: HP530 and VirB8 (Buhrdorf *et al.* 2003), HP529 and VirB6 (Bourzac and Guillemin 2005) and HP523 and VirB1 (Rohde *et al.* 2003) have been identified.

Although some components are conserved, the *H. pylori cag* T4SS is unique in several ways. The gene order is not conserved among T4SSs and in *H. pylori* some orthologues such as the *virB10* encode novel functions (reviewed by Bourzac and

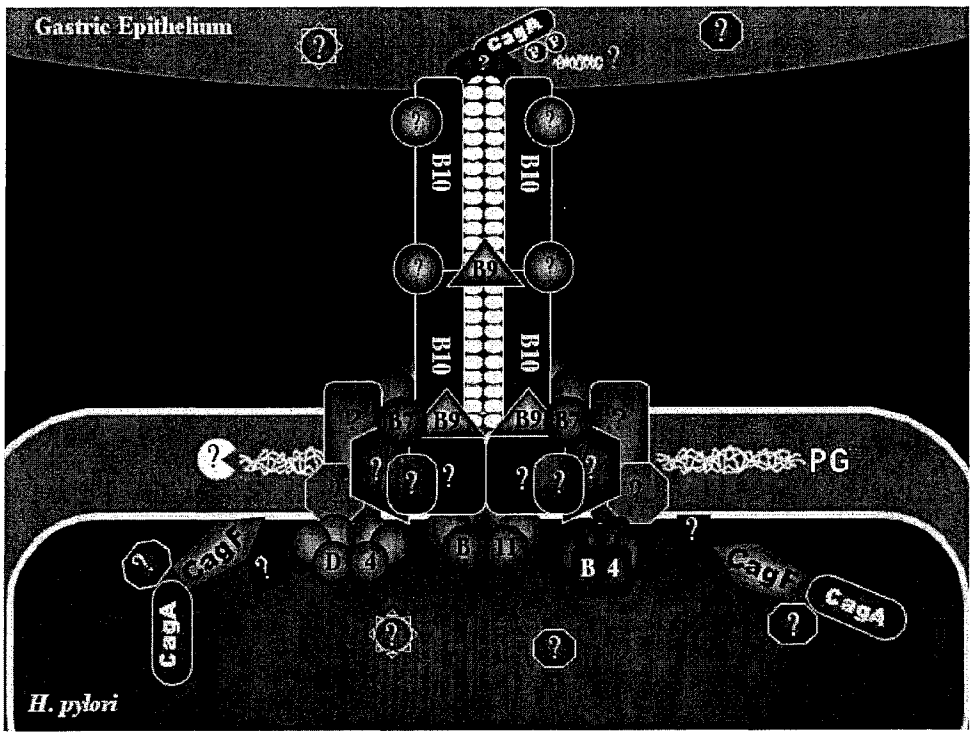


Figure 1.3 - Model of the type four secretion system of *H. pylori* including putative orthologues of the Vir system of *A. tumefaciens*. (Figure courtesy of M. Couturier)

Guillemin 2005). The *H. pylori* pilus is also much larger, (Rohde *et al.* 2003), than the *A. tumefaciens* T pilus, 40 versus 8-16 nm in diameter, respectively (Cascales and Christie 2003). Additionally, the T pilus is primarily composed of a single pilin subunit that is absent from *H. pylori.* The structure of the *cag* T4SS is poorly understood compared to the *vir* T4SS.

Not all of the proteins encoded on the *cag* PAI are required for CagA translocation or IL-8 induction, only eighteen and fourteen of twenty-seven, respectively (Fischer *et al.* 2001). The *A. tumefaciens* homologs VirB4, VirD4 and VirB7-11 are essential for CagA translocation, but so are several proteins with no known orthologues (CagF, CagH, CagI and CagM) (Fischer *et al.* 2001). IL-8 induction requires the same proteins as CagA translocation with the exception of VirD4 (HP524), HP543, HP540 and HP542 (Fischer *et al.* 2001). HP520, HP521, HP526, HP534-HP536, HP538 and HP545 are not required for IL-8 induction or translocation of CagA into host cells (Fischer *et al.* 2001).

Several attempts have been made to find the *H. pylori* effector protein responsible for IL-8 induction. In 1998, Sharma *et al.* determined that a functional T4SS leads to activation of nuclear factor kappa B (NF-κB), which triggers IL-8 induction. Subsequently, no progress was made until a family of intracytoplasmic pathogen recognition molecules with homology to proteins involved in the hypersensitivity response in plants was identified (Chamaillard *et al.* 2003, Giardin *et al.* 2003). A member of this family expressed in epithelial cells, Nod-1, is activated by peptidoglycan from gram-negative bacteria (Giardin *et al.* 2003). This discovery prompted Viala *et al.* (2004) to test if Nod-1 recognized peptidoglycan from *H. pylori.* They demonstrated that

delivery of *N*-acetylglucosamine-*N*-acetylmuramic acid (GlcNAc-MurNAc) to host cells, a component of *H. pylori* peptidoglycan, was dependent on T4SS, activated Nod-1 and appeared to be responsible for IL-8 induction.

1.2.1.2 Cytotoxin Associated Gene A (CagA)

CagA (HP547) is a 128-144 kDa protein encoded within the *cagI* region of the *cag* PAI (Covacci *et al.* 1993). CagA is translocated by the T4SS into host cells where it is tyrosine phosphorylated (Odenbreit *et al.* 2001, Asahi *et al.* 2000, Backert *et al.* 2000, Stein *et al.* 2000, Segal *et al.* 1999). It subsequently interferes with a number of host cellular processes leading to actin polymerization, cytoskeletal rearrangements (Stein *et al.* 2002), aberrant apical-junctional protein complexes (Amieva *et al.* 2003), increased cellular motility (Higashi *et al.* 2004, Churin *et al.* 2003, Segal *et al.* 1999), cellular proliferation and an elongated cell morphology known as the "hummingbird phenotype" (Tsutsumi *et al.* 2003, Stein *et al.* 2002, Segal *et al.* 1999). CagA expression is upregulated by low environmental pH (Allan *et al.* 2001, Karita *et al.* 1996).

The precise mechanism and signal region required for CagA translocation are unknown. After translocation CagA is recruited to the inner surface of the host membrane at the base of the pedestal at the site of bacterial attachment (Odenbreit *et al.* 2001, Stein *et al.* 2000, Segal *et al.* 1996). CagA is then phosphorylated on tyrosine residues by host Src kinases (Selbach *et al.* 2002a, Stein *et al.* 2002). Phosphorylated tyrosines are contained in a five amino acid motif consisting of glutamate, proline, isoleucine, tyrosine and alanine (EPIYA) (Stein *et al.* 2002, Backert *et al.* 2001). The EPIYA motifs are located in the C terminus of CagA, in a section of 102 bp repeats (Covacci *et al.* 1993). Size variation of CagA, 128-144 kDa, is due to the number of 102 bp repeats (Covacci *et*

al. 1993). Strains expressing CagA with a molecular weight of 128 kDa contain no repeats and are translocated, but not phosphorylated (Stein *et al.* 2002). CagA that is 144 kDa contains six 102 bp repeats and may be phosphorylated six times. The number of EPIYA motifs correlates with the amount of tyrosine phosphorylation that occurs on CagA and the ability of CagA to activate tyrosine phosphatase SHP-2 (Higashi *et al.* 2002). Cellular elongation and motility are also dependent on the SHP-2 CagA interaction activating extracellular signal-related kinases (ERKs) (Higashi *et al.* 2004). CagA also associates with receptor tyrosine kinases (RTKs) including hepatocyte growth factor receptor (c-Met) and the phospholipase effector PLCγ, promoting cell motility (Churin *et al.* 2003) (Figure 1.4).

Phosphorylated CagA also directly inhibits the kinase responsible for CagA phosphorylation, c-Src (Selbach *et al.* 2003) and activates C-terminal Src kinase (Csk) (Tsutsumi *et al.* 2003). Csk has been documented to inactivate Src kinases that tyrosine phosphorylate cortactin and ezrin (Selbach *et al.* 2004, Selbach *et al.* 2003). Tyrosine dephosphorylation of cortactin results in increased actin crosslinking (Bourzac and Guillemin 2005). The main function of ezrin is to link F-actin to membrane proteins, but it is not known if tyrosine dephosphorylation interferes with this capability (Bretscher *et al.* 2002). The CagA mediated effects on ERK, c-Met and cortactin are responsible for inducing cell elongation and cell scattering.

CagA disrupts epithelial cell tight junctions independent of tyrosine phosphorylation (Amieva *et al.* 2003). Amieva *et al.* found that CagA associates with the junctional adhesion molecule (JAM) and the scaffolding protein ZO-1 (2003). This alters the apical-junctional complex and leads to disruption of the epithelial cell barrier.

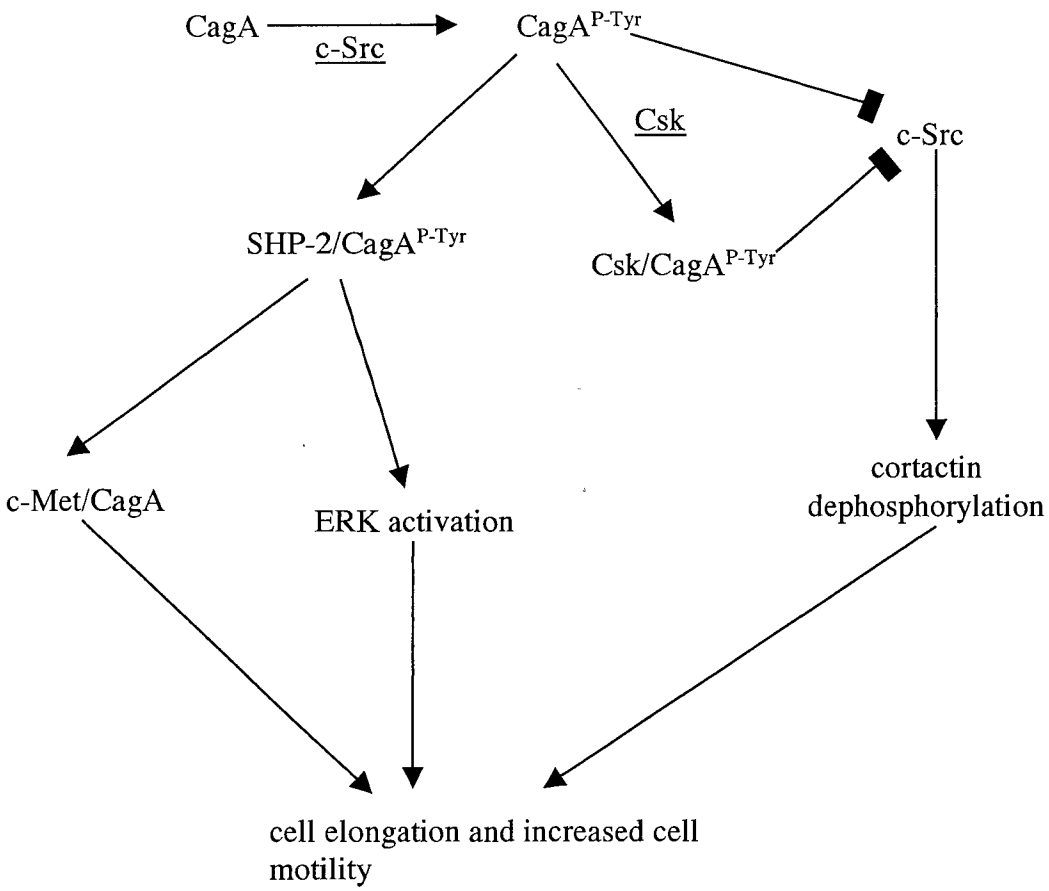


Figure 1.4- Effects on host epithelial cells mediated by tyrosine phosphorylated CagA. Enzymes mediating reactions are underlined and proteins that interact are indicated with backslashes.

1.2.2 Vacuolating Cytotoxin (VacA)

VacA is a 94 kDa protein derived from the 140kDa precursor (Telford *et al.* 1994). VacA has been primarily regarded as an epithelial cell layer cytotoxin, which also has immunomodulatory activities (Gebert *et al.* 2004). In the presence of VacA, endosomal compartments in gastric epithelial cells enlarge (Molinari *et al.* 1997) and cellular trafficking is altered (Satin *et al.* 1997). The vacuoles created are of late endosomal origin and contain some lysosomal markers (Molinari *et al.* 1997). The mechanism of vacuolation is not completely understood (Gebert *et al.* 2004), but is likely the result of aberrant cation transport (Leunk 1991). Alleles associated with increased vacuolating activity have been correlated with severe clinical manifestations (Atherton *et al.* 1995), but other reports have found no correlation (Pan *et al.* 1998).

VacA also induces apoptosis in epithelial cells (Cover *et al.* 2003) and interferes with antigen processing in B cells and subsequent antigen presentation to CD4+ T cells (Molinari *et al.* 1998b). *In vivo* purified and concentrated VacA produced ulceration and gastric lesions in mice (Telford *et al.* 1994) and has been correlated with increased risk of ulcers in gerbils (Ogura *et al.* 2000). VacA expression is not dependent on CagA (Xiang *et al.* 1995).

1.2.3 Adhesins

H. pylori is capable of binding to a number of compounds produced by host cells including phosphatidylethanolamine (Lingwood *et al.* 1993) and laminin (Valkonen *et al.* 1997, Valkonen *et al.* 1994). The most well studied adhesins bind fucosylated ligands expressed on the gastric epithelium.

Le^b on glycoproteins expressed by the gastric epithelium mediates *H. pylori* attachment (Boren *et al.* 1993). The bacterial adhesin responsible is bacterial blood group antigen binding A (BabA) (Ilver *et al.* 1998). There are two alleles: *babA2* encodes the complete adhesin, whereas *babA1* is transcriptionally defective (Ilver *et al.* 1998). BabA is arguably the most important adhesin and inhibition studies have documented that it is responsible for greater than 80% of adherence (Mahdavi *et al.* 2002). Other *in vitro* studies support this data (Ilver *et al.* 1998, Boren *et al.* 1993), but evidence of a primary role in adhesion is controversial when biopsies are used (Celik *et al.* 1998, Taylor *et al.* 1998, Clyne and Drumm 1997, Umlauft *et al.* 1996) suggesting multiple adherence factors are important *in vivo*.

1.2.4 Lewis Antigens and Fucosyltransferases

Several gram-negative bacteria express human ABO blood group antigens (Springer *et al.* 1961). *H. pylori* produce human Lewis antigens that are fucosylated oligosaccharides structurally related to human ABO blood group antigens (Rasko *et al.* 2001, Rasko et al. 2000a, Wang *et al.* 1999a, Wirth *et al.* 1997, Aspinall and Monteiro 1996, Aspinall *et al.* 1996, Simoons-Smit *et al.* 1996, Wirth *et al.* 1996, Chan *et al.* 1995, Sherburne and Taylor 1995, Aspinall *et al.* 1994). In mammals, fucose, a deoxyhexose (6-deoxy-L-galactose), is an important component of glycans that mediate selectin-dependent leukocyte-endothelial adhesion and other types of cell adhesion, host-microbe interactions and oncogenic events (Becker and Lowe 2003, de Vries *et al.* 2001). Changes in the expression of fucosylated oligosaccharides on the surface of cells can lead to cancer and atherosclerosis (reviewed by Becker and Lowe 2003, Miyake *et al.* 1992).

H. pylori produce four main types of Lewis antigens, two type II determinants, Lewis X (Le^x) and Lewis Y (Le^y), and two type I determinants, Lewis A (Le^a) and Lewis B (Le^b). Over 85% of isolates express at least one Lewis antigen (Simoons-Smit *et al.* 1996, Wirth *et al.* 1996). Greater than 80% of strains produce Le^x or Le^y (Simoons-Smit *et al.* 1996) and fewer than 5% synthesize Le^a or Le^b (Wirth *et al.* 1997). Le^x is synthesized by the addition of a fucose to the third carbon of the type II precursor (galactose β1-4 *N*-acetylglucosamine, Gal β1-4 GlcNAc) and Le^a is synthesized by the addition of a fucose to the fourth carbon of the type I precursor (Gal β1-3 GlcNAc) (Figure 1.5). Le^y and Le^b are produced by the addition of a fucose to the second carbon of Le^x and Le^a respectively. Other blood group determinants that can be produced by *H. pylori* include i-antigen (Monteiro *et al.* 2000a, Monteiro *et al.* 1998a), sialyl-Le^x ($SiLe^x$) (Monteiro *et al.* 2000a), H type I (Monteiro *et al.* 2000a, Monteiro *et al.* 1998a), blood group A (Moran *et al.* 1999), linear B (Monteiro *et al.* 2002), Lewis D (Le^d) (Monteiro *et al.* 1998a), and *N*-acetyl-lactosamine (LacNac) (Monteiro *et al.* 1998b).

Lewis antigens are expressed as components of the O-antigen of lipopolysaccharide (LPS). LPS in gram-negative bacteria is comprised of Lipid A that anchors the LPS to the outer membrane, an oligosaccharide core and O-antigen. The O-antigen is a polysaccharide containing repeat units consisting of 3-6 sugars. Generally, the O-antigen is variable and Lipid A and the core have a conserved structure. *Salmonella* sp. all have identical Lipid A and core regions, but can produce 40 forms of O-antigen (Wyk and Reeves 1989). *H. pylori* LPS is unusually unique because the core also varies (Aspinall and Monteiro 1996) and it is less proinflammatory than the LPS of other gram-

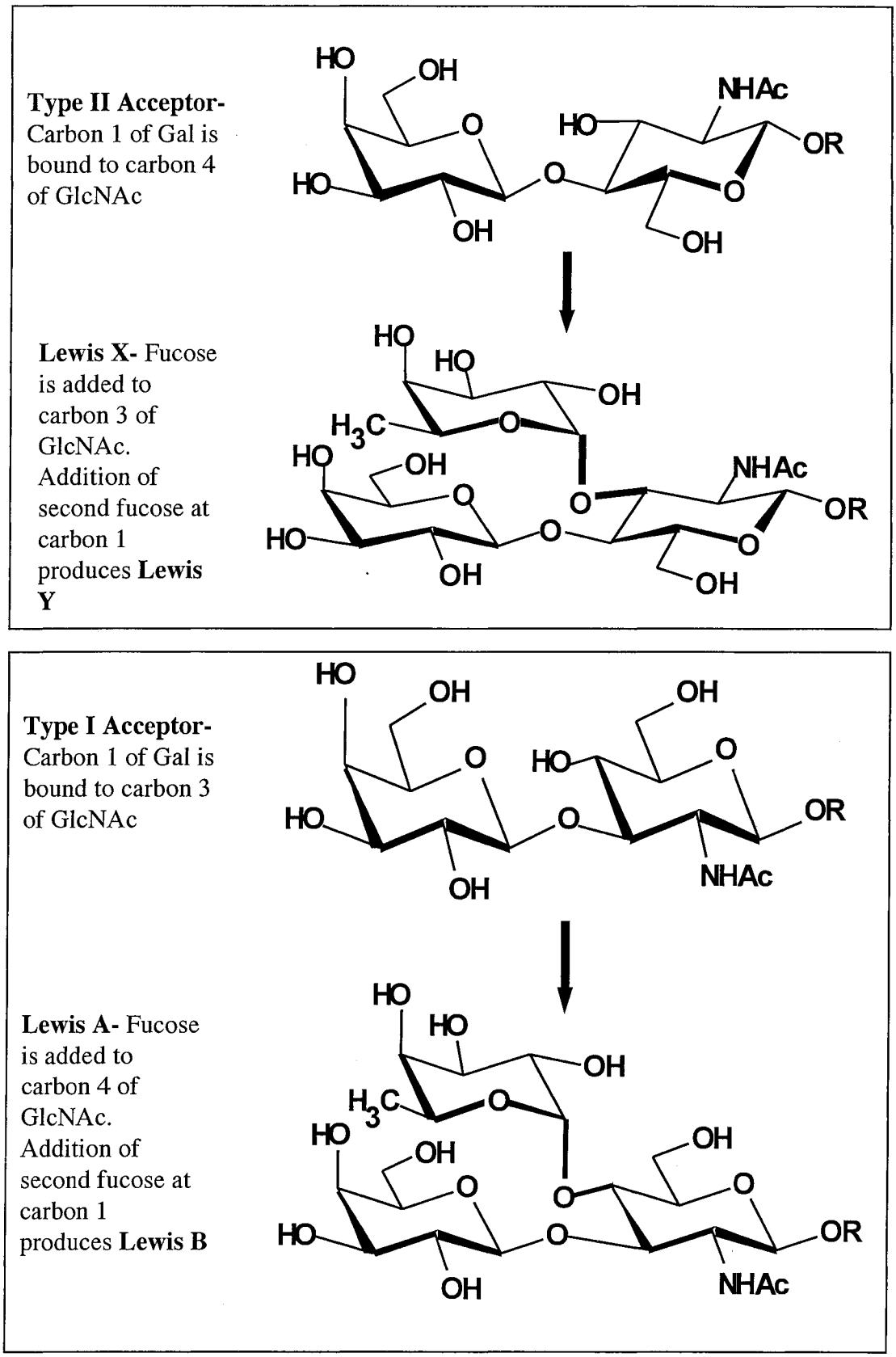


Figure 1.5- Lewis antigen synthesis from Type II and Type I precursors.

negative bacteria (reviewed by Torres *et al.* 2000). LPS is an essential component of the outer membrane.

Lewis antigens are synthesized by specific glycosyltransferases known as fucosyltransferases (FucTs). Glycosyltransferases transfer the glycosyl group from an activated sugar-nucleotide substrate to a specific acceptor (reviewed by Qasba *et al.* 2005). FucTs transfer the monosaccharide fucose from the GDP-fucose substrate to the acceptor (Qasba *et al.* 2005). Thirteen FucTs have been identified in the human genome (reviewed by Qasba *et al.* 2005). Each mammalian FucT has a unique acceptor and catalyzes the synthesis of a range of products (de Vries *et al.* 2001). *H. pylori* encodes three FucTs and several glycosyltransferases necessary for synthesizing precursors.

FutA (HP0379) and FutB (HP0651) exhibit α1-3 activity, producing Le^x, and may also possess α1-4 activity, producing Le^a (Appelmelk *et al.* 1999, Rasko *et al.* 2000b). α1-3 refers to the ability of the FucT to add a fucose to carbon 3 of GlcNAc, whereas α1-4 is the ability to add a fucose to carbon 4 of GlcNAc (Figure 1.6). In a single strain FutA and FutB can each have different substrate specificities. In 4187E, FutA is responsible for synthesizing polymeric Le^x and FutB synthesizes the majority of monomeric Le^x (Appelmelk *et al.* 1999).

Le^y and Le^b are synthesized from Le^x and Le^a, respectively, by FutC (Figure 1.6) (HP0094-0093) (Wang *et al.* 1999). FutC exhibits α1-2 activity enabling the addition of a fucose to carbon 2 of GlcNAc. While α1-2 FucT activity must precede α1-3 activity in mammals to produce Le^y and Le^b, *H. pylori* FucTs operate primarily in the reverse order (Wang *et al.* 2000). Genes required for the synthesis of precursors and the three FucTs are distributed throughout the genome (reviewed by Wang *et al.* 200). However, the three

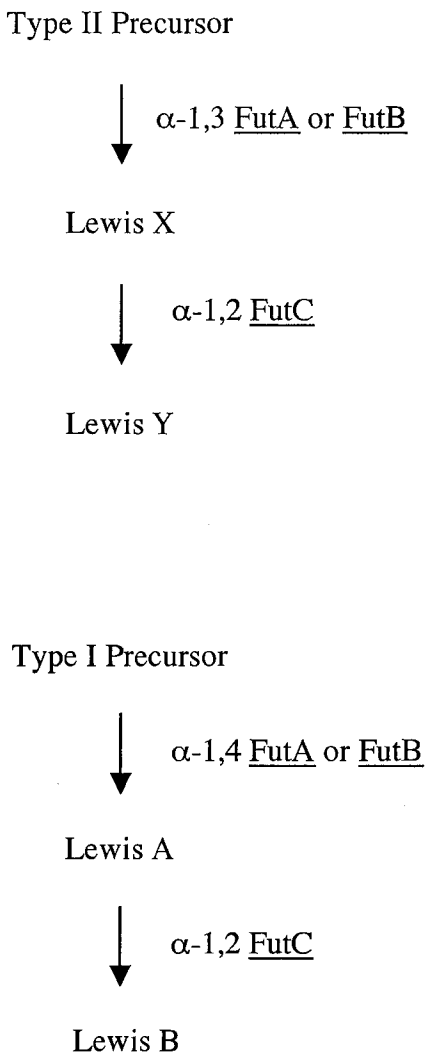


Figure 1.6- Lewis antigen synthesis in *Helicobacter pylori*. Enzymes catalyzing reactions are underlined and preceded by the type of activity they exhibit. *H. pylori* synthesizes Lewis Y and Lewis B directly from Lewis X and Lewis A, respectively, whereas human FucTs cannot add a fucose to a monofucosylated structure (Wang *et al.* 2000).

genes responsible for the synthesis of GDP-fucose *rfbM, rfbD* and *wbcJ* are grouped together (Wang *et al.* 2000). Bacterial FucTs are of interest to pharmaceutical companies because they may be used to produce therapeutic or nutritive oligosaccharides (Dumon *et al.* 2004).

Lewis antigen expression is regulated by phase variation and pH. Phase variation is the high frequency reversible switching of phenotype (Appelmelk *et al.* 2000a). *In vitro* Lewis antigen expression changes at a rate of 0.2-0.5%, but this rate is likely to be higher *in vivo* (Appelmelk *et al.* 1998). Passage of Sydney Strain 1 (SS1) through a mouse yields an altered LPS profile (Janvier *et al.* 1999), and multiple genetically identical, but phenotypically distinct isolates can be cultured from a single primary human gastric biopsy (Rasko *et al.* 2000a, Wirth *et al.* 1999, Gibson *et al.* 1998). *futA* and *futB* contain a short sequence of single nucleotide repeats capable of mediating slipped-strand mispairing (Appelmelk *et al.* 1999, Appelmelk *et al.* 1998). These regions are generally polyC tracts, but may also be polyA (Appelmelk *et al.* 1999). During replication, transcription or translation a repeat unit may be added or lost resulting in a translational frameshift. The *futC* gene contains multiple regulatory sites. Slipped strand mispairing is mediated by polyC tracts and imperfect TAA repeats (Wang *et al.* 1999). *futC* also contains a ribosomal frameshift sequence present in *E. coli* (Wang *et al.* 1999). In addition to the three FucTs, two galactosyltransferases required for Lewis antigen synthesis also contain single nucleotide repeats (Appelmelk *et al.* 2000a). Between the five enzymes there are thirty-two possible on/off combinations making large scale phenotypic variation possible (Appelmelk *et al.* 2000a). Bacterial phase variation is not

unique and has also been well documented in *Neisseria* sp. and *Haemophilus influenzae* (reviewed by Appelmelk *et al.* 2000b, reviewed by van Putten 1993).

pH also regulates Lewis antigen expression resulting in qualitative differences in *H. pylori* LPS at pH 5.0 and pH 7.0 (McGowan *et al.* 1998). WbcJ is an enzyme necessary for the conversion of GDP-mannose to GDP-fucose. Its expression is induced by lowering the pH (McGowan *et al.* 1998). A *wbcJ* null mutant is incapable of expressing Lewis antigens and is more sensitive to acid stress (McGowan *et al.* 1998). This suggests Lewis antigens may play an adaptive role at low pH.

The role of Lewis antigen expression on the LPS of *H. pylori* is controversial. Some reports suggest Lewis antigen expression plays a large role in the ability to cause chronic disease (Appelmelk *et al.* 1996, Moran *et al.* 1996), but the mechanism is unknown. Bacterial Le^x expression is consistently correlated with severe clinical manifestations whereas isolates expressing no Lewis blood group antigens, non-typable *H. pylori* (NtHP), are associated with a lack of symptoms (Heneghan *et al.* 2000, Rasko 2000, Marshall *et al.* 1999, Heneghan *et al.* 1998). In a study of 108 patients from Singapore, isolates producing two or more Lewis antigens were significantly correlated with ulcers, whereas *cagA, vacA* and *iceA1* were not (Zheng *et al.* 2000). Lewis antigens may function by playing a role in molecular mimicry, adhesion or immunomodulation.

Lewis antigens produced by *H. pylori* mimic Lewis antigens produced by the human gastric epithelium. Molecular mimicry may result in immune evasion or autoimmune damage to the host. *N. gonorrhoeae* (Mandrell *et al.* 1988) and *C. jejuni* (Aspinall *et al.* 1993) LPS also contain structures that mimic host molecules. It seems unlikely that Lewis antigen molecular mimicry in *H. pylori* is influenced by the Lewis

antigen phenotype in humans because most studies find no relationship between the *H. pylori* and host phenotype (Heneghan *et al.* 2000, Taylor *et al.* 1998). Wirth *et al.* 1997 found that host Le^a and Le^b expression was related to the microbial phenotype, but they typed humans using red blood cells, which may not reflect human secretor genotype (Henry *et al.* 1995). A study using monkeys also determined that there was a correlation between Lewis antigen expression of the host and the pathogen (Wirth *et al.* 1998). It has been shown that *H. pylori* LPS elicits a smaller inflammatory response compared to other enterobacterial LPSs (Moran 1995). However, Yokota *et al.* (2000, 1998) demonstrated that many Japanese strains produce LPS that is highly immunogenic. Antigen mimicry would be considered pathogenic if it led to the production of antibodies that bound to the gastric epithelium resulting in complement fixation and subsequent tissue injury. There is evidence that anti-Le^x and Le^y mAbs react with Le^x and Le^y on proton pumps and in the corpus glands (Appelmelk *et al.* 1996) and that high levels of anti-Le^y can cause gastric damage (Negrini *et al.* 1991). Appelmelk *et al.* (1996) and Negrini *et al.* (1996) independently determined that autoimmunity results in gastritis, gastric ulcers and duodenal ulcers. However, other research suggests that Lewis antigens are not autoimmune targets and the anti-LPS response is directed towards other carbohydrate epitopes (Claeys *et al.* 1998, Faller *et al.* 1998 Yokota *et al.* 2000, Yokota *et al.* 1998, Amano *et al.* 1997). Anti-Lewis antigen antibodies have been detected in individuals without *H. pylori* providing further evidence that Lewis antigens are unlikely autoimmune targets (Chmiela *et al.* 1999).

Lewis antigens may also play a role in cell to cell adhesion. Le^x is associated with the adhesion pedestal (Taylor *et al.* 1998) and anti-Le^x antibodies inhibit binding to

gastric cell lines (Appelmelk and Vandenbroucke-Grauls 2000, Osaki *et al.* 1998). Additionally, synthetic Le^x bound to polystyrene beads is also capable of attaching to human gastric epithelial cells (Edwards *et al.* 2000). Mahdavi *et al.* (2003) found that in 50% of biopsies examined isogenic Le^x negative mutants of strain 11639 bound less well to human gastric epithelium from symptomatic patients than their wild type counterparts. A major role for Lewis antigens in adherence was inferred because the Lewis antigen or Le^x negative mutants did not adhere to gastric mucosa tissue sections (Edwards *et al.* 2000). *In vivo* adherence data in mice is controversial. Martin *et al.* (2000) and Logan *et al.* (2000) showed Le^x and Le^y expression is crucial for colonization; Logan *et al.* (2000), Martin *et al.* (2000), and Moran *et al.* (2000) determined that Lewis negative mutants colonize less well in mice; while Takata *et al.* (2002) found that Lewis knock out strains were still able to colonize mice. Data from human gastric xenografts in nude mice suggests colonization rates are independent of Lewis antigen expression (Lozniewski *et al.* 2003). As a result, there are conflicting data on the importance of Lewis antigens on adherence.

Monomeric Le^x and Le^y bind to the C-type lectin DC-SIGN on dendritic cells (Bergman *et al.* 2004). This blocks Th1 T cell development, increases IL-10 production, and shifts the immune response towards a Th-2 mediated response. *H. pylori* that did not express Lewis antigens induced a strong Th-1 response. Conversely, 100% of isolates from seven asymptomatic patients did not express any blood group O-side chains suggesting that Lewis antigen expression is proinflammatory (Monteiro *et al.* 2001). This was supported by Heneghan *et al.* (2000) who demonstrated that bacterial Le^x and Le^y expression are correlated with neutrophil infiltration.

1.2.5 Other Virulence Factors

Other virulence factors that interfere with the adaptive and innate immune system have been identified. A recently identified 30-60 kDa protein or protein complex inhibits the proliferation of T and B lymphocytes by arresting the cell cycle in the G1 phase (Gerhard *et al.* 2005). Reduced phosphorylation of the retinoblastoma protein is suggestive of an inhibition of G1 cyclin-dependent kinase activity. A 150 kDa protein composed of 15 kDa subunits, *H. pylori* neutrophil activating protein (HP-NAP), attracts and activates neutrophils (Evans *et al.* 1995). HP-NAP is encoded by *napA* and although the sequence is well conserved among strains, activity levels vary (Evans *et al.* 1995). Purified HP-NAP attracts neutrophils (Satin *et al.* 2000) inducing the expression of β_2-integrins promoting transportation across the epithelium (Evans *et al.* 1995). HP-NAP bound to the bacterial surface can also act as an adhesin attaching to sphingomyelin on leukocytes (Teneberg *et al.* 1997). The release of proinflammatory IL-6 by mast cells is attributed to HP-NAP (Montemurro *et al.* 2002). HP-NAP mediated inflammation may cause nutrients to be released from tight junctions (reviewed by Dhar *et al.* 2003).

Chapter Two

Use of an Adenylate Cyclase Fusion to search for Novel Effector Molecules Transported by the Type IV Secretion System of *Helicobacter pylori*

2.1 Preface

CagA is the only known effector molecule transported into host cells by the type four secretion system (T4SS) of *H. pylori*. Other pathogenic bacteria including *Legionella pneumophila* and *Agrobacterium tumefaciens* employ their T4SS to translocate a number of effector molecules. Based on homology with these systems and unexplained host cellular responses, it is believed that additional effector molecules must exist. Therefore adenylate cyclase was used as a reporter gene and fused to several genes in the *cag* PAI and ten CagA mutants. Proteins translocated into host cells would cause an increase in cAMP levels, which could be detected by ELISA. No novel effector molecules were identified with this method.

2.2 Introduction

2.2.1 General Introduction

Helicobacter pylori is a gastric pathogen that is present in greater than fifty percent of the human population (Czinn and Nedrud 1997). It is known to play a causative role in the development of chronic gastritis, gastric ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma and gastric adenocarcinoma (Czinn and Nedrud 1997, Blaser and Parsonnet 1994, Lee *et al.* 1993, Parsonnet *et al.* 1991).

Strains of *H. pylori* can be typed by the virulence factors they express. Type I strains of *H. pylori* encode the *cag* PAI and a cytotoxic form of VacA, whereas Type II strains possess a nontoxic form of VacA and no *cag* PAI. Type I strains have been associated with severe clinical manifestations and Type II strains are more likely to be either asymptomatic or to cause only gastritis (Fischer *et al.* 2001, Allen *et al.* 2000, Naumann *et al.* 1999). However, other virulence factors may be produced because CagA and VacA do not explain all of the cellular responses attributed to Type I strains. Research effort is presently being directed towards discovering novel effector proteins.

Adenylate cyclase has been used successfully as a reporter gene to identify effector molecules secreted by several type three secretion systems (T3SS) (Petnicki-Ocwieja *et al.* 2005, Choy *et al.* 2004, Sory and Cornelis 1994) and the T4SS of *Legionella pneumophila* (Bardill *et al.* 2005, Nagai *et al.* 2005). To date, no publication has reported an attempt to identify novel effector molecules in *H. pylori* using this system.

2.2.2 Evidence Supporting Additional Effector Molecules

The existence of additional effector molecules secreted by the T4SS is supported by microarray experiments, the ability of Type I strains to inhibit phagocytosis (Ramarao *et al.* 2000) and the number of T4SS translocated effector molecules produced by other pathogens.

cDNA microarray data from G27 infections of AGS cells, a gastric adenocarcinoma cell line, suggests that undiscovered effector molecules secreted by the T4SS exist. AGS cells infected with G27 upregulated the expression of genes for the innate immune response, cell shape regulation and signal transduction (Guillemin *et al.* 2002). A CagA null mutant upregulated the same gene set as wild type G27 with the exception of the genes involved in the cytoskeleton. Mutants with non-functional T4SS failed to upregulate the same genes as the CagA mutant and several genes related to the immune response. A mutant with no T4SS failed to upregulate the same genes as the non-functional T4SS mutant in addition to some transiently expressed genes. The secretion-deficient mutant and the mutant lacking the entire T4SS failed to upregulate the same immune response genes suggesting failure to induce expression of these genes is not contact dependent, but is due to a secretion deficit. Since this is not due to a lack of CagA secretion, an additional effector molecule secreted by the T4SS is probably responsible.

Several reports have indicated that *H. pylori* can evade phagocytosis, but the mechanism is controversial. Ramarao *et al.* found that Type I *H. pylori*, strains that are positive for the cytotoxin VacA and the *cag*PAI, can evade uptake by polymorphonuclear (PMNs) monocytes, whereas Type II strains, which do not encode cytotoxic VacA or the *cag*PAI, cannot (2000). Furthermore, Type I bacteria were phagocytosed when subjected

to treatment with chloramphenicol, a protein synthesis inhibitor, or when genes necessary for CagA translocation were knocked out. This suggested that a protein dependent on the T4SS is responsible for inhibiting uptake by PMNs. However, Allen *et al.* (2000) found that bacteria were phagocytosed, but not killed, by mononuclear phagocytes. This study found that it took longer for uptake of Type I strains than Type II strains; moreover Type I strains went on to form megasomes and survive intracellularly, whereas Type II did not. Since then, it has been shown that VacA is responsible for inhibiting phagosome maturation and preventing phagosome/lysosome fusion (Zheng and Jones 2003), although the factor responsible for delaying uptake has not been identified. Finally, Odenbreit *et al.* (2001) found that *H. pylori* was phagocytosed by PMNs and macrophages independent of the *cag*PAI. Studies with more strains and different cell lines need to be completed to gain a clearer understanding of the anti-phagocytic capabilities of *H. pylori*. Some evidence suggests that a protein associated with the T4SS may be responsible for this phenomenon.

Other pathogenic bacteria that encode functional T4SS can translocate several effector molecules. Two human pathogens, *Legionella pneumophila* and *Bartonella henselae*, are good examples of this. The Dot/Icm T4SS of *L. pneumophila*, the causative agent of Legionnaire's disease, exports RalF (Nagai *et al.* 2005), LidA (Conover *et al.* 2003), LepA and LepB (Chen *et al* 2004), SidC (Luo *et al.* 2004), and SdeA (Bardill *et al.* 2005). *B. henselae*, which is responsible for a variety of clinical manifestations including cat-scratch disease, translocates *Bartonella*-translocated effector proteins (Beps) A-G *via* its VirB T4SS (Schulein *et al.* 2005). Based on these findings it seems likely that *H. pylori* expresses and translocates additional effector molecules.

2.2.3 Adenylate Cyclase as a Reporter Gene

The adenylate cyclase portion of the *cyaA* gene from *Bordetella pertussis*, the causative agent of whooping cough, has been widely used as a reporter gene. When adenylate cyclase (AC) associates with calmodulin (CaM), cAMP is produced (Wolff *et al.* 1980). CaM is only produced by eukaryotic cells thus CyaA is only active inside host cells (Botsford *et al.* 1992). AC fusions have been successfully used to identify effector molecules transported into plant and animal cells by both T3SSs and T4SSs.

CyaA, a 188 kDa protein composed of 1706 amino acids, possesses AC and haemolysin activity (Guo *et al.* 2005, Glaser *et al.* 1988). The AC and haemolysin domains can function independently (Sakamoto *et al.* 1992), with the haemolysin domain essential for binding and internalization of the toxin (Ladant and Ullman 1999, Bellalou *et al.* 1990). The AC domain is encoded by amino acids 1-400 of which amino acids 1-224 comprise the catalytic site and amino acids 225-399 form the CaM binding domain (Ladant and Ullmann 1999).

A crystal structure of the AC portion of CyaA and the C terminus of CaM has provided further insight into the function of this toxin (Guo *et al.* 2005). CaM exists in a closed state until it is bound by four calcium ions, which are important intracellular secondary messengers (Guo *et al.* 2005, Berridge *et al.* 2003). Upon binding of Ca^{2+}, CaM undergoes a structural transition exposing a binding pocket. This binding pocket is responsible for binding to AC and activating the toxin. Although AC has low level activity alone, and subsequently low activity level in bacterial cells (Sory and Cornelis 1994), its catalytic ability is increased from 1000 to greater than 10 000 fold by CaM (Shen *et al.* 2002, Ladant and Ullman 1999).

CyaA was first used as a reporter gene in *Yersinia enterocolitica* to demonstrate YopE (Yersinia outer protein E) T3SS-mediated translocation into host cells (Sory and Cornelis 1994). This system has also been used to document the translocation domains of YopE and YopH (Sory *et al.* 1995). SipC and its translocation domain (Chang *et al.* 2005), Spt-P and its translocation domain (Fu and Galan 1998), and SseK1 and SseK2 (Choy *et al.* 2004) have been identified as effector molecules using CyaA as a reporter gene in *Salmonella enterica.* AC has also been used to document *Pseudomonas syringae* mediated translocation of HrpK into tomato cells (Petnicki-Ocwieja *et al.* 2005) and several T3SS translocated Xops produced by *Xanthomonas campestris* pr. *Vesicatoria* have been confirmed in the cells of pepper plants (Roden *et al.* 2004).

AC has been used several times to identify effector molecules secreted by the Dot/Icm T4SS of *L. pneumophila.* This is a primarily intracellular pathogen and replicates inside phagosomes in alveolar macrophages. Translocated effectors identified using CyaA include: SdeA (Bardill *et al.* 2005), LepB and LepA (Chen *et al.* 2004) and RalF (Nagai *et al.* 2005). The domain of RalF necessary for translocation was also determined with an AC fusion (Nagai *et al.* 2005).

2.2.4 Relevance

Identification of novel effector molecules will supplement our understanding of *H. pylori* biology and possibly act as a target for novel therapeutics. CagA is primarily associated with increasingly severe pathology in the Western hemisphere, although not in Asian countries where the vast majority of *H. pylori* isolates are CagA positive (Yamaoka *et al.* 1999). Although CagA is likely required for induction of severe pathology, it cannot be used to identify which individuals will progress to duodenal ulcers or gastric

adenocarcinoma. A novel effector molecule may be more strongly associated with disease, allowing identification of individuals likely to develop severe clinical manifestations. These individuals could be recommended for *H. pylori* eradication therapy preventing progression to a symptomatic condition. For example, the correlation between production of cholera toxin (CT) and toxin-coregulated pili (TCP) by *Vibrio cholera* and disease is much stronger than CagA and severe disease (Waldor and Mekalanos 1996, Taylor *et al.* 1987).

2.3 Experimental Procedures

2.3.1 Bacterial Strains and Growth Conditions

Escherichia coli strain DH5α was grown on Luria broth (LB) plates [25.0 g/l LB powder (Invitrogen, Burlington, OT, Canada), 7.5 g agar (Becton, Dickinson and Company, Sparks, MD, USA)] supplemented with either ampicillin (100 μg/ml) or kanamycin (50 μg/ml) depending on the resistance marker on the plasmid. *H. pylori* strain G27 was plated on Brucella broth (BB) plates [28.0 g/l BB powder (Becton, Dickinson and Company, Sparks, MD, USA), 5% fetal bovine serum (FBS), 5% horse serum (HS), 14.0 g/l of agar, 8 μg/ml amphotericin B, 100 μg/ml cyclohexamide, 5 μg/ml trimethroprim and 8 μg/ml vancomycin]. *H. pylori* transformants were grown on BB plates containing 25 μg/ml of kanamycin. Plates were incubated at 37°C under microaerobic conditions for a maximum of 48 hours.

H. pylori was grown in liquid culture prior to infection of AGS cells. A 1 ml loop was used to scrape a plate of competent *H. pylori*. The cells were then placed in 10 ml of BB liquid [28.0 g/l BB, 5% FBS, 5% HS, 8 μg/ml amphotericin B, 100 μg/ml

cyclohexamide, 5 μg/ml trimethroprim and 8 μg/ml vancomycin] and grown to an OD of 1.6-2.9 at 600 nm. Liquid cultures were shaken at 165 rpm at 37°C for 14-16 hours under microaerobic conditions.

2.3.2 Cloning

Due to ease of manipulation all cloning was done in *E. coli*. All ligations, transformations and restriction endonuclease digestions were carried out using standard techniques as described by Sambrook and Russell (2001). Plasmid isolation was performed using the Mini-Prep kit from Sigma-Aldrich (Oakville, ON, Canada). Constructs engineered in *E. coli* were transformed *via* natural recombination into the recombinase gene of *H. pylori*. The adenylate cyclase fusion, used as a reporter gene, is toxic to *E. coli* so IG9, a promoter from *Campylobacter jejuni* that is inactive in *E. coli,* was used (Wosten *et al.* 1998). The IG9 promoter was sequenced with the IG9+ oligonucleotide primer (5'-ATATCTCGAGATGCGTATTTTTCCCTTGAGT-3') to ensure no restriction sites were present that needed to be used in vector construction. The IG9 promoter is inactive in *E. coli*, but active in *H. pylori* (M. Couturier, personal communication). This promoter was amplified from *C. jejuni* and cloned into the PCR-Blunt vector. Sequencing revealed that no XhoI, NdeI, XbaI or BamHI cut sites were present (Appendix 1).

2.3.2.1 Plasmid Construction

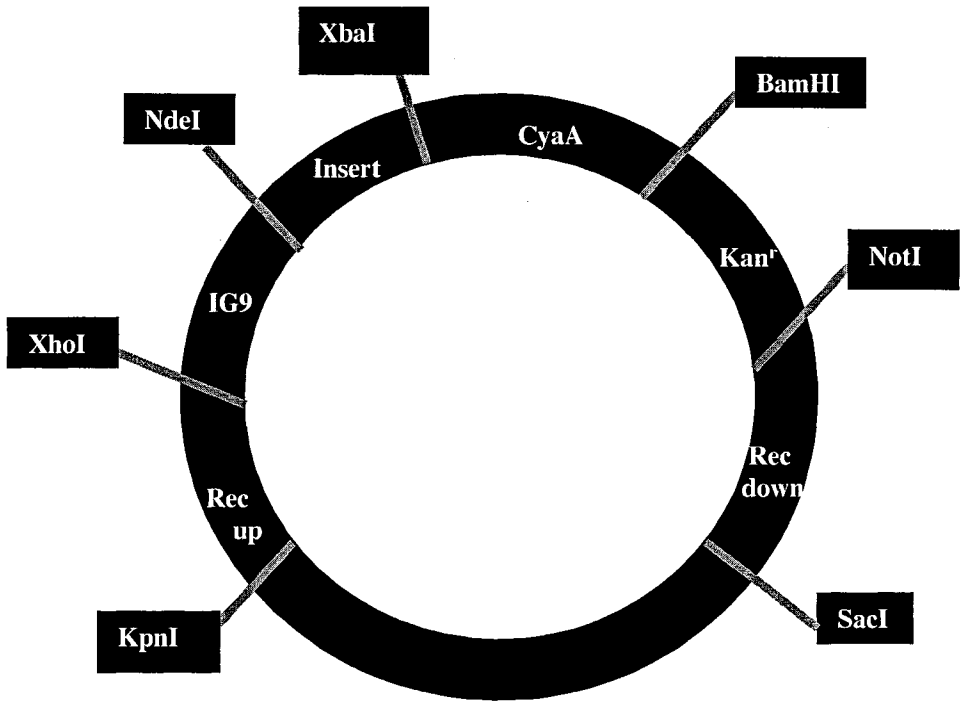
A commercially available plasmid, pBluescript SK+ (Stratagene, La Jolla, CA, USA), was modified by Couturier *et al.* (*In Press*) to create the plasmid SK+ rec. Briefly, the upstream portion of the G27 recombinase gene (KpnI/XhoI), the CagA promoter (XhoI/NdeI), *orf8* (NdeI/XbaI), the Flag tag (XbaI/BamHI), a kanamycin resistance

cassette (BamHI/NotI) and the downstream region of the recombinase gene (NotI/SacI) were cloned into the multiple cloning site (MCS). Several changes were made to this construct. The CagA promoter was removed and replaced with the IG9 promoter. This promoter was PCR amplified from *Campylobacter jejuni* with the oligonucleotides IG9+ (5'-ATATCTCGAGATGCGTATTTTTCCCTTGAGT-3' {XhoI}) and IG9- (5'-ATATCATATGAGCCTTTCTTAAATGTTAATT-3' {NdeI}). The Flag tag was also removed and replaced with the catalytic domain of adenylate cyclase (CyaA). The primers Cya2+ (5'-AAAATCTAGAATGCAGCAATCGCATCAGGCTG-3' {XbaI}) and Cya2- (5'-AAAAGGATCCTCATCCCGATCCCACCCCATCA-3' {BamHI}) were used to PCR amplify *cyaA* from the p-EX-CyaA1-412 plasmid (Wolff *et al.* 1998). The new SK+ rec plasmid with the $IG9^{Pr}$ and CyaA insertions was called SK+Cya2 (Figure 2.1). A second plasmid, SK+Cya3, was engineered from SK+Cya2. *cyaA* was double digested out of SK+Cya2 with XbaI and BamHI. A CagA mutant, *cagAmutCya3* (creation of this mutant is discussed in section 2.3.2.2), was ligated in. *orf8* was removed from SK+Cya2 by double digestion with NdeI and XbaI and replaced by *cyaA*. *CyaA* was again amplified from p-EX-CyaA1-412, but the oligonucleotides Cya3+ (5'-AAAACATATGATGCAGCAATCGCATCAGGCTG-3' {NdeI}) and Cya3- (5'-AAAATCTAGATCATCCCGATCCCACCCCATCA-3' {XbaI}) were used.

2.3.2.2 PCR Amplification of *cag* PAI genes

DNA from strains G27, CCUG 17874 and 26695 was extracted by resuspending one plate containing bacteria in 500µl of STE buffer (10mM Tris-HCl (pH 8.0), 0.1M NaCl, 1mM EDTA). The solution was centrifuged for 5 mins at 14000 rpm and resuspended in 350 µl of STE. 2 µl of lysozyme (100 mg/ml in STE) and 4 µl RNAase

a)



b)

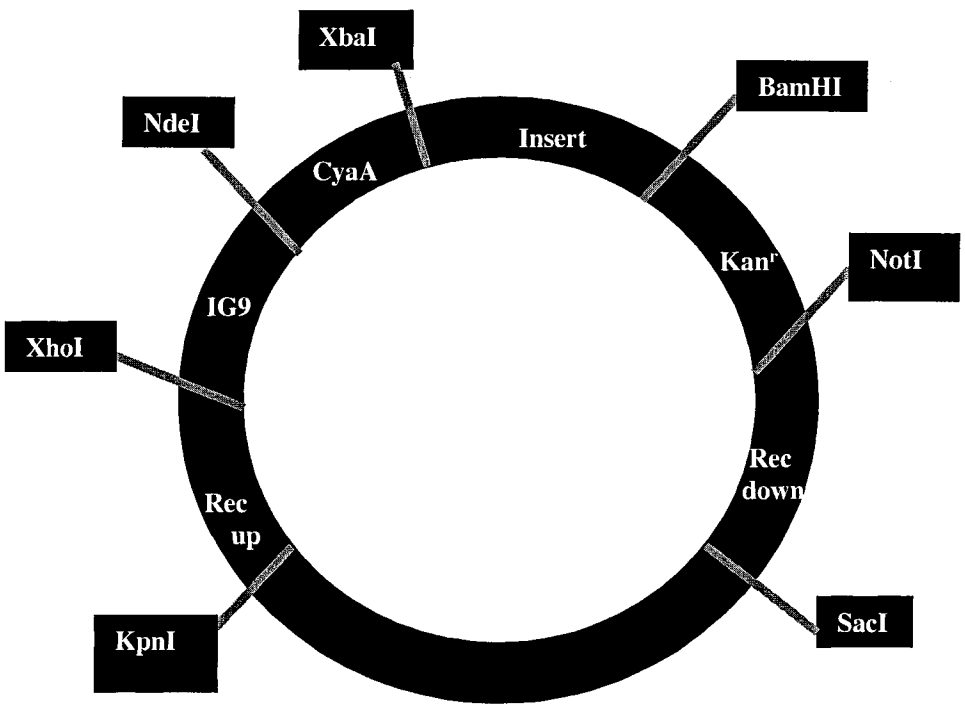


Figure 2.1- The two plasmids used to create the CyaA fusion proteins a) SK+Cya2 b) SK+Cya3. The *recA* upstream (Rec up) and downstream (Rec down) sequences facilitate homologous recombination into the *rec* gene on the *H. pylori* chromosome.

(10 mg/ml) was added and the tube was incubated at 37°C for 30 mins. 35 µl of 10% SDS was added and the solution was incubated at 65°C for 15 mins. Finally, 1 µl of Proteinase K (100 mg/ml in STE) was added and the tube was incubated for 90 mins at 50°C. The DNA was extracted using an equal volume of phenol/chloroform, washed with 77% absolute ethanol, pelleted and resuspended in 500 µl dd$H_2$0. *cagC, cagD, cagF, cagG, cagH, cagI, cagL, cagT, cagU,* HP522 (ORF8), HP524 *(virD4),* HP528 *(virB9)* and ten *cagA* mutants were PCR amplified from G27 genomic DNA using PWO polymerase (Roche Diagnostics, Laval, QC, Canada) The oligonucleotide primers used are listed in Table 2.1. HP529 (ORF16) was amplified from CCUG genomic DNA and CagA was amplified from genomic 26695 genomic DNA. ORF16 was amplified from CCUG as the G27 sequence for this gene is unavailable. CagA was amplified from 26695 since G27 contains an XbaI site, used in the SK+Cya2 vector, at the 3' end of the sequence. Products were blunt end ligated into the PCR-Blunt vector (Invitrogen, Burlington, ON, Canada) using the manufacturer's instructions.

PCR products were excised from the PCR-Blunt vector using restriction enzymes and ligated into the insertion site in the SK+Cya2 vector. *cagC, cagD, cagF, cagH, cagI, cagL, cagT, cagU, orf8, cagAmutΔ1, cagAmutΔ2, cagAmutΔ3, cagAmutΔ4, cagAmutT1, cagAmutT2, cagAmut150, cagAmut251* and *cagAmut350* were double digested with NdeI and XbaI. *cagA* and *orf16* were digested with XbaI overnight and then NdeI for 15 mins. These genes contain an NdeI cut site so partial digestion was necessary in order to obtain full length product. *cagG* was digested with NdeI and SpeI and *virD4* and *virB9* were double digested with NdeI and NheI. The cut sites of NheI and SpeI can be ligated to the NdeI. Five CagA truncation mutants (*cagAmutT1, cagAmutT2, cagAmut150, cagAmut251*

Table 2.1 - Oligonucleotide primers used for PCR amplification of *cag* genes and CagA mutants to be fused with adenylate cyclase

Gene Amplified	Primer Sequences	Restriction Sites
cagA	(AF+) 5' TATCTCGAGCATATGCTAACGAAACCATTAACCA 3' (AF-) 5' TATTCTAGAAGATTTTTGGAAACCACCTTTTG 3'	NdeI XbaI
cagC	(CF+) 5' TATATATCATATGAAATTTTTTACAAGAATCAC 3' (CF-) 5' TATTCTAGAGCTACGTCCTCCACCCTCGC 3'	NdeI XbaI
cagD	(DF+) 5' TATATATCATATGTTGATCAACAATAATAATAATAG 3' (DF-) 5' TATTCTAGATAGATATACCGCTTCACATGTAAT 3'	NdeI XbaI
cagF	(FF+) 5' TATATATCATATGAAACAAAATTTGCGTGAAC 3' (FF-) 5' TATTCTAGAATCGTTATTTTTGTTTTGATTTTT 3'	NdeI XbaI
cagG	(GF+) 5' TATATATCATATGAAAACGAATTTTTATAA 3' (GF-) 5' GGACTAGTATACCCTAAGATCGGTGGTAA 3'	NdeI SpeI
cagH	(HF+) 5' TATATATCATATGGCAGGTACACAAGCTATA 3' (HF-) 5' TATTCTAGACTTCACGATTATTTTAGTCTGC 3'	NdeI XbaI
cagI	(IF+) 5' AATTATTCATATGGGTGAAATGTTTTTTAAGTATA 3' (IF-) 5' AATTCTAGATTTGACAATAACTTTAGAGCTAGC 3'	NdeI XbaI
cagL	(LF+) 5' TATATATCATATGAGAACACTCGTAAAAAAT 3' (LF-) 5' TATTCTAGATTTGACAATGATCTTACTTGA 3'	NdeI XbaI
cagT	(HP532+) 5' TATATATCATATGAAAGTGAGAGCAAGTGTTTTA 3' (HP532-) 5' ATATTCTAGACTTACCACTGAGCAAACTTCTGAT 3'	NdeI XbaI
cagU	(U+) 5' AAACATATGAACGATACAACAGAGCATC 3' (U-) 5' AAATCTAGATTGCTCTTGTTTCTTTG 3'	NdeI XbaI
ORF8	(HP522+) 5' ATAATACATATGTTTAGAAAACTAGCAACC 3' (HP522-) 5' ATCTCTAGACTTTGAATCTTTCAGTAACGC 3'	NdeI XbaI
ORF16	(16+) 5' TATCATATGTTTAATATTAAAAGGACT 3' (16-) 5' TATACTATGTCTAGATCCTTTAAACATAGATCCACC 3'	NdeI XbaI
virB9	(HP528+) 5' TATATATCATATGGGGCAGGCATTCTTTAAAAAAATTG 3' (HP528-) 5' ATATATGCTAGCTTTATCTCTGACAAGAGGGAGCTT 3'	NdeI NheI
virD4	(HP524+) 5' TATATATCATATGGAAGACTTTTTGTATAACACC 3' (HP524-) 5' ATATATGCTAGCCAGTTCGCTTGAACCCACAGGCAC 3'	NdeI NheI
cagAmut Δ1	(DA1+) 5' ATACTGCAGTTCACAAGTTGGGTGTCCCAT 3' (DA1-) 5' ATACTGCAGATCTGGTGTTCTTGTTTGATC 3'	PstI PstI
cagAmut Δ2	(DA2+) 5' ATACTGCAGGGAGACAAACACGATTGGAAC 3' (DA2-) 5' ATACTGCAGATCCAACCAATCCCCACCAGT 3'	PstI PstI
cagAmut Δ3	(DA3+) 5' ATACTGGACGTTTCCCATTTAGAAGCAGGC 3' (DA3-) 5' ATACTGCAGTTGTGCAAGAAATTCCATGAA 3'	PstI PstI
cagAmut Δ4	(DA4+) 5' ATACTGCAGTCGGTGAAAGATTTAGGTATC 3' (DA4-) 5' ATACTGCAGCTCGTCATAGTTGCCTGTGCT 3'	PstI PstI
cagAmut T1	(AF+) 5' TATCTCGAGCATATGCTAACGAAACCATTAACCA 3' (T1-) 5' ATATCTAGAGAGAGCTGTGGCCTCTATTCC 3'	NdeI XbaI
cagAmut T2	(AF+) 5' TATCTCGAGCATATGCTAACGAAACCATTAACCA 3' (T2-) 5' ATATCTAGATTCTGATACCGCTTGATTGAG 3'	NdeI XbaI
cagAmut 150	(AF+) 5' TATCTCGAGCATATGCTAACGAAACCATTAACCA 3' (A150cya-) 5' TATCTAGAAGGGATAGGGGGTTGTATGATATT 3'	NdeI XbaI
cagAmut 251	(AF+) 5' TATCTCGAGCATATGCTAACGAAACCATTAACCA 3' (A250cya-) 5' TATCTAGAAGGCGGTAAGCCTTGTATGTCGG 3'	NdeI XbaI
cagAmut 350	(AF+) 5' TATCTCGAGCATATGCTAACGAAACCATTAACCA 3' (A350cya-) 5' TATCTAGAATGCACATTAATTATTGTAGCCAC 3'	NdeI XbaI
cagAmut Cya3	(CagACya3+) 5' AAAATCTAGAATGGTGCCTGCTAGTTTGTCAGCG 3' (CagACya3-) 5' AAAAGGATCCTCAAGATTTTTGGAAACCACCTTTTG 3'	XbaI BamHI

and *cagAmut350*) were created by using the forward CagA primer (AF+) and novel reverse primers (T1-, T2-, A150cya-, A251cya- and A350cya-) (Figure 2.2). Four other CagA mutants (*cagAmutΔ1, cagAmutΔ2, cagAmutΔ3* and *cagAmutΔ4*) were deletion mutants and were amplified in two parts and ligated separately into the PCR-Blunt vector (Figure 2.3). The first portion was amplified using AF+ and one of four reverse primers (D1-, D2-, D3- and D4-), and the second part using the wild type *cagA* reverse primer (AF-) and the forward primer corresponding to the novel reverse primers (D1+, D2+, D3+ and D4+). The eight internal primers (D1+, D2+, D3+, D4+, D1-, D2-, D3- and D4), each contain a PstI cut site. The first portion (AF+ with a D(X)- primer) was digested with NdeI and PstI and the second part (AF- with a D(X)+ primer) was digested with PstI and XbaI. The two pieces were then ligated together at the PstI site and cloned into the SK+Cya2 vector. A final *cagA* mutant, *cagAmutCya3*, was amplified with the forward primer CagACya3+ containing a XbaI cut site, from the carboxy-terminus of the protein and the reverse *cagA* primer with a BamHI site replacing the XbaI site (CagACya3-). This mutant was double digested from the PCR-Blunt vector with BamHI and XbaI and ligated into the SK+Cya3 vector. Correct insertion of PCR products was confirmed with restriction endonuclease digestion Taq PCR using the original primer pair, and visualization after electrophoresis on a 1% agarose gel after stained with ethidium bromide.

2.3.2.3 Transformation of *H. pylori*

G27 was streaked on a BB plate and incubated at 37°C for 16-24 hours. The plate was examined for maximally competent cells, which are light tan colored and not sticky. These cells were scraped off with a 10 μl loop and spread in a 4cm wide circle on a new

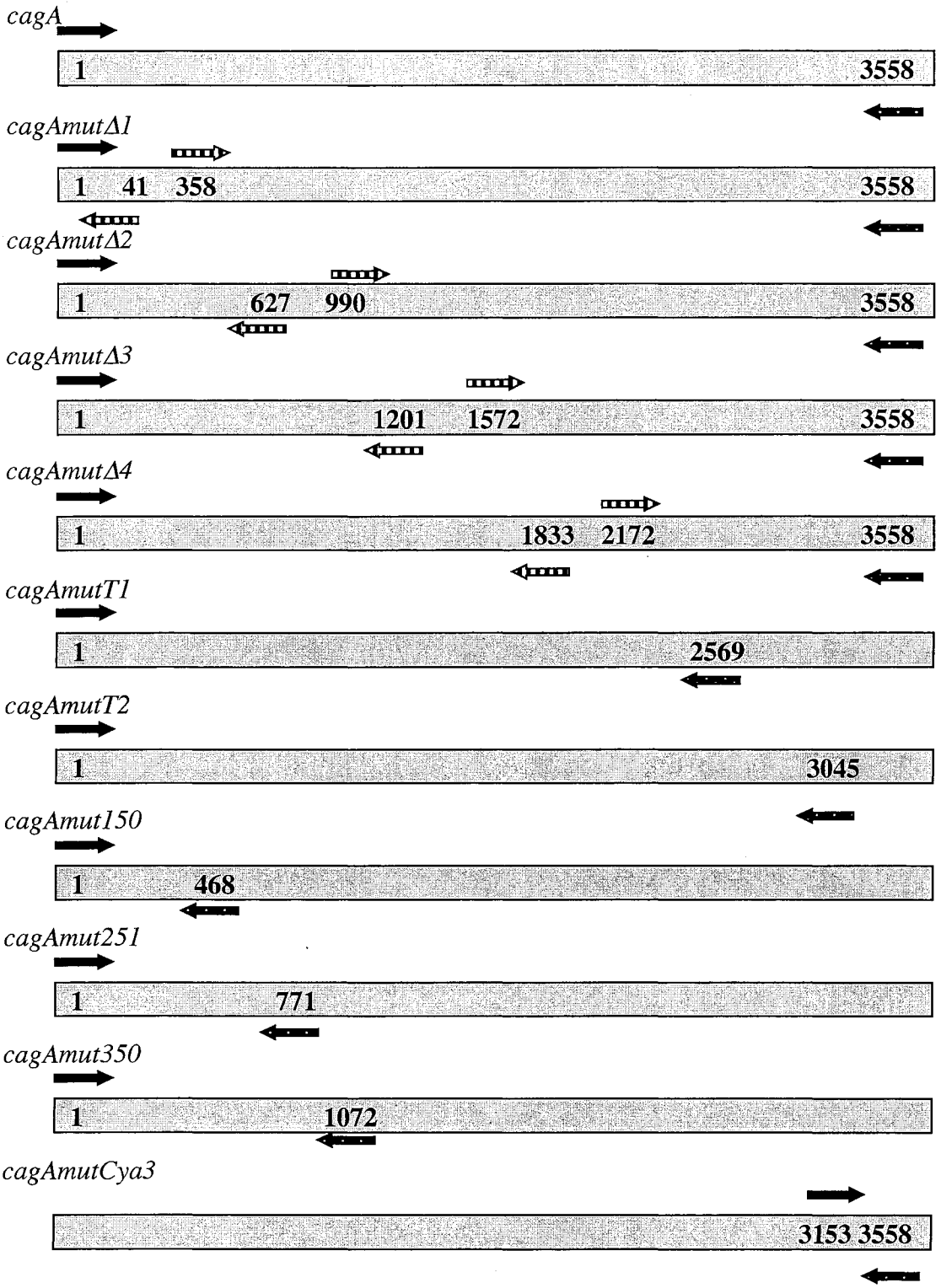


Figure 2.2- Primers (represented by arrows) used for cloning CagA deletion and truncation mutants. The grey bar represents the G27 CagA sequence and the bold numbers indicate the first (for forward primers) or last (for reverse primers) base pair amplified by that primer. CagA is 3558 base pairs long. Primers represented by black arrows (➡) contain NdeI cut sites, polka dot arrows (➡) contain XbaI cut sites and dashed arrows (➡) contain PstI cut sites.

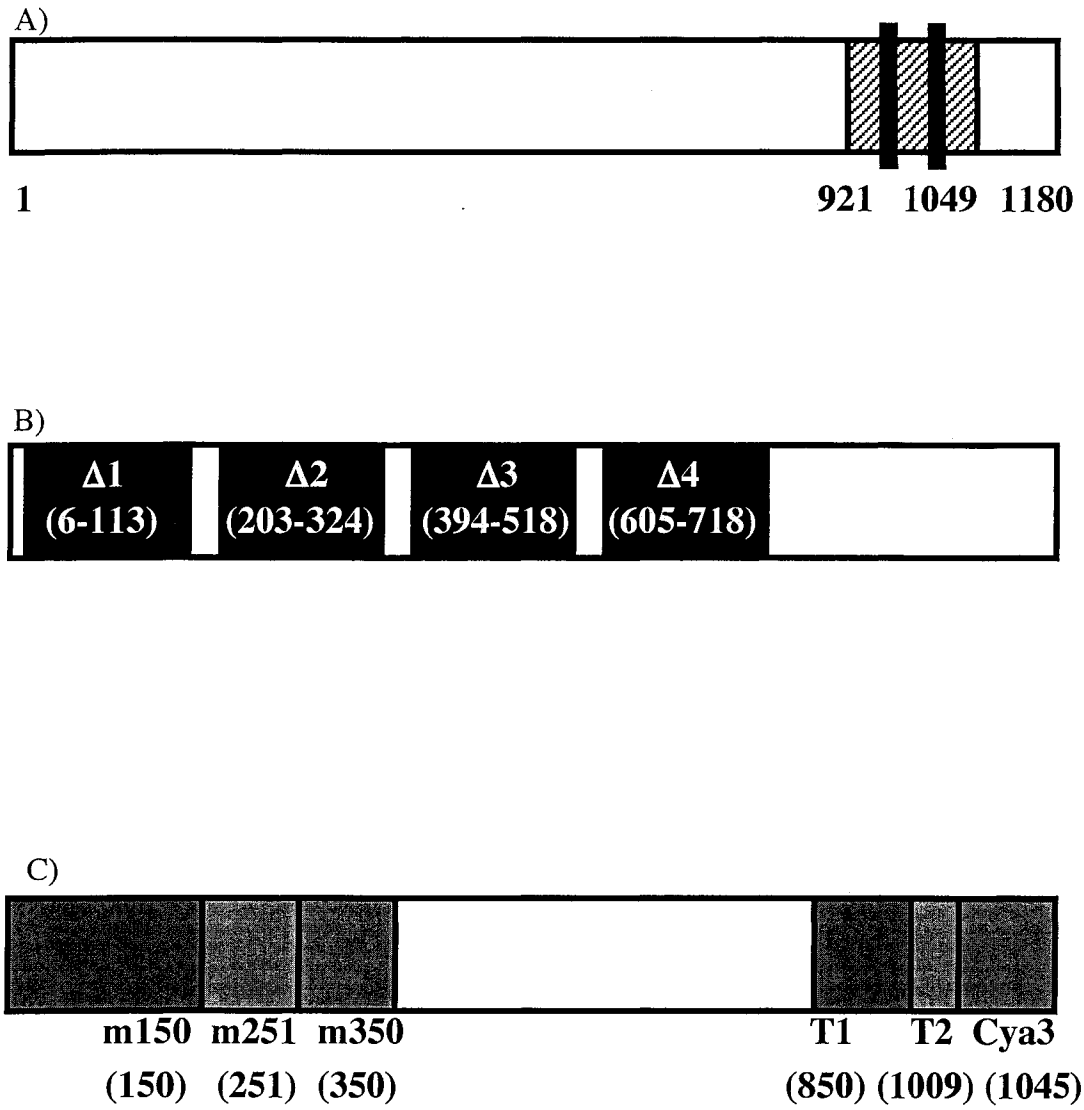


Figure 2.3- Domain structure of the CagA deletion and truncation mutants. A) Wild type CagA from strain G27. The repeat region (amino acids 921-1049) is indicated by the hatched box and the EPIYA motifs are represented by grey bars. Numbers below the bar indicate the amino acids included in each region. B) Deletion mutants. The area excised is black and the amino acids deleted are indicated by the numbers in brackets. C) Truncation mutants. Regions deleted are shaded and the first amino acid of each mutant is indicated by the number in brackets below the bar.

BB plate. The plate was incubated for 3-6 hours and 15 μl of plasmid was pipetted onto the cells. The cells were incubated overnight and then streaked across a BB plate containing 25 μg/ml of kanamycin. Single colonies appeared 4 to 5 days later and were screened for expression.

2.3.3 Western Blotting

Protein lysates for Western blotting were made as follows. Single colonies were streaked across one quarter of a BB plate and incubated for 48 hours. A 10 μl loop was scraped across the plate until it was full and the cells were placed in 100 μl of 1X PBS, vortexed, centrifuged for 7 mins at 2000 rpm at 4°C, and resuspended in 100 μl of 1X PBS. 40 μl of 6X SS was added and the cells were boiled for 10 mins and then frozen at -20°C for future use. Alternatively, a 10 μl loop of cells was grown in 10 ml of liquid BB overnight. Cultures were grown in microaerobic conditions, with shaking at 165 rpm until they reached an OD of 1.5-2.0 at 600 nm. Equivalent numbers of cells were centrifuged for 7 mins at 2000 rpm at 4°C, the supernatant was poured off and the bacteria were resuspended in 100 μl of 1X PBS. Equal numbers of cells were used instead of adding equal amounts of protein because equivalent amounts of cells, not protein, were used for each cAMP assay. 40 μl of 6X SS was added and the cells were boiled for 10 mins and then frozen at -20°C for future use.

Lysates were run on a 10% SDS-PAGE gels and then transferred at 70 V, without exceeding 280 milliamps, for 90 mins onto an Immobilon membrane (Millipore, Bedford, MA, USA). The membrane was blocked in 1X PBS containing w/v 15% skim milk powder at 4°C for a minimum of 12 hours. Membranes were then incubated at room temperature for one hour in PBS with 5% skim milk containing 1:4000 polyclonal rabbit

α-CagA (M. Stein), 1:2500 polyclonal rat α-CagF (M. Stein) or 1:2000 mouse 3D1 (List Biological Laboratories, Hornby, ON, Canada). 3D1 recognizes the distal portion of the catalytic domain of adenylate cyclase. The membrane was washed for five minutes two times in 1X PBS. The secondary antibodies, goat anti-rabbit conjugated to HRP (Sigma-Aldrich) for CagA, goat anti-rat conjugated to HRP (Sigma-Aldrich) for CagF, and rabbit anti-mouse conjugated to HRP (Sigma-Aldrich) for 3D1 were all diluted to 1:5000 in 5% skim milk. Membranes were incubated in the secondary antibody for one hour at room temperature and then washed two times for five minutes in PBS. Blots were developed using the Enhanced Chemiluminescence (ECL) kit (Amersham Biosciences, Piscataway, NJ, USA).

2.3.4 Tissue Culture and Infection

AGS cells (European Çollection of Cell Cultures 89090402), a gastric adenocarcinoma cell line, were grown in RPMI 1640 with 25 mM HEPES and L-glutamine (Invitrogen, Burlington, OT, Canada). The medium was supplemented with 10% (vol/vol) FBS and cells were grown in 600 ml polystyrene tissue culture flasks (Becton-Dickinson, Franklin Lakes, NJ, USA) at 37°C in 5% CO_2. Every 48 hours the cells were split by removing the medium, washing with 13 ml of PBS without calcium or magnesium (Invitrogen, Burlington, OT, Canada) and incubating at 37°C for 5 mins with 4 ml of trypsin. The cells were then divided, placed in four flasks and the final volume was adjusted to 35 ml.

For infection, 1 ml of cells was pipetted into each well in a 24 well plate. An equal number of cells were added to each plate. Cells were counted using a hemocytometer. The cells were incubated overnight at 37°C in 5% CO_2. Then the cells

were gently washed two times with 1 ml of RPMI 1640. 1 ml of RPMI 1640 supplemented with 5% FBS was added to each well along with the bacteria. The plate was incubated at 37°C in 5% CO_2 for 3-4 hours. The infection was halted when the hummingbird phenotype was uniformly seen in wells containing infected cells. Uninfected AGS cells or AGS cells infected with wild type G27 acted as negative and positive controls respectively.

2.3.5 Cell Fractionation

To determine if weakly expressed *cag* PAI proteins were upregulated during AGS cell infection, cell fractionation was performed. AGS cells were infected with wild type G27, mutant G27 expressing CagH-CyaA, G27 expressing Flag tagged CagH (Generously supplied by M. Couturier), and an isogenic CagA-CyaA mutant as a positive control at a multiplicity of infection (MOI) of 100:1. Cells were washed at 4°C three times with 10 ml of 1X PBS containing 2 mM vanadate. Cells from four dishes were scraped and combined in a 15 ml Falcon-tube. Cells were pelleted at 200 X *g* for five minutes and the supernatant was discarded. Cells were resuspended in 8 ml of homogenization buffer (pH 7.4 250 mM sucrose, 3 mM imidazole, 0.5 mM EDTA), spun down at 1500 X *g* for 10 mins, and resuspended in 600 µl homogenization buffer. The cells were mechanically lysed by pushing them through a 0.22 gauge needle 4-5 times. The cells were pelleted again at 1500 X *g* for 10 mins and the supernatant was transferred to a centrifuge tube. The pellet, containing bacteria, unlysed AGS cells and the cytoskeletal fraction was resuspended in 600 µl of 1X SS. The supernatant was centrifuged at 41 000 X *g* for 30 mins and the supernatant was transferred to a 1.5 ml eppendorf. Then 50 µl of 5X SS was added to 200 µl of supernatant containing the host

cell cytosol. Finally, the pellet, containing the host cell membranes, was resuspended in 200 μl 1X SS. The samples were boiled for 7 mins and frozen at -20°C.

2.3.6 Calmodulin Control

Wild type G27 and the isogenic mutant with CagA-CyaA fusion homologously recombined into the recombinase gene were sonicated and incubated with calmodulin. 500 μl of bacterial culture containing 2.5 X 10^8 bacteria in 2 ml eppendorf were sonicated on ice for five 1 min intervals with 10 sec rests at 35% output (Fisher Sonic Dismembrator, amplitude 1.4). 1 μM PMSF and either 12 μM or 1.3μM calmodulin were added and the tubes were incubated on ice for 45 mins.

2.3.7 ELISA Protocol

A commercially available cAMP ELISA kit (Amersham Biosciences) was used to quantify cAMP in infected AGS cells. The manufacturer's instructions were followed with two modifications. Tissue culture was performed using a 24 well plate instead of a 96 well plate and 250 μl of lysis buffer 1B was added to lyse the cells instead of 200 μl.

For each construct, a minimum of two separate AGS cell infections were run per plate. A minimum of two wells per ELISA plate were inoculated from each tissue culture well and the average amount of cAMP produced per infection (a minimum of four wells from two separate infections with the same isogenic mutant) was determined. Each construct was used for a minimum of three separate assays with the exception of CagU-CyaA and CagAmut251-CyaA which were tested twice and Cya3-CyaA which was tested once.

2.4 Results

2.4.1 Expression of Adenylate Cyclase Constructs

Adenylate cyclase was fused to genes in the *cag*PAI and *cagA* mutants to identify novel effector molecules and the translocation domain of CagA. Correct insertion of all twenty-four constructs was verified by restriction endonuclease digestion and PCR. Only fourteen constructs (CagA-CyaA, CagD-CyaA, CagF-CyaA, CagG-CyaA, CagH-CyaA, CagU-CyaA, ORF8-CyaA, CagAmutΔ2-CyaA, CagAmutΔ3-CyaA, CagAmutΔ4-CyaA, CagAmutT1-CyaA, CagAmutT2-CyaA, CagAmut251-CyaA and CagAmutCya3-CyaA) expressed protein as determined using a monoclonal anti-AC antibody, 3D1, in *H. pylori* (Figures 2.4 and 2.5). Of these fourteen constructs, three (CagH-CyaA, CagG-CyaA and CagAmutCya3-CyaA) expressed protein very weakly and it could only be detected on western blots that had been overexposed. The CagH-CyaA strain was incubated with AGS cells to determine if protein expression is upregulated during cell infection. The fusion protein of approximately 80 kDa was barely detectable in the insoluble fraction containing the bacteria (Figure 2.6). CagH-CyaA was not detected in any of the host cell fractions indicating it was not translocated.

Levels of wild type protein expression were compared to two fusion proteins, CagA-CyaA and CagF-CyaA, which expressed well. Levels of wild type CagF and CagA expression were much higher than expression from the other constructs (Figure 2.7). The anti-CagF western blot had to be overexposed for 30 mins before the CagF-CyaA (~72 kDa) fusion protein could be detected.

At least three attempts were made to transform the remaining ten constructs (CagC-CyaA, CagI-CyaA, CagL-CyaA, CagT-CyaA, ORF16-CyaA, VirB9-CyaA,

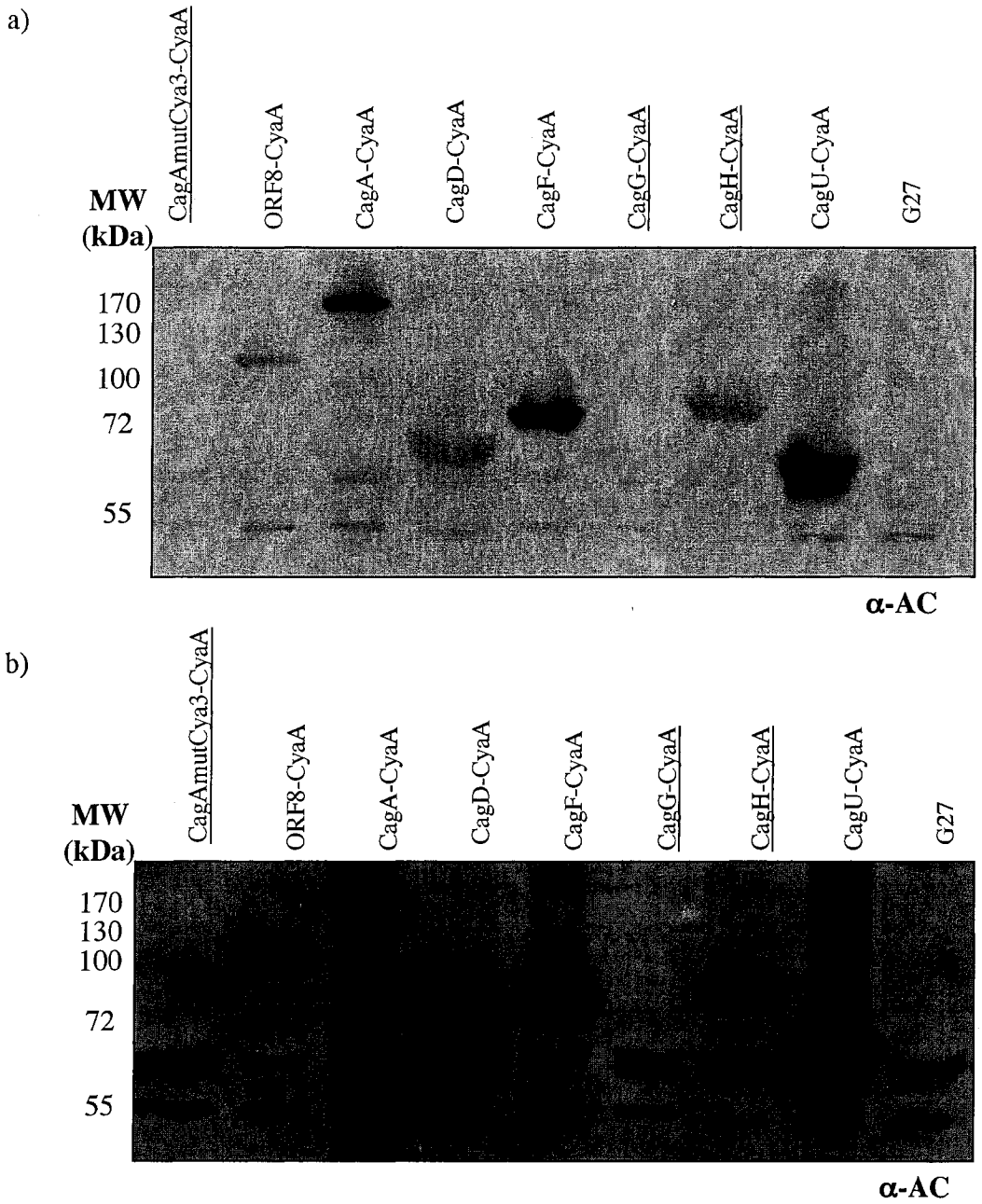


Figure 2.4 - Western blot analysis of CyaA fusions: *H. pylori* lysates were separated by SDS PAGE and proteins transferred to PVDF membranes. CyaA fusion proteins were visualized with a monoclonal anti-CyaA antibody. (a) was exposed for 45 secs and (b) was overexposed for 30 mins to confirm expression of CagAmutCya3-CyaA (~62kDa) and CagG-CyaA (~64kDa). Two times as many cells was loaded in underlined lanes.

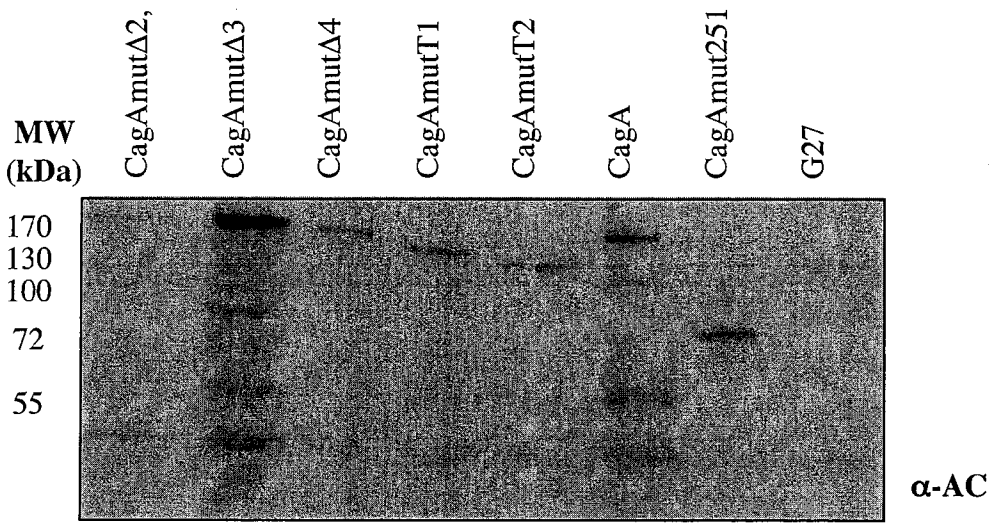


Figure 2.5 - Western blot analysis of CagA and CagA mutant CyaA fusions: *H. pylori* lysates were separated by SDS PAGE and proteins transferred to PVDF membranes. CyaA fusion proteins were visualized with a monoclonal anti-CyaA antibody.

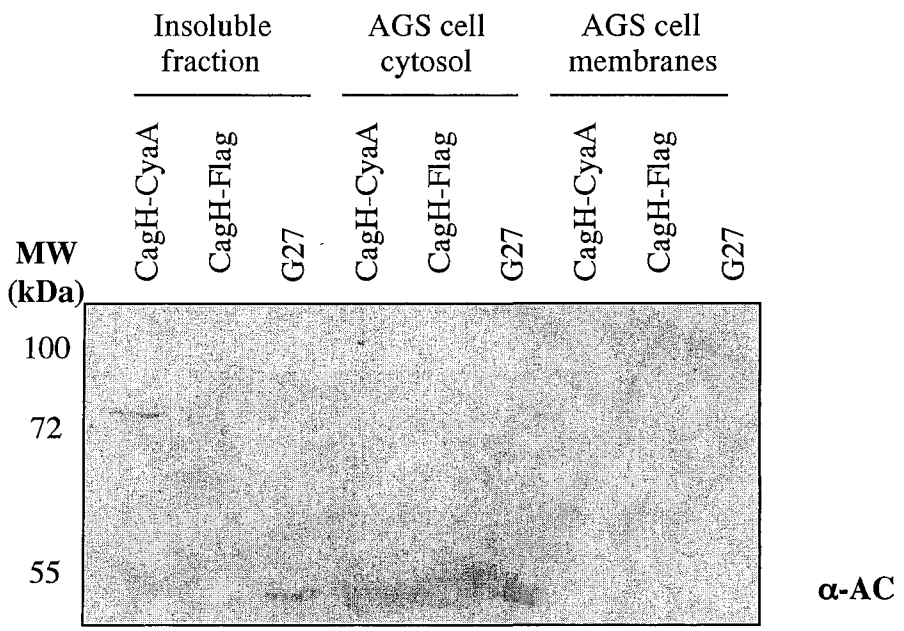


Figure 2.6- Cell fractionation of AGS cells infected with *H. pylori*. Cell fractions were separated by SDS PAGE and proteins transferred to PVDF membranes. CyaA fusion proteins were visualized with a monoclonal anti-CyaA antibody. Cell fractions include the insoluble fraction containing the bacteria, the AGS cell cytosol and the AGS cell membranes. An identical fractionation experiment was preformed using CagA-CyaA as a positive control.

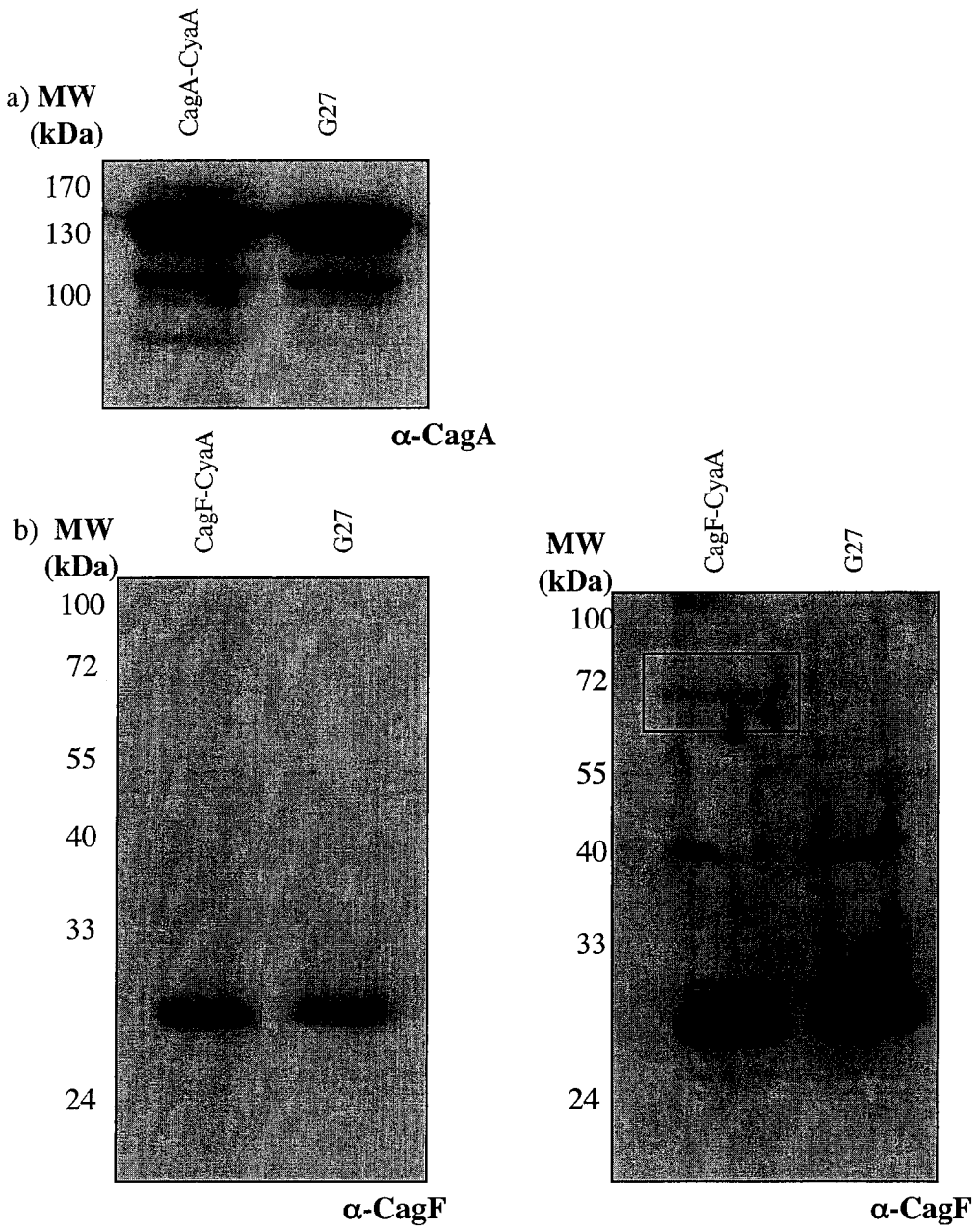


Figure 2.7 - Western blot analysis of CyaA fusions: *H. pylori* lysates were separated by SDS PAGE and proteins transferred to PVDF membranes. CyaA fusion proteins were visualized with polyclonal anti-CagA and anti-CagF antibodies. a) Anti-CagA western blot. The CagA-CyaA fusion protein is slightly larger than wild type CagA and much less abundant. b) Anti-CagF western blot exposed for 45 seconds and 30 mins. The CagF-CyaA fusion protein is 70 kDa, outlined, and is only visible after 30 mins.

VirD4-CyaA, CagAmutΔ1-CyaA, CagAmut150-CyaA and CagAmut350-CyaA) into *H. pylori*. Three attempts were also made to transform CagA-CyaA into an isogenic G27 CagA null mutant (strain courtesy of M. Stein). No kanamycin resistant colonies were obtained during ORF16-CyaA transformations. This construct was also very difficult to clone in *E. coli* and may have some toxic properties. Multiple resistant colonies grew for all of the other fusion proteins, but none expressed CyaA (Figure 2.8). Three of these were CagA mutants, two in the N terminus of the protein suggesting that this region may play an important role in protein stability. Homologs of the *vir* T4SS (CagT, VirB9 and VirD4) also were not expressed as fusion proteins in *H. pylori*.

2.4.2 Adenylate Cyclase Activity Control

Bacterial lysates were incubated with CaM to insure that the CyaA fusions were catalytically active. The isogenic CagA-CyaA mutant displayed 3.95 times higher relative activity at 1.3 μM CaM and 4.46 times relative activity at 12 μM CaM than wild type G27. These results suggest that CaM is activating the CyaA portion of the fusion protein leading to the production of cAMP. A concentration of cAMP could not be determined because the reaction could not be completed in the lysis buffer supplied with the kit preventing subtraction of the background and comparison to the standard curve.

2.4.3 Adenylate Cyclase as a Reporter Gene in *H. pylori* AGS cell infection

AGS cells were infected with isogenic mutants expressing *cag*PAI proteins or *cagA* deletion and truncation mutants fused to AC. AC fusion proteins translocated into host cells will result in an increase in intracellular cAMP. Uninfected AGS cells and AGS cells infected with G27 were the negative controls and CagA fused to AC was the positive control. An initial test with a cell density of 200 000 cells/well and an MOI of

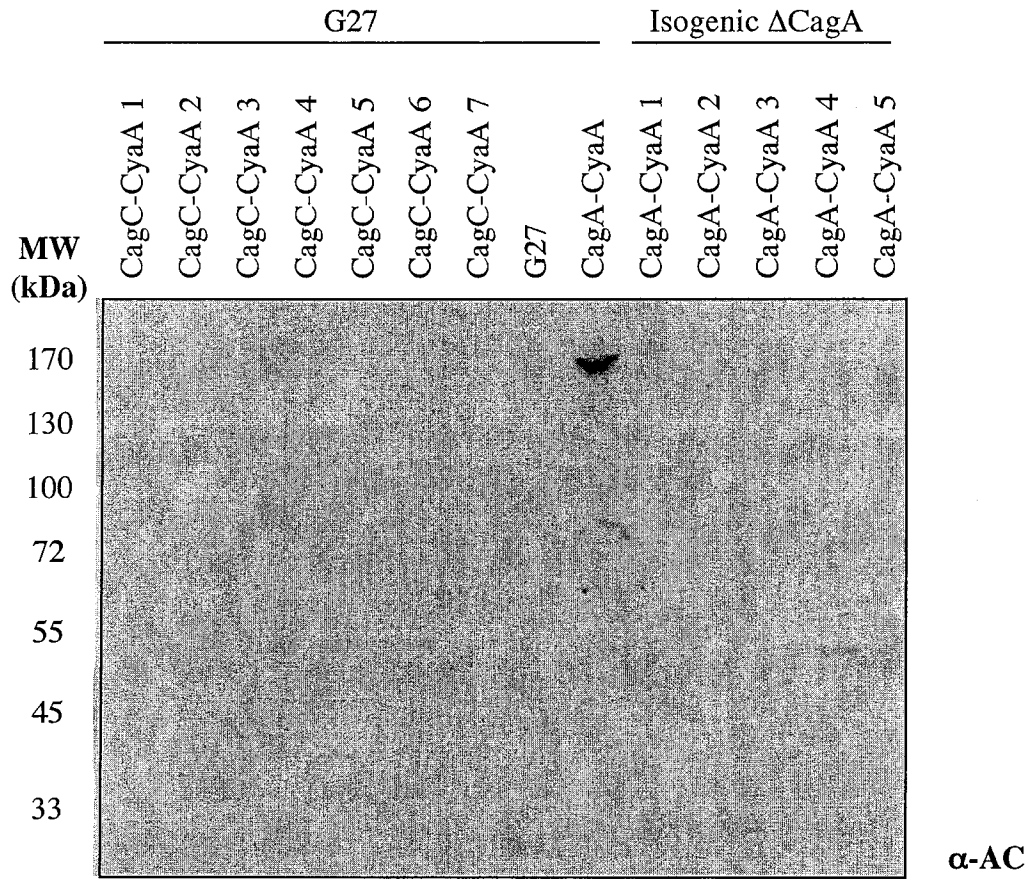


Figure 2.8- Western blot analysis of CyaA fusions: *H. pylori* lysates were separated by SDS PAGE and proteins transferred to PVDF membranes. CyaA fusion proteins were visualized with a monoclonal anti-CyaA antibody. These constructs did not express and the background band at 55 kDa can be seen in each lane if the blot is over exposed. Wild type G27 is the negative control and CagA-CyaA is the positive control.

400:1 was completed to confirm the system was functional in *H. pylori*. The initial run included CagA-CyaA as a positive control, ORF8-CyaA an unknown, G27 as a negative control, and AGS cells alone. To insure that different colonies expressing the same fusion protein induced similar levels of cAMP in host cells, two isogenic mutants expressing CagA-CyaA were compared. Uninfected, G27 infected and ORF8-CyaA infected cells contained 84.2, 35.9, 89.0 fmol cAMP respectively while the two CagA-CyaA isogenic colonies produced 1358 and 1242 fmol. This suggests that CagA is positive for translocation and ORF8 is not.

Isogenic mutants containing the CyaA constructs that were expressed well (CagA-CyaA, CagD-CyaA, CagF-CyaA, CagU-CyaA, ORF8-CyaA, CagAmutΔ2-CyaA, CagAmutΔ3-CyaA, CagAmutΔ4-CyaA, CagAmutT1-CyaA, CagAmutT2-CyaA andCagAmut251-CyaA) were infected at a MOI of 200:1 at an AGS cell density of 250 000 cells/well. CagA (3463 fmol cAMP) and CagAmutΔ3 (2354 fmol cAMP) were the only clones that appeared positive for translocation (Figure 2.9). This suggests that base pairs 1201-1572 of CagA are not required for translocation. The amount of cAMP in the G27 infection (254 fmol) was again lower than AGS cells alone (487 fmol).

Since CagG-CyaA, CagH-CyaA and CagAmutCya3-CyaA expressed weakly the experiment was performed with an increased MOI of 2750:1 and a cell density of 200 000 cells/well. At this MOI the amount of cAMP produced correlates with the expression level of the construct (Figures 2.4 and 2.5). CagF-CyaA and CagU-CyaA were negative for translocation at a MOI of 200:1, but at the increased MOI were positive producing 1267 and 1370 fmol cAMP/ per well, respectively (Figure 2.10). CagAmutΔ3 and CagA still appeared to be positive for translocation. Since an MOI that is too high may produce

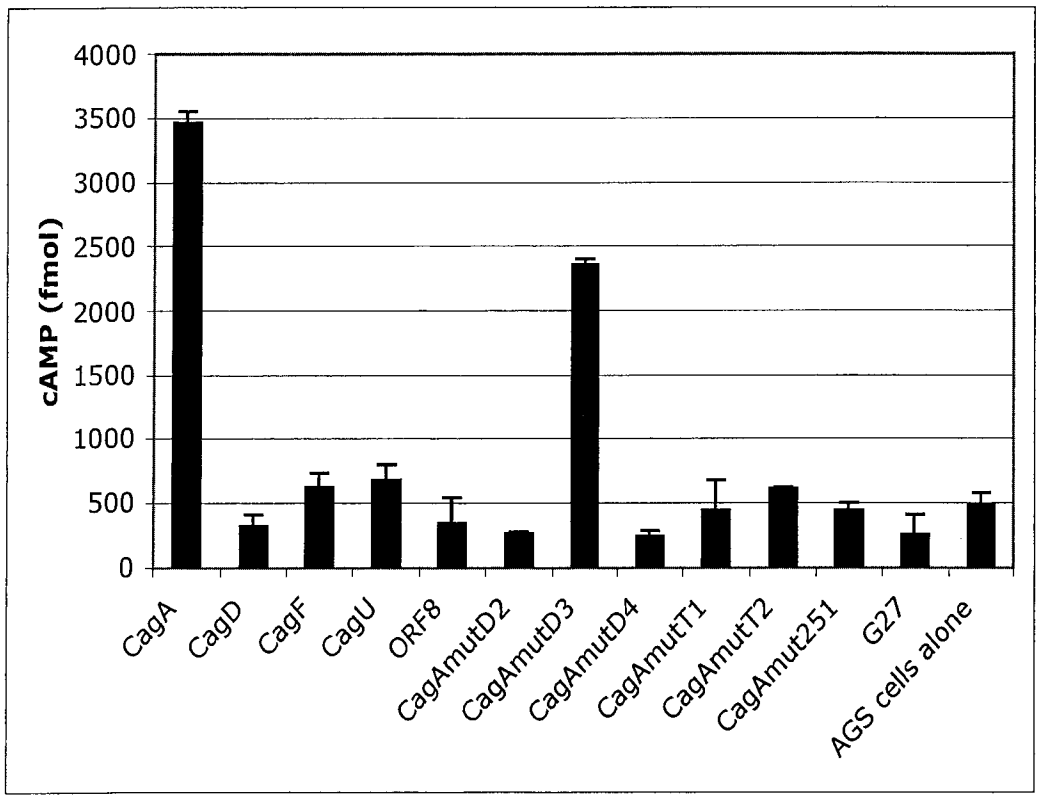


Figure 2.9 - Amount of cAMP produced by AGS cells infected with isogenic G27 mutants expressing genes from the *cag* PAI or *cagA* mutants fused to adenylate cyclase at a MOI of 200:1. Error bars represent the standard deviation.

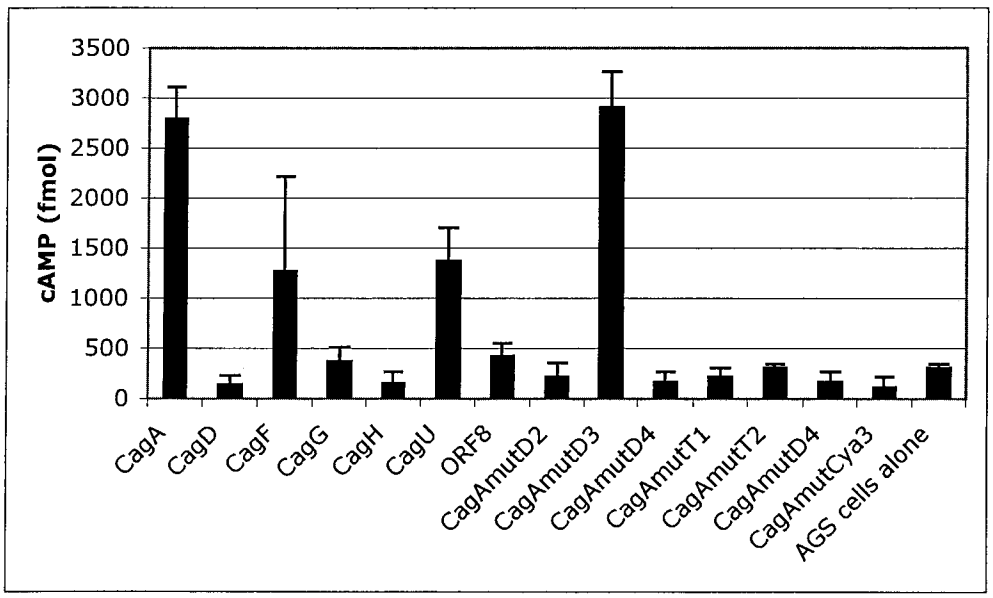


Figure 2.10 - Amount of cAMP produced by AGS cells infected with isogenic G27 mutants expressing genes from the *cag* PAI or *cagA* mutants fused to adenylate cyclase at a MOI of 2750:1. Error bars represent the standard deviation.

false positives it did not seem wise to perform additional experiments at even higher MOIs.

2.5 Discussion

This study demonstrated that adenylate cyclase could be successfully used as a reporter gene to identify effector molecules transported by the *cag* PAI encoded T4SS as long as protein expression is consistent. CagA was correctly identified as a translocated effector molecule, but no novel translocated proteins were identified. Using a series of *cagA* mutants it was possible to identify 371 bp in the central region of the protein (amino acids 400-524) that are not necessary for CagA translocation into host cells. Unfortunately there was a lack of expression of some constructs, variable expression levels of the constructs that did express the fusion protein, and expression of the wild type protein far exceeds expression of the fusion protein. The MOI of infection was increased to try to compensate for poor expression, but increasing the MOI too would appeared to produce false positive results. To unequivocally state whether other Cag proteins are also effector molecules expression of the CyaA fusion proteins would need to be optimized.

Unfortunately, ten fusion proteins (CagC-CyaA, CagI-CyaA, CagL-CyaA, CagT-CyaA, ORF16-CyaA, VirB9-CyaA, VirD4-CyaA, CagAmutΔ1-CyaA, CagAmut150-CyaA and CagAmut350-CyaA) were not expressed in *H. pylori*. Little is known about CagC, CagI and CagL. Work in our laboratory has shown that these constructs can be Flag-tagged and stably expressed in *E. coli*. The three *vir* homologs VirB9, VirD4 and CagT (VirB7) are likely to be integral components of the T4SS. Since we do not have an

antibody for these cag proteins it is not possible to say, whether or not they are indeed expressed. Moreover it is possible that post-transcriptional processing may have resulted in cleavage of the fusion protein.

It was not possible to clone ORF16 into the PCR blunt cloning vector. Since this vector has an active promoter, the inability to clone ORF16 may have been due to toxicity of this protein in *E. coli* as abnormal colony growth was observed. Replated single colonies grew very thickly and were sticky. Another possibility is that expression may be contact dependent. CagY expression is contact dependent (Rohde *et al.* 2003), although this is not the case for CagF (Seydel *et al.* 2002). Since the CagG and CagH fusion proteins were expressed poorly relative to the other *cag* proteins, their expression may be contact dependent too. This possibility was explored using CagH-CyaA because CagG-CyaA is the same size as a background band in western blots. Since CagH-CyaA is not expressed from its natural promoter only contact dependent protein stability can be analyzed. The instability of CagH-CyaA protein expression does not appear to be contact dependent. Further investigation would require a CagH antibody. Finally, deletion or truncation mutants of CagA may affect protein stability leading to dramatic reductions in protein expression.

Difficulty in obtaining transformants could also be credited to CyaA toxicity. CyaA toxicity has been documented for a few species of bacteria including *Y. enterocolitia* (Sory and Cornelis 1994). Sory and Cornelis induced fusion protein expression when they used CyaA as a reporter gene to circumnavigate this problem (1994). Their constructs were encoded by a multi-copy plasmid pMS111. Using this method YopE-CyaA was detectable and capable of increasing cAMP levels one hour post

induction. However, post induction pMS111 was unstable and reduced bacterial virulence. Potential toxicity of CyaA in *H. pylori* needs to be further investigated.

The greatest problem encountered with this methodology was inconsistent levels of protein expression between constructs. Compensating for this by increasing the number of bacteria used for infections with weakly expressed constructs is not an option because the bacterium interferes with AGS cell signaling processes. Therefore it could not be determined if increases in cAMP were due to construct translocation or the MOI.

Another significant problem is that wild type protein expression dramatically overshadowed the amount of fusion protein produced. To overcome this problem Choy *et al.* (2004) knocked out the gene of interest in *Salmonella typhimurium* and then complemented it with the fusion protein. This complementation approach led to increased amounts of cAMP produced by translocated substrates resulting in greater discriminatory powers. Knockouts with minimal downstream effects in *H. pylori* can be achieved using homologous recombination to effect replacement mutagenesis. This procedure was used successfully in *H. pylori* without affecting secretion system biogenesis by Fischer *et al.* (2001).

A combination of the procedures used by Choy *et al.* (2004), who knocked genes out and complemented them in *S. typhimurium*, and Sory and Cornelis (1995), who induced expression of their fusion proteins from a multi-copy plasmid, may improve the discriminatory power of this assay. Selbach *et al.* (2002b) used the *E. coli/H. pylori* shuttle vector pHe12 to complement CagA and VirD4 expression. pHel2 is not functional in G27 so either another strain or shuttle vector would need to be used (M.Stein, personal communication). Other *E. coli/H. pylori* shuttle vectors, such as pBHP489K are

available, but have not been used in G27 (Lee *et al.* 1997). This system would be an improvement on the methodology used in this screening assay because cloning could still be done in *E. coli*, induction of expression would allow for a more active promoter to be used, constructs would not have to compete with wild type protein for translocation and a multi-copy plasmid would allow for greater protein expression.

Various portions of CagA were fused to CyaA to identify the region required for translocation. It was possible to show that amino acids 400-524 are not required for secretion. In contrast, secretion signals in the *vir* system have been discovered in the C termini of VirE2 (Simone *et al.* 2001) and VirF (Vergunst *et al.* 2000). Identifying the signal sequence necessary for translocation could also be important in determining if reporter genes should be fused to the C-terminus or N-terminus. Nagai *et al.* determined that the secretion signal is in the C terminus of RalF from *Legionella pneumophila* (2005). It was determined that a CyaA-RalF construct led to the production of five times more cAMP than RalF-CyaA (Nagai *et al.* 2005). As a result, production of cAMP by CyaA fused to the N terminus of CagA was compared with CyaA fused to the C terminus. The C terminal fusion protein had very weak expression and was not positive for translocation.

Adenylate cyclase may not be a good system for defining secretion domains. Three CagA mutants were not expressed and the other seven exhibited varying levels of protein expression. Sory *et al.* (1995) found that even though a series of YopE mutants fused to CyaA were all translocated they produced varying amounts of cAMP (0.5 nmol/mg-1.5 nmol/mg). It is not known if this is due to a higher secretion efficiency or steric interference of the CyaA active site.

In addition to the problems discussed above, no clearly defined standard has been determined in previous studies to identify what level of cAMP production constitutes a translocated substrate. Effector molecules in some studies, for example YopE examined by Sory and Cornelis (1995), produced one thousand times more cAMP than negative controls. In contrast, SseK2 was designated as a translocated substrate even though the positive control produced seven times more cAMP (Choy *et al.* 2004). A series of recommended standards needs to be generated before this assay can be used to define what constitutes translocation. In addition, one study contained no negative control fused to adenylate cyclase (Choy *et al.* 2004). Their only negative control was the wild type *S. enterica*. This is not adequate since CyaA has low-level activity without calmodulin. Is SseK2 positive because some cAMP was produced in the bacterium 21 hours post infection or because the fusion protein was translocated? Other reporter genes that have been used to identify secreted substrates including Cre (Schulein *et al.* 2005) and TEM-1 β-Lactamase (Charpentier and Oswald 2004) may be more useful. GFP variants designed for prokaryotes (Josenhans *et al.* 1998) or a biotinylation tag (Beckett *et al.* 1999, Cronan 1990) could also be used. Each system has different advantages. For example, fusion proteins could be visualized using GFP, whereas the biotinylation tag is smaller and would potentially interfere less with the T4SS.

One problematic finding was that cAMP levels were higher in AGS cells alone than in AGS cells infected with G27. It has been previously determined that *H. pylori* infection of gastric mucosal epithelial cells (GMEC) transiently increases the concentration of calcium ions (Marlink *et al.* 2003). Mediation of the concentration of calcium ions, which CaM requires to activate AC, leads to an increase in intracellular

cAMP (Beil *et al.* 1998). cAMP levels of infected cells may have been lower than non-infected cells if bacterial infection may lead to AGS cell lysis. Qualitative observation supports this hypothesis. In the center of infected wells there were occasionally areas with no adherent cells at the conclusion of the 3 hour infection period.

To summarize, adenylate cyclase, CyaA, shows promise as a reporter gene in *H. pylori*. This system correctly identified CagA as a translocated molecule. A deletion mutant of CagA was also successfully translocated suggesting base pairs 1201-1572 are not necessary for translocation. Caution must be taken in interpreting results obtained with this system because at high cell densities or MOIs false positives may be generated. This system should only be used to indicate which molecules may be translocated and not to exclude negatives as possible translocated effector molecules. Proteins may appear not to be translocated if the reporter gene is removed during post-transcriptional processing, the active site of the reporter gene is not available due to steric interference or the protein associates with the membrane in an orientation that would force adenylate cyclase out of the cytoplasm and into the plasma membrane.

Chapter Three

Characterization of Virulence Factors Expressed by *Helicobacter pylori* Isolated from Pediatric Patients

Portions of this work will be part of the following publication:

Skipper, V.E., Simala-Grant, J.L, Keelan, M., Bourke, B., Drumm, B., Stein, M., and Taylor, D.E. Characterization of *Helicobacter pylori* clinical isolates from symptomatic children: Virulence factor expression and antibiotic susceptibility. *Infection and Immunity*. In preparation.

3.1 Preface

Significant research effort has been directed towards determining virulence factors and understanding the immunological response in adult patients infected with *H. pylori*. The clinical significance of infection in the pediatric population remains poorly understood. It is believed that several virulence factors such as the adhesin BabA, the cytotoxin VacA, the effector molecule CagA and the potentially immunomodulatory Lewis antigens may not play as significant a role in pediatric infection as in adult infection. A study of virulence factors produced by *H. pylori* isolates from symptomatic pediatric patients was undertaken. Lewis antigen expression in pediatric patients was significantly different from asymptomatic and symptomatic adults, suggesting Lewis antigens may play a different role in pediatric patients. Lewis X expression was correlated with CagA expression and the *iceA1*+ genotype. Isolates that express Lewis X and Lewis Y were also significantly correlated with CagA expression.

3.2 Introduction

3.2.1 General Introduction

H. pylori was first identified as a causative agent of pediatric gastritis in 1986 (Hill *et al.* 1986). At this time many epidemiological studies on adults had been published, but there were few pediatric studies. Even now, the number of publications on adult disease still far exceeds the number of pediatric studies even though the clinical manifestations, cellular response and important virulence factors differ between the two groups.

Childhood infection is widespread, but the mode of transmission is unknown. In the developing world the majority of children under 10 years of age are colonized by *H. pylori* (Megraud *et al.* 1989). Although children in underdeveloped countries have very high infection rates, even children in developed countries are at risk for infection. For example, in rural villages near Maiduguri, Nigeria, 82% of children aged 5-9 are *H. pylori* positive (Holocombe *et al.* 1992), while 17% of school age children in London (O'Donohoe *et al.* 1996) and 30% of a population of Italian children (Dominici *et al.* 1999) were positive for infection. The mode of transmission of infection in unknown, but low socioeconomic status is a risk factor (Malaty *et al.* 2001, Lin *et al.* 1999, Mitchell *et al.* 1992). Additionally, crowded living conditions (Malaty *et al.* 2001, Sherman and Macarthur 2001, Lin *et al.* 1999, Mitchell *et al.* 1992), multiple siblings (Yang *et al.* 2005, Yang *et al.* 2003, Goodman and Correa 2000) and an *H. pylori* positive mother (Yang *et al.* 2005, Sherman and Macarthur 2001) increased the risk of acquiring *H. pylori*. For example, children with *H. pylori* infected mothers were 4.6 times more likely to acquire *H. pylori* (Yang *et al.* 2005).

3.2.2 *H. pylori* Associated Clinical Manifestations in Children

Infection usually occurs in early childhood (Nogueira *et al.* 2004) and like adults, most children are asymptomatic. However, symptomatic pediatric patients display different clinical manifestations from adults (Chelimsky and Czinn 2000, Celik *et al.* 1998). Association of duodenal ulcers, peptic ulcers and lymphomas with *H. pylori* infection are extremely rare in children (Krauss-Etschmann *et al.* 2005, Chelimsky and Czinn 2000, Torres *et al.* 2000). These manifestations, in addition to non-ulcer dyspepsia, are more common in symptomatic adults (Torres *et al.* 2000, Gold 1999). Additionally, children exhibit novel manifestations, but clinical data is limited and attributing these symptoms to *H. pylori* is controversial. Both the immediate and long term health consequences of childhood *H. pylori* infection are not well understood. Progress is hampered by inaccurate diagnosis of infection in early childhood (Kindermann *et al.* 2000).

Chronic gastritis is associated with infection in all ages, but the majority of macroscopic nodular gastritis detected in the antral mucosa is observed in children (Kato *et al.* 1997, Sorrentino *et al.* 1996, Ashorn *et al.* 1994, Hassall and Dimmick 1991, Czinn *et al.* 1986). Like adults, chronic gastritis can be reversed with eradication of the bacteria (Yeung *et al.* 1990). Unlike adults, where men exhibit more severe gastric pathology than women, male and female children are equally likely to develop symptomatic gastritis (Blecker *et al.* 1994).

Approximately ten percent of infected adults develop duodenal ulcer, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma (Dunn *et al.*

1997, Blaser *et al.* 1995a), but *H. pylori* associated ulcers and cancers are rare in children. The latter observation is expected since it is the long term chronic infection that is believed to increase one's risk of the development of gastric cancer. Peptic ulcers are uncommon, but recurrent abdominal pain (RAP) associated with *H. pylori* infection is a predictor for development of a pediatric peptic ulcer (Gormally *et al.* 1995). RAP alone is not enough to suspect *H. pylori* infection since only one case study has found a positive correlation between RAP and *H. pylori* (Chong *et al.* 1995) and several clinical studies have documented no correlation (Macarthur 1999, Hardikar *et al.* 1996, O'Donohue *et al.* 1996, Macarthur *et al.* 1995, McCallion, *et al.* 1995, Van der Meer *et al.* 1995). Duodenal ulcers are rare in children less than ten years of age (Torres *et al.* 2000), but are correlated with infection (Drumm *et al.* 1990). Gastric ulcers are much less common than duodenal ulcers in children (Chong *et al.* 1995, Gold 1999). Only 25% of childhood gastric ulcers are associated with the presence of *H. pylori*, the majority being attributed to aspirin or non-steroidal anti-inflammatory drugs (NSAIDS) (Torres *et al.* 2000).

There are few documented cases of *H. pylori* related cancers in children. However, early onset of infection dramatically increases the risk of developing gastric cancer as an adult (Chelimsky *et al.* 2001, Torres *et al.* 2000, Blaser *et al.* 1995a). Associated cancers include gastric adenocarcinoma and MALT or Burkitt's lymphoma. Due to the rarity of the condition, the causative role of *H. pylori* in development of pediatric cancers is not well defined. In a study of 135 pediatric patients with non-Hodgkins Lymphoma (NHL) only two had primary gastric NHL and only one of these patients was positive for infection with *H. pylori* (Moschovi *et al.* 2003).

Iron deficiency anemia (IDA) may be a common extragastrointestinal manifestation of *H. pylori* infection in children. Low serum ferritin and hemoglobin levels have been documented to increase upon eradication of the pathogen (Choe *et al.* 2000, Konno *et al.* 2000, Mutsuko *et al.* 2000, Annibale *et al.* 1999, Barabino *et al.* 1999, Choe *et al.* 1999, Maliyevsky and Nijevitch 1996, Brueil *et al.* 1993, Dufour *et al.* 1993). Iron is an essential element during growth and untreated anemia could lead to growth retardation or impaired cognitive function (Deinard *et al.* 1986, Oski 1979). Anemia impacts young girls more severely because females have higher iron requirements than males (Konno *et al.* 2000). Yang *et al.* found that *H. pylori* infected girls had significantly lower ferritin levels than uninfected, age and sex matched controls (2005). It is not known why infection causes anemia, but the most likely explanations are decreased iron absorption due to reduced acid secretion or a reduced pool of available iron due to iron uptake by the pathogen (Sherman and Macarthur 2001, Chelimsky and Czinn 2000, Konno *et al.* 2000). Lower serum ferritin levels and IDA have also been observed in colonized adults (Milman *et al.* 1998, Marignani *et al.* 1997).

Childhood *H. pylori* infection has been correlated with growth retardation and infantile failure to thrive. Other pathogens that cause chronic infections have also been linked to reduced growth rates (Stephensen 1999). Decreased linear growth, compared to age matched controls, was observed for infants greater than six months of age who were infected with *H. pylori* by Yang *et al.* (2005), but decreases in the rate of weight gain were only observed in children older than two by Passaro *et al.* (2002). The weight of infected children was reduced by an average of 24% and the height by 31% compared with noninfected controls (Passaro *et al.* 2002). In a study of 347 infants monitored for

two and a half years, infection resulted in a 0.042 cm/month reduction in growth velocity. A correlation between infection and slower growth has also been supported by Bravo *et al.* (2003), Passaro *et al.* (2002), Demir *et al.* (2001), Dale *et al.* (1998), Fall *et al.* (1997), Perri *et al.* (1997), Patel *et al.* (1994) and Raymond *et al.* (1994). Again, girls were more adversely affected resulting in a greater attenuation of growth compared to age matched boys (Passaro *et al.* 2002, Patel *et al.* 1994). Conversely, Rothenbacter *et al.* (2000) and Sullivan *et al.* (1990) did not find a significant correlation between *H. pylori* infection and growth retardation. Quinonez *et al.* (1999) and Oderda *et al.* (1998) only noted a small effect on growth reduction after socioeconomic status was accounted for. Conflicting results may be due to not comparing individuals that became infected at the same age. Growth retardation may be the result of iron deficiency, gastric inflammation, dyspepsia, diarrhea or decreased nutritional intake (Yang *et al.* 2005).

Diarrhea and malnutrition are the main postulated pediatric symptoms of *H. pylori* infection. Clinical studies have documented a correlation between diarrhea and *H. pylori* (Rothenbacher *et al.* 2000, Sullivan *et al.* 1990), but Bravo and colleagues (2003) and Castro-Rodriquez *et al.* (1999) found no correlation between infection and diarrhea. This clinical manifestation is especially problematic in the developing world where childhood diarrheal diseases have high mortality rates. Diarrhea may be due to hypochloridia, a loss of the gastric acid barrier, resulting in secondary infection from enteric pathogens (Dale *et al.* 1998, Howden and Hunt 1987). Malnutrition, likely related to diarrhea and secondary enteric infections, is also a clinical outcome of pediatric infection by *H. pylori* (Bravo *et al.* 2003, Sullivan *et al.* 1990).

Progress in understanding the clinical significance of infection by *H. pylori* in young children is hampered by the lack of a reliable method to determine if the child is infected. The urea breath test (UBT) is the most popular method of detection, but it is not recommended for use in children less than six years of age. In a study of 1499 children, all under 18 years old, individuals less than six years old had a higher proportion of inconclusive results and false positives (Kindermann *et al.* 2000). More research needs to be done to determine if other methods such as the stool antigen test are more reliable.

Pediatric clinical manifestations differ from those of adults. Understanding childhood symptoms will help to understand the early infection process, which could lead to the development of novel therapeutics. Additionally, problems caused by secondary infections could result in increased morbidity in developing countries. Symptoms that significantly impact the overall health of the individual need to be identified so infection can be eradicated in these individuals.

3.2.3 Pediatric Immune Response

Post-colonization there is a local and systemic immune response. Clinical and histopathological observations suggest the immune response is different in children than adults (Krauss-Etschmann *et al.* 2005). More is known about the adult immune response, but investigators are only beginning to study the pediatric response. The reduced occurrence of severe pathologies such as ulcers (Torres *et al.* 2000, Gold 1999) and the ability of children to spontaneously clear infections (Granstrom *et al.* 1997, Klein *et al.* 1994) are likely due to these differences.

In adults the immune response is characterized by an inflammatory infiltrate in the lamina propria consisting of neutrophils, monocytes, lymphocytes, macrophages,

eosinophils and plasma cells (Crabtree 1996, Dixon 1995, Kuipers *et al.* 1995a, Kuipers *et al.* 1995b, Genta and Graham 1994, Getna and Hamner 1994). Gastric epithelial cells secrete IL-8 that attracts polymorphonuclear cells (PMNs) (Galustian *et al.* 2003) and activates the Th-1 immune response (Dixon 1994). Major histocompatibility class two (MHC class II) antigens are expressed on epithelial cells (Ermak *et al.* 1998, Dixon 1994) and anti-*H. pylori* IgA and IgG antibodies are produced (Crabtree *et al.* 1993a). CD25+ cells are an integral component of the adult immune response (Seifarth *et al.* 1996). Different groups have documented CD4+ and CD8+ T cells in the lamina propria (Sommer *et al.* 1998, Ye *et al.* 1997, Hatz *et al.* 1996, Hood and Lesna 1993). More research needs to be done to determine the nature of the T cell response. Histologically, there are signs of chronic inflammation including superficial epithelial cell injury (Dixon 1995).

The degree of gastritis and extent of colonization are significantly lower in children (Meining *et al.* 1996). Although the number of neutrophils is increased during pediatric infection there are significantly fewer than in an adult infection (Torres *et al.* 2000, Whitney *et al.* 1998, Ashorn 1995, Husson *et al.* 1995, Quieroz 1991). Nevertheless, there are also substantially fewer plasma cells and eosinophils (Torres *et al.* 2000). There is no significant difference in the number of lymphocytes. In children, peripheral blood monocytes are the most abundant antigen presenting cells, but colonization and subsequent infection still results in an increase in T cells (Crabtree *et al.* 1993a). CD4+ and CD8+ T cells are present, in addition to B cells, CD23+ and CD1a/b+ cells (Krauss-Etschmann *et al.* 2005). There are significantly fewer CD25+ cells (Seifarth *et al.* 1996). CD1a/b+ is indicative of dendritic cells that may play an immunomodulatory

role in *H. pylori* infection. There is no significant difference in the number of dendritic cells found in the gastric tissue of adults and children gastric tissue (Krauss-Etschmann *et al.* 2005, Dixon 1994).

The role and abundance of macrophages in the anti-*H. pylori* immune response is not well described. Krauss-Etschmann *et al.* determined that macrophages are rare compared to other cell types and there is no difference in macrophage levels between adults and children (2005). Dixon (1995) found that macrophages were a component of the adult inflammatory infiltrate and Torres and colleagues (2000) determined that macrophages played an important role in the pediatric immune response.

The most convincing evidence of a differential immune response is the ability of children to spontaneously clear an infection. This is a frequent occurrence in childhood, but is rarely observed in adults (Granstrom *et al.* 1997, Klein *et al.* 1994). Spontaneous clearance occurs primarily between 6 and 30 months (Klein *et al.* 1994). In a survey of 101 children aged three to twelve months, 73 were positive and reverted to negative and 22% of these children were later reinfected (Dale *et al.* 1998). It is not known if the ability to clear infection is the result of a different immune response, differential expression of host cellular receptors or differences in the infecting strains of *H. pylori.* Young children have reduced numbers of immunocompetent lymphocytes and plasma cells compared with adults (Torres *et al.* 2000, Gold 1999). A less developed immune system may allow children to subvert *H. pylori* immunomodulation that favors persistence.

3.2.4 Virulence Factors

3.2.4.1 Factors Essential For Colonization

Urease production and motility are essential for survival and colonization (Blaser *et al.* 2004). Urease catalyzes the hydrolysis of urea to ammonia and carbon dioxide protecting the pathogen from low pH (Dundon *et al.* 1998, Eaton and Krakowka 1994, Eaton *et al.* 1991). Seven genes are involved in urease production. UreA and UreB form the urease enzyme and *ureE-ureI* incorporate nickle into the enzyme (Mobley *et al.* 1995). In adults, increased urease activity is correlated with the degree of gastritis and the anti-*H. pylori* IgG response (Nijevitch 1998). Urease may not be as immunogenic in children. Less than 20% of children produce antibodies directed against urease (Leal-Herrera 1999).

The rapid urease test (RUT) is a commonly used clinical diagnostic tool, but urease is also produced by other gastric *Helicobacter* sp. (Mobley *et al.* 1995). Of the twenty-four members of the genus *Helicobacter*, seven are associated with human disease and may produce a false positive *H. pylori* diagnosis (Pena *et al.* 2002). These include *H. cinaedi*, which is associated with erysipelas, diarrhea, bacteremia and proctocoloitis (Van Genderen *et al.* 2005, Solnick and Schauer 2001) and *H. heilmannii* that causes gastritis (Solnick and Schauer 2001). Primers for *ureC,* a gene located upstream of *ureAB* in the same open reading frame, are specific for *H. pylori* (Lage *et al.* 1995, Bickley *et al.* 1993). It was subsequently determined that *ureC* is a phosphoglucosamine mutase involved in the synthesis of glucosamine-1-phosphate, a precursor of peptidoglycan and LPS (Reuse *et al.* 1997). *ureC,* renamed *glmM,* PCR is still used diagnostically to

distinguish between *H. pylori* and other Helicobacters (Pena *et al.* 2002). In addition, catalase and oxidase activity tests are also commonly used (Gold 1999).

Multiple unipolar flagella are responsible for locomotion (O'Toole *et al.* 1994). It is essential that *H. pylori* reach and move through the mucous layer lining the gastric epithelium to establish infection. Although more than sixty genes are involved in motility (reviewed by Torres *et al.* 2000), mutations in the genes *flaA* and *flaB,* the major flagellar proteins, drastically compromise the ability to colonize (Andrutis *et al.* 1997, Eaton *et al.* 1996). Motility may also be responsible for inducing a cytokine response (Jung *et al.* 1997).

3.2.4.2 Cytotoxin Associated Gene (CagA) and the *cag*PAI

CagA is a major virulence determinant translocated into host cells by a type four secretion system (T4SS) encoded by the *cag* pathogenicity island (*cag* PAI) (Odenbreit *et al.* 2001, Asahi *et al.* 2000, Backert *et al.* 2000, Stein *et al.* 2000, Segal *et al.* 1999). In western Caucasian populations CagA is correlated with severe clinical manifestations including intestinal metaplasia (Warburton *et al.* 1998), chronic inflammation (Warburton *et al.* 1998), duodenal ulcer (Covacci *et al.* 1993, Crabtree *et al.* 1991), peptic ulcer (Cover *et al.* 1995, Crabtree *et al.* 1991) and gastric cancer (Blaser *et al.* 1995b, Crabtree *et al.* 1993b). Cover *et al.* estimated that 80-100% of adults with *H. pylori* associated duodenal ulcers are colonized by *cagA* positive strains (1990). There is not a strong correlation between CagA and pathogenesis in non-Western populations. In Japan, close to one hundred percent of individuals are CagA positive and *cagA* is not associated with clinical presentation (Kato *et al.* 2000). A study of 108 patients in Singapore, determined that 86.6% of patients with a peptic ulcer were *cagA* positive, whereas 90.2%

of non-ulcer patients were also *cagA* positive (Zheng *et al.* 2000). Several other reports have documented no correlation between CagA and severe clinical manifestation (Yamaoka *et al.* 1999, Pan *et al.* 1997, Miehlke *et al.* 1996).

Studies examining the association between CagA and clinical manifestation in children are contradictory. In one pediatric study *cagA* was correlated with macroscopic gastritis and an increased inflammatory infiltrate (Queiroz *et al.* 2000). All children in this study with duodenal ulcers were *cagA* positive, but 62.3% of children without ulcers were also positive (Queiroz *et al.* 2000). An association between CagA and gastric inflammation in another pediatric population was also observed by Husson *et al.* (1995). Conversely, three out of five biopsies with maximal inflammation examined by Celik and associates (1998) and one biopsy from a patient with a duodenal ulcer were *cagA* negative. Celik *et al.* (1998) found that in symptomatic children there was no correlation between the degree of inflammation and the presence of CagA; VacA; and the ability to bind Lewis B (Le^b), which is attributed to the adhesin BabA. This finding was supported by Kato *et al.* (2000), Gold *et al.* (1999) and Loeb *et al.* (1998). In addition, other studies have found older children are more likely to be CagA positive (Ramadan *et al.* 2001, Gusmao *et al.* 2000, Queiroz *et al.* 2000 Alarcon *et al.* 1999) or have a duodenal ulcer (Queiroz *et al.* 2000).

3.2.4.3 Vacuolating Cytotoxin (VacA)

The *vacA* gene is present in all strains of *H. pylori*, but is highly polymorphic (Prinz *et al.* 2001). It was first discovered in 1988 by Leunk *et al.* (1988). VacA holotoxin comprises an N-terminal signal region (s), the middle region containing the toxin domain (m) and a C-terminal extension that possesses autotransporter activity

(Fischer *et al.* 2001a, Schmitt and Haas 1994). There are four signal region alleles (s1, which can be further subdivided into s1a, s1b, s1c (van Doorn *et al.* 1999, van Doorn *et al.* 1998, Atherton *et al.* 1997); and s2) and two middle region alleles (m1 or m2) (Yamaoka *et al.* 1999, Atherton *et al.* 1995). The genotype s1/m1 is associated with greatest cytotoxicity, whereas s2/m2 is not associated with cytotoxicity (Atherton *et al.* 1995). s1/m1 *vacA* is more abundantly expressed than s2/m2 (Forsyth *et al.* 1998). Subtyping of s1 is useful for determining the geographic/ethnic origin of the isolate and the associated level of cytotoxicity. s1a is associated with peptic ulcer disease and increased severity of gastritis (Prinz *et al.* 2001, Atherton 1997) and is the primary genotype found in northern Europe, whereas, s1b is related to lower toxigenicity and is more common in South America (van Doorn *et al.* 1999, van Doorn *et al.* 1998). The s1c isolates are primarily found in eastern Asia (van Doorn *et al.* 1999, van Doorn *et al.* 1998). As in adults, *vacA* alleles in children are correlated with the geographic origin of the isolate (Gold *et al.* 2001), but there is also a correlation between the cytotoxic form of *vacA* and increasing age (Gusmao *et al.* 2000, Queiroz *et al.* 2000, Alarcon *et al.* 1999).

3.2.4.4 Adhesins

BabA is arguably the most important adhesin and inhibition studies have documented that it is responsible for greater than 80% of adherence (Mahdavi *et al.* 2002). BabA has been correlated with ulcers (Gerhard *et al.* 1999), gastric adenocarcinoma (Gerhard *et al.* 1999) and severity of gastritis (Prinz *et al.* 2001). Additionally, Gerhard and associates found more ulcer patients express BabA than patients with gastritis alone (1999). However, in Japanese isolates Mizushima *et al.* (2001) found no correlation between *babA* and pathogenesis, which was supported by

Yamaoka *et al.* (2002) who determined that *babA* was not related to duodenal ulcer. BabA may enable effective delivery of CagA and VacA to the host cell. The *cagA* and *vacA* genes are only correlated with severity of gastritis if *babA* is present (Prinz *et al.* 2001), but Le^b binding does not require *cagA* (Su *et al.* 1998). Several studies have determined that there is correlation between *cagA* or the *cag* PAI and *babA* (Prinz *et al.* 2001, Gerhard *et al.* 1999, Ilver *et al.* 1998). For example, Ilver *et al.* (1998) determined that 73% percent of *cagA* positive strains were also *babA* positive, whereas only 5% of *cagA* negative strains were *babA* positive. Furthermore, *cagA* positive, *babA* positive strains are associated with duodenal ulcer (Prinz *et al.* 2001) and only 24% of isolates from asymptomatic patients bind Le^b (Celik *et al.* 1998). In a large study of patients from Japan, Korea, Colombia and the USA *babA* was a candidate for a universal virulence factor, whereas *cagA* and *vacA* were excluded (Yamaoka *et al.* 2002).

Young children do not express the same amount of Le^b on their gastric mucosa and *H. pylori* are less capable of binding to this surface (Celik *et al.* 1998). The lower frequency of *cagA* positives in pediatric patients could be explained by the correlation between *babA* and *cagA*. Celik and colleagues found that only two out of 32 isolates from children expressed BabA (1998).

Another adhesin, sialic acid binding adhesin (SabA), attaches to the sialyl-dimeric-Lewis X glycosphingolipid receptor on the surface of the gastric epithelium (Mahadavi *et al.* 2002). Adherence due to this receptor is not significant if *babA* is present.

3.2.4.5 IceA

Transcription of the methyltransferase M.*Hpy*I is initiated in the upstream gene *iceA* (Xu and Blaser 2001), which is induced by contact with gastric epithelial cells (Peek *et al.* 1998). Two main allelic variants of *iceA, iceA1* and *iceA2*, have been identified. A minority of *iceA1* alleles encode a functional endonuclease, but the majority contain inactivating mutations (Figueiredo *et al.* 2000). *iceA2* has no known or predicted function (Figueiredo *et al.* 2000). Prevalence of *iceA1* is correlated with increased peptic ulcer disease in the USA (Peek *et al.* 1998) and the Netherlands (van Doorn *et al.* 1998). Also *iceA1* was associated with acute inflammation and increased induction of IL-8 (Peek *et al.* 1998). However, in four other countries outside Europe and North America, *iceA1* was not correlated with peptic ulceration (Ito *et al.* 2000, Mukhopadhyay *et al.* 2000, Yamaoka *et al.* 1999). In Brazil, *iceA2* was associated with ulcers, adenocarcinoma, patients older than seven and the male sex (Ashour *et al.* 2001). A study of 424 patients from Colombia, the United States, Korea and Japan concluded that the allele alone or in any combination with *vacA* and *cagA* was not predictive of clinical presentation and that the predominate genotype varied among countries (Yamaoka *et al.* 1999). *iceA* expression may be coregulated with M.*Hpy*I or a yet to be identified virulence factor.

3.2.4.6 Lewis Antigens and Fucosyltransferases

Like *H. pylori* isolates, not all humans express all Lewis antigens. Lewis antigen expression depends on the individual, the tissue type and their developmental stage (Becker and Lowe 2003, de Vries *et al.* 2001). 70-90% percent of adults, but only 44% of children, express Le^b on the superficial epithelium of the antrum or corpus (Nogueira *et al.* 2004). Le^a is expressed more rarely in adults than Le^b, but more commonly in children

(64%) than Le^b (Nogueira *et al.* 2004). Le^a expression has been predominantly observed on the apical surface of the superficial epithelium of the antrum and the corpus (Nogueira *et al.* 2004, Taylor *et al.* 1998, Koyobashi *et al.* 1993), but has also been documented on the deep glands (Celik *et al.* 1998). Celik *et al.* also observed Le^a superficially expressed on surface mucous cells in children, but not adults (1998). In addition, the amount of Le^a and Le^b produced varies among individuals (Clyne and Drumm 1997). Le^x and Le^y are uniformly expressed in the deep glands of children and adults (Nogueira *et al.* 2004, Taylor *et al.* 1998, Koyobashi *et al.* 1993), but expression on the superficial epithelium varies. 43% and 84% of children express superficial Le^x and Le^y, respectively (Nogueira *et al.* 2004). Le^b and superficial Le^y expression are correlated with increased age (Nogueira *et al.* 2004). This work agrees with data gathered from rats. Post-weaning there is an increase in the fucose biosynthetic capacity and the amount of fucosylated glycans (Becker and Lowe 2003). Le^x expression is significantly associated with pediatric duodenal ulcer (Nogueira *et al.* 2004). In children, Le^b expression is more closely related to chronic gastritis than duodenal ulcer (Nogueira *et al.* 2004).

In vitro, Lewis antigen expression in *H. pylori* increases during the logarithmic phase of growth (Rasko *et al.* 2001). Le^x is maximally produced at the end of the logarithmic phase, but Le^y expression is highest during the stationary phase (Rasko *et al.* 2001). In addition, Lewis antigens are shed into the cultural supernatant (Rasko *et al.* 2001).

Analysis of all four major Lewis blood group antigens combined with the expression of other virulence factors from pediatric isolates has not been examined to date.

3.2.4 Relevance

By reducing childhood infection rates the occurrence of adult onset ulcers and gastric cancer could be reduced. In addition, pediatric manifestations such as malnutrition, diarrhea and growth retardation may result in irreversible effects. The cellular response, differences in virulence factor production and the ability to spontaneously clear infection indicate the pediatric *H. pylori* infection is significantly different from adult infection. Research effort should be focused on pediatric studies because infection predominantly occurs in childhood. Data gathered could further our understanding of the infection process and possible transmission routes. In addition the strains of bacteria in pediatric patients and the immune response appear to be distinct. In order to better understand these differences, the frequency of several virulence factors was examined and compared to data from adult populations.

3.3 Experimental Procedures

3.3.1 Bacterial Strains and Growth Conditions

Biopsy samples from 110 pediatric patients from Toronto (Canada), Edmonton (Canada) and Ireland were plated on Brucella broth (BB) plates [28.0 g/l BB powder (Becton, Dickinson and Company), 5% fetal bovine serum (FBS), 5% horse serum (HS), 14.0g/l of agar, 8 μg/ml amphotericin B, 5 μg/ml cefsulodin, 100 μg/ml cyclohexamide, 0.2149 μg/ml polymyxin B, 5 μg/ml trimethroprim and 8 μg/ml vancomycin]. Plates were incubated for 48-72 hours at 37°C under microaerobic conditions .

To insure that Lewis antigen expression was not dependent on culture conditions, selected isolates were also grown on brain heart infusion plates (BHI) [37 g/l BHI powder

(Becton, Dickinson and Company), 5.0 g/l yeast extract, 14 g/l agar and 5% horse serum), on trypticase soy agar plates (TSB) [40 g/l TSB, 14 g/l agar and 5% sheep blood), in BHI liquid culture [37 g/l BHI, 5 g/L yeast extract and 5% HS] and in BB liquid culture [28.0 g/l BB, 5% FBS, 5% HS, 8 μg/ml amphotericin B, 100 μg/ml cyclohexamide, 5 μg/ml trimethroprim and 8 μg/ml vancomycin]. Liquid cultures were shaken at 165 rpm at 37°C for 24-48 hours.

Cultures were confirmed to be *H. pylori* by light microscopy and PCR amplification of the *flaA* and *glmM* genes. Cultures exhibiting unusual phenotypes were further tested with the urease, catalase and oxidase tests. (Simala-Grant *et al.* 2001).

3.3.2 Genomic DNA Extraction and PCR Typing

Genomic DNA was extracted using the Loop Out procedure as described by Simala-Grant *et al.* (2004). Briefly, one entire plateful of cells was scraped and placed in a microfuge tube containing 200 μl of extraction buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0). The microfuge tube was then vortexed until the solution was homogeneous. 20 μL of SDS was added and the tube was gently inverted 3-4 times until the solution cleared. An equal volume of phenol chloroform (1:1) was added and the tube was centrifuged at 14000 rpm. The supernatant was extracted and placed in 1000 μl of -20°C 99% ethanol. A loop was used to remove the resulting DNA precipitate, which was placed in 100 μL ddH_2O and stored at -20°C.

Genomic DNA preparations were used for PCR amplification of 13 primer sets (Table 3.1). The primer set for the *glmM* gene was used as a positive control since this primer pair is unique to *H. pylori* (Bickley *et al.* 1993). Annealing temperatures, elongation times and magnesium chloride concentrations for each primer pair are listed in

Table 3.1- Oligonucleotide primers used for PCR typing of 58 isolates of *H. pylori* from symptomatic pediatric patients from Toronto, Edmonton and Ireland.

Gene Amplified	Primer Sequences	50 mM MgCl2 (µl/50µl reaction)	Annealing Temperature (°C)	Elongation Time (secs)	Expected Size (kb)	Reference
babA	+ 5' AATCCAATTTAATCCAAA 3' - 5' ATAGTTGTCTGAAAGATC 3'	1.5	50	45	0.2	Provided by Dr. Monika Keelan
cag PAI+	+ 5' ATACGCTTTTGTGCATAGAATTGCGC 3' - 5' GGTTCGACGCATTTTCCCTTAATC 3'	1.0	55	45	0.4	Akopyants *et al.* 1998
cag PAI-	+ 5' ACATTTTGGCTAAATAAACGCTG 3' - 5' TCTCCATGTTGCCATTATGCT 3'	1.5	52	45	0.6	Akopyants *et al.* 1998
cagA	+ 5' AGTAAGGAGAAACAATGA 3' - 5' AATAAGCCTTAGAGTCTTTTTGGAAATC 3'	1.5	52	120	1.4	Provided by Dr. Monika Keelan *
cagE	+ 5' TCTATAAAGAGAGGGGTGTT 3' - 5' GGCTAATCTTTGGTAATCAG 3'	1.5	50	180	2.7	Maeda *et al.* 1999
cagT	+ 5' TATATATCATATGAAAGTGAGAGCAAGTGTTTTA 3' - 5' ATATTCTAGACTTACCACTGAGCAAACTTCTGAT 3'	1.5	55	60	700	This thesis
flaA	+ 5' ATGGCTTTTCAGGTCAATAC 3' - 5' CCTTAAGATATTTTGTTGAACG 3'	1.5	50	120	1.6	Provided by Dr. Monika Keelan
futA	+ 5' CGGGATCCCGGCGTGAATTACTACCTTTCTG 3' - 5'CGGAATTCCGCAAAACCCTCCTTTCTAATG3'	1.5	50	120	1.7	Rasko 2000
futB	+ 5' CGGGATCCCGAGCGACCAATCATTACAG 3' - 5' CGGAATTCCGACCTGGCAATTAGACAAC 3'	1.5	50	120	2.0	Rasko 2000
futC	+ 5' GAACACTCACACACGCGTCTT 3' - 5' TAGAATTAGACGCTCGCTAT 3'	1.5	50	60	1.0	Provided by Dr. Ge Wang
glmM	+ 5' AAGCTTTTAGGGGTGTTAGGGGTTT 3' - 5' AAGCTTACTTTCTAACACTAACGC 3'	1.5	60	45	0.3	Bickley *et al* 1993
iceA1	+ 5' GTTGGGTAAGCGTTACAGAATTT 3' - 5' CATTGTATATCCTATCATTACAAG 3'	1.5	50	45	0.5	Provided by Dr. Monika Keelan
vacA	+ 5' GCTTCTCTTACCACCAATGC 3' - 5' TGTCAGGGTTGTTCACCATG 3'	1.5	55	90	1.1	Xiang *et al* 1995

Table 3.1. Each time genomic DNA was extracted *glmM* was amplified to insure the DNA was PCR quality. Each PCR reaction was repeated in duplicate. PCR products were resolved on ethidium bromide stained 1% agarose gels and DNA was visualized with ultraviolet light.

Clinical strains that had been previously determined to be positive or negative for each primer pair were used as positive and negative controls.

3.3.3 Western Blotting

Bacterial cell lysates were made by either collecting a 1 μl loopful of bacteria from a plate or by centrifuging 1 ml of liquid culture (1.0-2.0 OD 600). 100 μl of PBS and 20 μl of 6X SS were added and the solution was boiled for 10 minutes. Lysates were stored at -20°C. A minimum of three lysates from different plates were made from each isolate.

Western blots were performed to identify the presence of Le^a, Le^b, Le^x, Le^y and $SiLe^x$. Lystates were separated by 10% SDS-PAGE at 32 mAmps for 60 mins, and then transferred onto an Immobilon membrane at 70 V for 80 mins (Millipore). Membranes were incubated in the primary antibody, diluted 1:1000 in 5% skim milk in 1X PBS, for 12 hours at room temperature. Primary antibodies included α-Le^a (MAb BG-5), α-Le^b (MAb BG-6), α-Le^x (MAb BG-7) and α-Le^y (MAb BG-8) from Signet Laboratories Inc. (Dedham, MA, USA). The membranes were washed 2 times for 5 minutes in PBS. The secondary antibody, rabbit anti-mouse conjugated to horse radish peroxidase (HRP) (Sigma-Aldrich), was diluted 1:7500 in 5% skim milk in 1X PBS and incubated for 1 hour at room temperature. The membrane was rewashed 2 times for 5 minutes in PBS. Blots were developed using an enhanced chemiluminescence (ECL) kit (Amersham

Biosciences). A minimum of two lystates from each isolate were analyzed for each primary antibody. The following strains were used as positive controls: UA948 for Le^a and Le^x, UA1111 for Le^b and G27 for Le^y (Rasko 2000). UA948 FutTα was used as a negative control (Rasko 2000).

Membranes used for Le^a, Le^b, Le^x and Le^y western blots were stripped by washing them in ddH_2O at room temperature for a minimum of 3 hours. These blots were reprobed with either an α-SLe^x MAb (Calbiochem, Missussaga, Ontario, Canada) or a α-CagA polyclonal antibody (Provided by Dr. Antonio Covacci, Sienna, Italy). Where stripped blots were not used, the procedure used for α-SLe^x blots was identical to the one described in the previous paragraph except that strain P466 was used (Monteiro *et al.* 2000a) as a positive control. Blots to be probed with α-CagA were blocked in 5% PBS milk at room temperature for twelve hours. Membranes were then incubated with the primary antibody diluted 1:4000 in 5% skim milk in 1X PBS. The membranes were washed 2 times for five minutes in PBS and then incubated in a goat anti-rabbit antibody conjugated to HRP (Sigma-Aldrich). The membrane was again washed 2 times in PBS and then developed with the ECL kit. UA948 FutTα was used as a positive control. Each isolate was tested a minimum of two times for CagA expression.

3.3.4 Statistical Analysis

Data was compared using either a chi square test or a Fisher's exact test and p values of <0.05 were considered statistically significant. Chi square tests were used for data sets where all frequencies of occurrence were greater than five, otherwise a Fisher's exact test was utilized.

3.4 Results

3.4.1 Bacterial Strains

One hundred and ten isolates from symptomatic pediatric patients from Canada and Ireland were grown on BB plates and tested to insure they were *H. pylori*. Twenty-seven (24.5%) isolates would not grow under culture conditions used and 10 (9.1%) were irreversibly contaminated. Seventeen strains (15.4%) had severely retarded growth and required a minimum of 14 days to fill a plate or alternatively would not survive colony expansion. The remaining 56 (50.9%) isolates were confirmed to be positive by *glmM* PCR.

3.4.2 PCR Typing

PCR typing of twelve genes was completed for all 56 strains to confirm that isolates were *H. pylori* and identify virulence factors encoded by each isolate. Five of the 56 isolates were *flaA* negative with the selected primers. These isolates were positive for several other *H. pylori* associated genes and virulence factors, in addition to *glmM*, and were confirmed to be *H. pylori*. The *cagE* primer pair was discarded from further use because in initial trials only 16/42 isolates were positive. Three other individuals in the Department of Medical Microbiology and Immunology have used the *cagE* primer set with minimal success (M. Keelan, J. Simala-Grant and T. Magis personal communication). Although multiple PCR conditions were utilized, including lowering the annealing temperature and adjusting the concentration of magnesium chloride, the number of positive isolates did not increase. The number of *cagE* positive isolates (38.1%) should be approximately close to the number of *cagA* (62.5%), *cag+* (78.6%), *cag-* (66.1%) and *cagT* (57.1%) isolates, but is comparably lower. It is possible that *cagE*

has been deleted from a larger proportion of these strains, but it is more likely that this primer pair is too specific for PCR typing of isolates from multiple geographic locations.

In total, 62.5% of isolates were *cagA* positive (Table 3.2). The *cag*+ primer pair amplifies part of the upstream region of the *cag*PAI and the *cag*- primer pair amplifies a portion of the downstream region not inside the *cag*PAI. The T4SS required for CagA translocation is encoded by the *cag*PAI. A PAI encoding the upstream and downstream regions amplified is suggestive of a complete PAI encoding the entire T4SS. There was some size variation in the *cag*+ products (Figure 3.1), but the primers were very specific and the bands were very strong with no additional bands being present. This is not unexpected given the genomic diversity of *H. pylori*. The *cag* upstream region was amplified from 78.6% of isolates. The *cag* downstream primers clearly amplified 66.1% of isolates, but there was a substantial amount of laddering at all annealing temperatures where product was formed (45-52°C). c*agE* and *cagT* are components of the T4SS required for CagA translocation. The *cagT* primer pair amplified product from 57.1% of the isolates. This primer worked well for the North American isolates, but only amplified product in 25.0% of the Irish isolates. It appears this primer pair is not useful for amplifying isolates from disparate geographic locations.

Primer pairs for the three FucT genes amplified *futA, futB* and *futC* from 73.2%, 87.5%, and 91.1% of isolates, respectively. These primers were designed for cloning FucTs from strains isolated from adults, not PCR typing. These results suggest that these primers would be useful for cloning pediatric FucTs from North America and Europe. The PCR FucT typing results are not completely consistent with the Lewis antigen expression of three strains. Strain PU20 was positive for Le^y, but not *futC*, strain Sh.Sm.

Table 3.2- PCR typing results of 56 isolates of *H. pylori* from symptomatic pediatric patients from Toronto, Edmonton and Ireland. a) Percentage of positive isolates at each of the twelve loci. b) Genotyping of the four clinically relevant virulence factors. c) Percentage of isolates positive for three clinically significant combinations of virulence factors.

a)

	flaA	*glmM*	*babA*	*cagA*	*iceA1*	*vacA*	*cag+*	*cag-*	*cagT*	*futA*	*futB*	*futC*
% of Positive Isolates	91.1	100	57.1	62.5	76.8	67.9	78.6	66.1	57.1	73.2	87.5	91.1

b)

	babA+ cagA+ iceA1+ vacA+	*babA+ cagA+ iceA1+ vacA-*	*babA+ cagA+ iceA1- vacA+*	*babA+ cagA- iceA1+ vacA+*	*babA- cagA+ iceA1+ vacA+*	*babA+ cagA+ iceA1- vacA-*	*babA+ cagA- iceA1+ vacA-*	*babA- cagA+ iceA1+ vacA-*	*babA- cagA+ iceA1- vacA+*	*babA- cagA- iceA1+ vacA+*	*babA+ cagA- iceA1- vacA-*	*babA- cagA+ iceA1- vacA-*	*babA- cagA- iceA1+ vacA-*	*babA- cagA- iceA1- vacA+*
% of Isolates	19.6	7.1	5.4	12.5	12.5	3.6	5.4	5.4	7.1	10.7	3.6	1.8	3.6	1.8

c)

	cagA vacA	*babA cagA vacA*	*cagA iceA1 vacA*
% of Positive Isolates	44.6	25.0	32.1

was negative for all three FucTs but positive for Le^y, and strain W02497 was negative for *futA* and *futB* and positive for Le^x and Le^y. Interestingly, one NtHP strain was negative for all three FucTs.

The primer pairs for the four major virulence factors *babA, cagA, iceA1* and *vacA* have all been used previously for PCR typing. 57.1% of isolates were *babA* positive, 62.5% were *cagA* positive, 76.8% were positive for *iceA1* and 67.9% were positive for *vacA*. The length of the amplified region of *vacA* varied indicating different subtypes of *vacA* may be present (Figure 3.1).

19.6% of isolates were positive for all four virulence factors and 44.6% of isolates were positive for *cagA* and *vacA* (Table 3.2). There was no significant correlation between any of the virulence factors (Table 3.3), but all of the Irish strains were positive for *iceA1* suggesting that this gene may be correlated with geographical location. Further investigation may include subtyping of *vacA* and clinical data including ethnicity.

3.4.3 CagA Expression

The presence of the *cagA* gene as determined by PCR was compared with CagA expression as determined by anti-CagA Western blots (Figure 3.2a). A total of 67.9% of the strains were positive for CagA expression. One isolate, 2176, was positive for the *cagA* gene, but CagA negative. In contrast, four isolates (A, M002, PU38 and T49180) were positive for CagA expression, but negative for amplification of *cagA*. These four isolates were positive for *cag+*, *cag-* and *cagT*, which suggests a complete PAI is more likely to be present. However, three other isolates (37080909, 40201313 and F22965) were also positive for *cag+*, *cag-* and *cagT*, but negative for *cagA* by PCR and CagA by

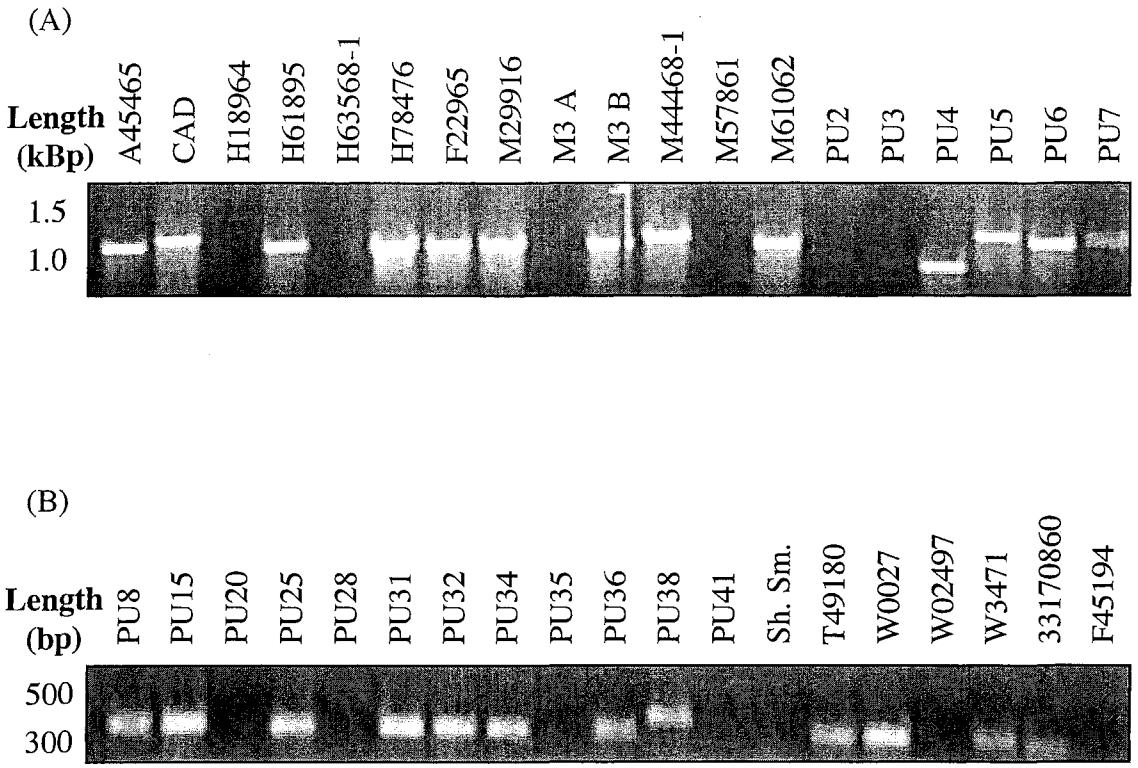


Figure 3.1- Agarose gel electrophoresis of PCR products generated from *vacA* primers (A) and *cag*+ primers (B) amplified from clincal isolates of *H. pylori*.

Table 3.3- Correlation and frequency of virulence factors produced by *H. pylori* isolates from symptomatic pediatric patients.

Genotype (n=56)	*babA+* (n=32)	*babA-* (n=24)	P value
cagA+	20	14	<1.0
iceA1+	25	18	<1.0
vacA+	20	18	<1.0

Genotype (n=56)	*cagA+* (n=35)	*cagA-* (n=21)	P value
babA+	20	12	<1.0
iceA1+	25	18	0.32
vacA+	25	13	<1.0

Genotype (n=56)	*iceA1+* (n=43)	*iceA1-* (n=13)	P value
babA+	26	7	<1.0
cagA+	25	8	<1.0
vacA+	30	8	<1.0

Genotype (n=56)	*vacA+* (n=38)	*vacA-* (n=18)	P value
babA+	20	11	<1.0
cagA+	25	10	<1.0
iceA1+	30	13	<1.0

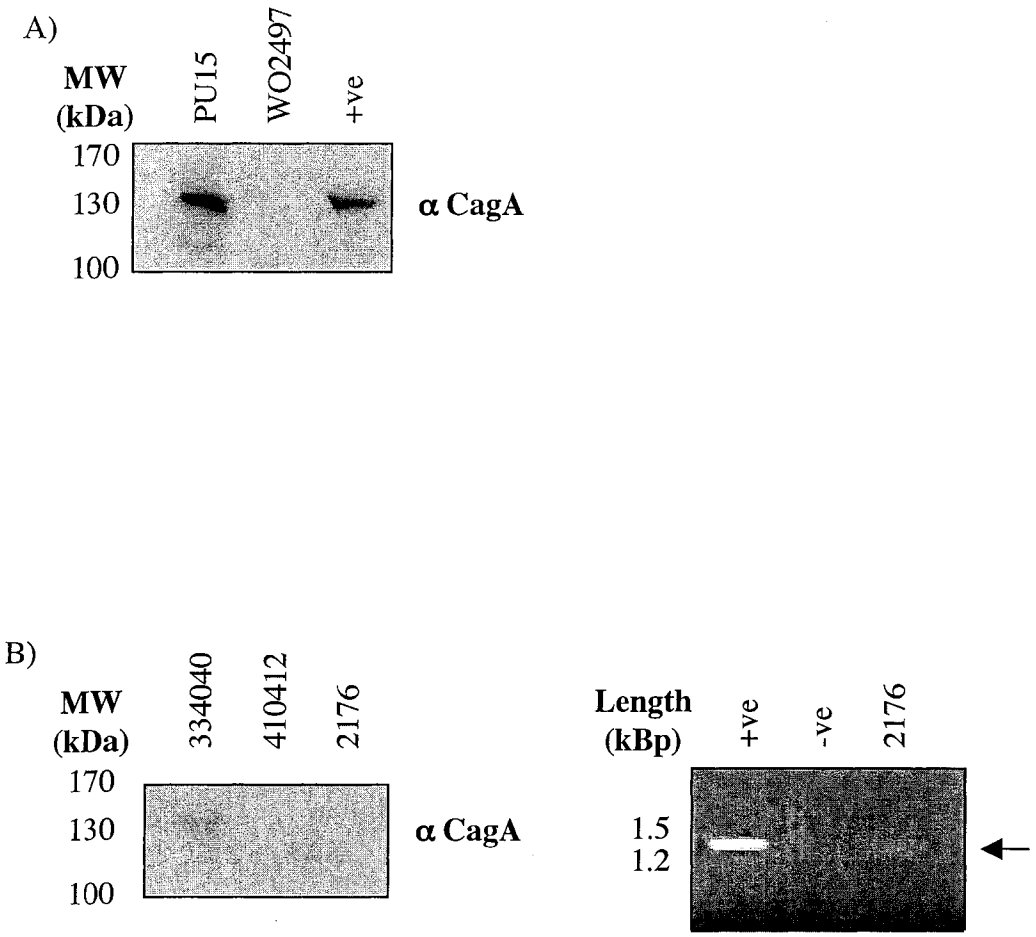


Figure 3.2- Western blot analysis of CagA produced by *H.* pylori isolates from symptomatic pediatric patients. *H. pylori* lysates were separated by SDS PAGE and proteins transferred to PVDF membranes. CagA was visualized with a polyclonal antibody. A) Representative sample of CagA positive and CagA negative isolate. B) Isolate 2176 is negative for CagA expression (left), but positive for *cagA* by PCR typing (right).

western blotting. This suggests that these three genes cannot be used to conclude that a complete *cag*PAI is present.

3.4.4 Lewis Antigen Phenotyping

The frequencies of each Lewis antigen phenotype were determined for the pediatric isolates and compared to an adult data set. For each of the isolates the same Lewis antigens were expressed regardless of the growth medium (Figure 3.3). However, the molecular weight range was variable indicating that the chain length of the Lewis antigens may be variable.

The presence of the genes responsible for Lewis antigen status *futA, futB* and futC were determined for 58 isolates. In addition to fifty-six isolates with reasonable growth that were used for PCR typing, two additional isolates were added that had retarded growth. Both isolates were confirmed to be *H. pylori*, but only enough chromosomal DNA was obtained to type *futA, futB, futC, glmM, cagA* and *vacA*. Two of the 58 isolates were Le^a positive, 3 were Le^b positive, 30 were Le^x positive, 42 were Le^y positive, and 12 were NtHP. One isolate, W3471, produced Le^a and not Le^x (Figure 3.4). No isolates, except the positive *H. pylori* control strain, P466, produced sialyl-Le^x.

The frequency of pediatric isolates expressing Le^b was significantly different from asymptomatic adults ($p<0.05$) (Table 3.4). Pediatric isolates also expressed significantly less Le^x than symptomatic adults ($p<0.05$). The frequency of isolates expressing Le^x and Le^y is significantly lower in asymptomatic adults compared to pediatric patients ($p<0.05$). The ratio of NtHP was significantly higher in isolates from symptomatic pediatric patients than symptomatic adult patients ($p<0.001$).

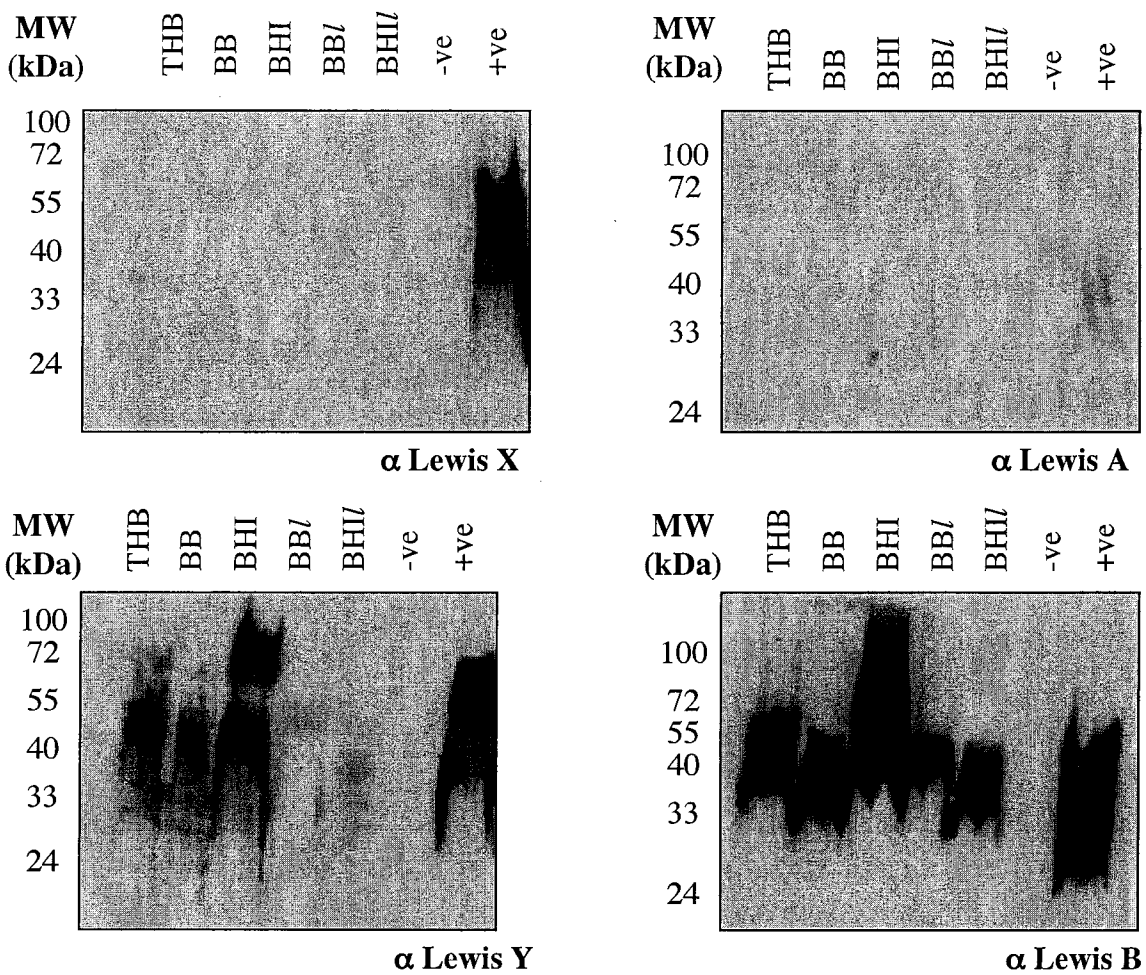


Figure 3.3 - Western blot analysis of Lewis antigens produced by pediatric isolate 4342 grown on three types of solid media and in two types of liquid media. *H. pylori* lysates were separated by SDS PAGE and proteins transferred to PVDF membranes. Lewis antigens were visualized using monoclonal antibodies.

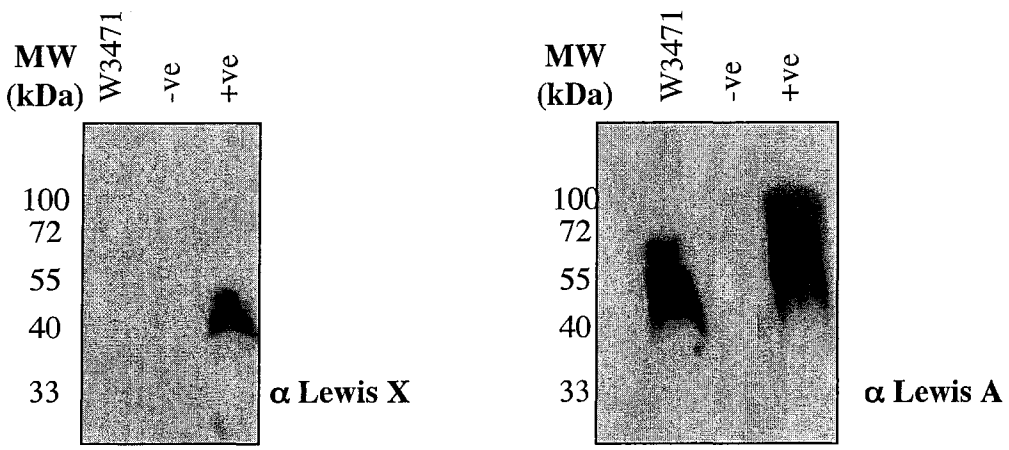


Figure 3.4 - Western blot analysis of Lewis antigens produced by pediatric isolate W3471. *H. pylori* lysates were separated by SDS PAGE and proteins transferred to PVDF membranes. Lewis antigens were visualized using monoclonal antibodies.

Table 3.4- Comparison of Lewis antigen expression from *H. pylori* isolates from symptomatic adults, asymptomatic adults and children.

Antigen	Symptomatic Adults	Asymptomatic Adults	Children
Lewis A	1.6% (4/156)	0% (0/159)	3.4% (2/58)
Lewis B[a]	3.2% (15/156)	0% (0/159)[c]	5.2% (3/58)
Lewis X[b]	58.1% (96/156)	39.6% (63/159)	51.7% (30/58)
Lewis Y	71% (118/156)	64.2% (102/159)	72.4% (42/58)
Sialyl Lewis X	-	-	0% (0/58)
A Only	-	-	1.7% (1/58)
B Only	-	-	0% (0/43)
X Only	14.5% (19/156)[d]	6.2% (10/159)	1.7% (1/58)
Y Only	30.6% (38/156)	35.8% (57/159)	22.4% (13/58)
X and Y[b]	41.9% (72/156)	33.3% (53/159)[c]	48.2% (28/58)
Non-Typable[b]	4.8% (3/156)[e]	30% (47/159)	20.7% (12/58)

a- Number of positive isolates may be artificially inflated because the mAb recognizes precursor structures.

b- Significant difference between symptomatic adults and asymptomatic adults ($p<0.05$). Data for symptomatic adults and asymptomatic adults from Rasko *et al.* 2001.

c- Statistically significant difference between asymptomatic adults and children ($p<0.05$)

d- Statistically significant difference between symptomatic adults and children ($p<0.05$)

e-Statistically significant difference between symptomatic adults and children ($p<0.001$)

Isolates expressing Le^x and a combination of Le^x and Le^y ($Le^{x/y}$) were significantly correlated with CagA expression ($p<0.05$) (Table 3.5). However, this correlation was not significant for *cagA* emphasizing that PCR typing should not be used to examine correlations between virulence factors. Le^x was also significantly associated with the *iceA1*+ gentotype ($p<0.05$). There is a relationship between an NtHP phenotype and a *babA*+ genotype, but it is not statistically significant ($p=0.07$). Nonetheless, this association may be clinically relevant. Le^y was not significantly correlated with any virulence factors.

3.4.5 Virulence Factors and Clinical Manifestation

The clinical manifestations were known for six symptomatic pediatric patients (Table 3.6). Both patients with ulcers and a patient with colitis were *babA*+, *iceA1*+ and CagA positive. The patient with colitis and the patient with duodenal ulcer both expressed Le^y and the patient with a peptic ulcer was NtHP. None of the patients with less severe clinical manifestations expressed all four virulence factors, but they all expressed Le^x and Le^y.

3.5 Discussion

The frequency of Lewis antigens is significantly different in *H. pylori* isolates from symptomatic pediatric patients (See Table 3.4) than isolates from symptomatic and asymptomatic adults. The proportion of isolates expressing $Le^{x/y}$ is significantly different from asymptomatic adult isolates and the proportion of NtHP isolates is signicantly different from symptomatic adult isolates. CagA and *iceA1* are both significantly correlated with Lewis X expression.

Table 3.5- Frequency and correlation between Lewis antigen phenotypes and virulence factor genotypes. For CagA correlation between the genotype is examined in (A) and the phenotype in (B).

(A)

Genotype (n=56)	Lewis A (n=2)	Other Phenotypes (n=54)	P value
babA +	1	29	1.00
cagA+	2	31	0.51
iceA1+	2	40	1.00
vacA+	1	36	1.00

Genotype (n=56)	Lewis B (n=3)	Other Phenotypes (n=53)	P value
babA +	1	29	0.59
cagA+	2	34	1.00
iceA1+	3	39	0.57
vacA+	2	34	1.00

Genotype (n=56)	Lewis X (n=30)	Other Phenotypes (n=26)	P value
babA +	17	11	0.42
cagA+	21	13	0.20[b]
iceA1+	25	14	0.012[a]
vacA+	21	14	0.27

Genotype (n=56)	Lewis X & Y (n=28)	Other Phenotypes (n=28)	P value
babA +	14	15	1.00
cagA+	20	14	0.20[b]
iceA1+	24	17	0.07[c]
vacA+	19	18	1.00

Genotype (n=56)	Non-Typable (n=10)	Other Phenotypes (n=46)	P value
babA +	8	21	0.07[c]
cagA+	5	28	0.72
iceA1+	7	34	1.00
vacA+	7	31	1.00

(B)

Genotype (n=56)	Lewis A (n=2)	Other Phenotypes (n=54)	P value
babA +	1	29	1.00
CagA+	2	33	0.52
iceA1+	2	40	1.00
vacA+	1	36	1.00

Genotype (n=56)	Lewis B (n=3)	Other Phenotypes (n=53)	P value
babA +	1	29	0.59
CagA+	2	32	1.00
iceA1+	3	39	0.57
vacA+	2	34	1.00

Genotype (n=56)	Lewis X (n=30)	Other Phenotypes (n=26)	P value
babA +	17	11	0.42
CagA+	23	13	<0.05[a]
iceA1+	25	14	0.012[a]
vacA+	21	14	0.27

Genotype (n=56)	Lewis X & Y (n=28)	Other Phenotypes (n=28)	P value
babA +	14	15	1.00
CagA+	22	14	<0.05[a]
iceA1+	24	17	0.07[c]
vacA+	19	18	1.00

Genotype (n=56)	Non-Typable (n=10)	Other Phenotypes (n=46)	P value
babA +	8	21	0.07[c]
CagA+	6	29	1.00
iceA1+	7	34	1.00
vacA+	7	31	1.00

a- Significant correlation between Lewis antigen phenotype and the indicated virulence factor.

b- Correlation between Lewis antigen phenotype and *cagA* genotype is not significant. Correlation between Lewis antigen phenotype and CagA phenotype is significant.

c- Lewis antigen phenotype and indicated virulence factor genotype appear to be related, but correlation is not significant.

Table 3.6- Characteristics of *H. pylori* isolates from six symptomatic pediatric patients.

Clinical Manifestation	*babA*	CagA	*iceA1*	*vacA*	Le^x	Le^y
Colitis	+	+	+	+	-	+
Duodenal Ulcer	+	+	+	-	+	+
Inflammation	-	+	+	+	+	+
Peptic Ulcer	+	+	+	+	-	-
Upper Gastrointestinal	+	+	+	+	-	+
Vomiting	-	+	+	-	+	+

In total 56% of pediatric *H. pylori* isolates that were not contaminated grew well under laboratory conditions. DNA was obtained from frozen cell culture for sixteen of the seventeen strains with severely retarded growth. Sixty-nine percent of these isolates were type II isolates, which are not positive for both *cagA* and *vacA*. This is significantly higher ($p<0.001$) than the estimated frequency of type II isolates in western populations of 10-40% (Prinz *et al.* 2001). These results as well as those previously published suggest Type II isolates do not grow as well as type I isolates under laboratory growth conditions (Xiang *et al.* 1995). As a result, growing clinical strains under laboratory conditions may select for type I *H. pylori.*

Oligonucleotides that had previously been used for PCR typing worked well with the exception of *cag-* and *cagE*. The *cagE* primers had been designed for use with Japanese isolates (Maeda *et al.* 1999). As it has been observed that isolates from disparate geographical locations are often genotypically distinct at select locations (Gold *et al.* 2001) it is not entirely unexpected that the *cagE* primer set only amplified a small percentage of isolates, when other primer pairs indicated the cagPAI was present. A similar situation occurred when *vacA* primers designed by Atherton and colleagues (1995) for use on American isolates did not amplify *vacA* from Chinese isolates well (Pan *et al.* 1998).

Results from this study show that PCR typing is indicative of the isolate's phenotype, but not completely reliable. The majority of clinical studies use only DNA based techniques, either PCR or Southern blotting, to determine correlations among virulence factors and clinical manifestations. A study examining reliability of DNA techniques in the *cag*PAI found a 7% discrepancy between their DNA microarray and

PCR data (Nilsson *et al.* 2003). The majority of the difference was ascribed to false negatives from the PCR. In this study, four isolates were positive for CagA expression, but negative for *cagA*. In contrast, one isolate that was *cagA*+ did not express CagA. Data from Maeda *et al.* (1999) confirms this finding. They found that 100% of their isolates were *cagA*+, but only 90% expressed CagA. In this study, false negatives produced abrogation of a significant correlation between Lewis antigen phenotype and CagA status.

If a strain is *cagA*+, other *cag*PAI genes should be typed to eliminate false estimates of cagPAI function. In their analysis of the *cag*PAI, Nilsson *et al.* (2003) found that 76% of strains encoded the entire *cag*PAI, 9% had no *cag*PAI genes and 15% possessed partial deletions of the *cag*PAI. Maeda *et al.* also determined that only 94% of *cagA*+ isolates possessed the complete *cag*PAI (1999). Unsurprisingly, PAIs are highly unstable and are lost from one in a million colonies under laboratory conditions (Blum *et al.* 1994). Genes required for a competent T4SS are located throughout the *cag*PAI (Fischer *et al.* 2001b), so it cannot be assumed that *cagA*+ strains translocate CagA. The *cag*PAI+ and *cag*PAI- primer pairs amplify the ends of the *cag*PAI. If borders of the *cag*PAI are not present it is indicative of an incomplete or absent *cag*PAI. The *cag*PAI+ oligonucleotide primers have been successfully used for PCR typing (Akopyants *et al.* 1998). Five strains that were CagA positive (M29916, M57861, PU2, PU20 and Sh.Sm.) were *cag*+ negative suggesting that the entire *cag*PAI, may not be present and CagA may not be translocated. Even if a strain encodes a complete *cag*PAI CagA may not be translocated. For example, an isogenic mutant of strain 26695 was incapable of translocating CagA due to a single point mutation in *cagT* (Fischer *et al.* 2001b).

No studies have tried to correlate CagA translocation with clinical manifestations, but several have correlated the vacuolating ability of VacA with several clinical factors. Maeda *et al.* determined that 59/68 isolates produced VacA, but only 54 of the 59 positive isolates displayed vacuolating ability (1998). In another study 100% of isolates were positive for the s1 signal region of *vacA* associated with cytotoxicity, but only 79% induced vacuoles in HeLa cells (Pan *et al.* 1998). A study determining the percentage of CagA+ strains that are actually capable of translocating CagA into AGS cells would be very informative. Additionally, the number of CagA motifs that are tyrosine phosphorylated should be quantified to detemine if there is any association with clinical manifestations. If no motifs are present and CagA is not phosphorylated, the protein would be incapable of interfering with some aspects of host cellular signaling, including the hummingbird phenotype, that phosphorylated isolates are capable of (Stein *et al.* 2002). Intragenomic recombination of *cagA* repeat sequences is also possible, resulting in duplication or deletion of tyrosine phosphoylation sites (Aras *et al.* 2003). Samples could be taken from children over an extended time course to determine if the number of phosphorylation sites is associated with increasing age. It would also be interesting to examine if the number of motifs differ between pediatric and adult isolates.

It is difficult to compare genotype frequencies among studies due to methodological differences, and due to different ethnic origins of patients, since there are genotypic differences from *H. pylori* from different geographical locations (Gold *et al.* 2001). A study of 151 adults, primarily German, found that 61% of isolates were *cagA+*, 38% were *babA* positive and 34% were *babA+/cagA+/vacA+* (Prinz *et al.* 2001). The frequency of *cagA+* isolates in the German study of adults was comparable to 62.5% of

cagA+ isolates in this study. Surprisingly, the pediatric isolates had a higher ratio of *babA*+ strains (57.1% versus 38%). Since children express less Le^b, the counterligand of *babA,* in all tissues tested, one might expect fewer *babA*+ strains in pediatric patients. However, only 25.0% of pediatric isolates were *babA*+/*cagA*+/*vacA*+ compared to 34% of adults in the German study. *vacA* subtyping would allow for better discrimination between isolates in pediatric and adult populations.

The frequency of *cagA, babA, iceA1* and *vacA* in strains isolated from children differs dramatically among studies. For example, in a study of Mexican children 76% were *cagA*+ (Cutler *et al.* 1995). Husson *et al.* (1995) determined only 40% of a subset of French children were *cagA*+, which is much lower than the estimated 80-95% of *cagA*+ children in Japan (Kato *et al.* 2000). The frequency of *iceA* alleles also varies geographically (Ashour *et al.* 2001). However, Ashour *et al.* (2001) determined that the frequency of *cagA*+ and *iceA2*+ positive isolates increases after seven years of age. Several other studies have also correlated *cagA* and cytotoxic alleles of *vacA* with increasing age (Gusmao *et al.* 2000, Queiroz *et al.* 2000, Alarcon *et al.* 1999). Studies genotyping virulence factors of strains from very young children with immature immune systems should be undertaken. Generally, older children have more severe symptoms suggestive of a preponderance of clinical isolates from this age group (Ashour *et al.* 2001). If data is pooled from all age groups, clinically significant differences in young children may be lost. In this study, clinical data was available for six patients and the youngest patient was nine years old. The mean age of the six patients was 12.7 years. It is difficult to obtain *H. pylori* isolates from very young children. The urea breath test (UBT) and the *H. pylori* stool antigen test (HpSA) often provide false positives in young

children (Yang *et al.* 2005, Imrie, *et al.* 2001, Kindermann *et al.* 2000), serology cannot be used in infants younger than six months because there is interference from maternal antibodies (Passaro *et al.* 2002), and the effectiveness of the fecal antigen test has not been extensively evaluated in children (Bravo *et al.* 2003). In addition, a greater proportion of isolates from very young children is necessary to determine what aspects of the immune response in infants are better adapted to clear *H. pylori* infection. Also, several virulence factors including HP-NAP have not been examined in children. Identifying aspects of the pediatric immune response and characteristics of isolates in younger children, who are generally asymptomatic or have less severe symptoms, may help us to understand what combinations of host and bacterial factors induce severe clinical manifestations.

The genotype for each virulence factor was not correlated with the presence or absence of any other virulence factor. All of the isolates from Edmonton were *iceA1*+ suggesting this study may be observing a correlation between geographic origin and genotype. It is well documented that subtypes of the signal region of *vacA* are associated with the geographic location (Yamaoka *et al.* 1999). In a study of pediatric isolates from North America, genotype was correlated with ethnic origin (Gold *et al.* 2001). To further examine this possibility it would be necessary to subtype *vacA*, determine the frequency of *iceA2*, have larger sample sizes from Edmonton and Ireland, and have the clinical data for all 56 isolates.

The Lewis antigen phenotype of each isolate was determined using Western blotting. Other studies such as Rasko *et al.* (2001) have used ELISA to determine the Lewis antigen status of *H. pylori* isolates from biopsies. Rasko and colleagues determined

there was no difference between ELISA and Western blotting for Lewis antigens (Rasko *et al.* 2001). However, ELISA may be more sensitive because it has been published that strain G27 is Le^a positive (Appelmelk *et al.* 2000a), but this was not confirmed using Western blotting in this study. Nonetheless, it is possible that the G27 used in this study is a phase variant. FABS-MS may also be utilized to characterize Lewis antigen expression. Trace amounts of Le^a expressed by 4178E are detectable by FABS-MS, but not ELISA (Appelmelk *et al.* 1999). The advantage of Western blotting is that gel mobility and staining characteristics of the Lewis antigens and LPS can be examined. The antibody used to detect Le^b by Rasko *et al.* (2001) was produced by the same manufacturer as the mAb used in this study. The antibody used by Rasko *et al.* (2001) is not ideal because it cross reacts with Le^b precursor structures (Monteiro *et al.* 1998). Since FucTs undergo phase variation PCR typing of FucTs cannot be used to determine the Lewis antigen phenotype. In addition, *futA* and *futB* may have α1,3 and/or α1,4 activity (Ma *et al.* 2003, Rasko 2000). However, none of the FucTs could be amplified from the NtHP strain 39250513 in this study. Southern blots would need to be performed to confirm that *futA, futB* and *futC* are indeed absent from this strain. It would be interesting to identify the structure of the O-side chain of this isolate and the glycotransferases necessary for the synthesis of precursor structures should be genotyped. Rasko *et al.* (2000a) discovered one NtHP strain of *H. pylori* that did not produce an O-side chain. It is not known if this strain encodes any of the three FucTs.

The amount of $Le^{x/y}$ is dependent on the culture medium, the growth rate and the growth phase (Taylor *et al.* 1996), but isolates remain positive or negative for $Le^{x/y}$ irrespective of cell age (Wirth *et al.* 1996). Although several studies have examined

Lewis antigen expression on a variety of media no studies have examined all types of media commonly used to grow *H. pylori*. For example, Wirth *et al.* (1996) examined Lewis antigen expression after growth on TSB plates, BB plates and BB liquid culture, but did not assess expression on BHI plates or in BHI liquid culture. In this study, seven isolates were grown on TSB, BB and BHI plates and in BB and BHI liquid culture. For each isolate, $Le^{a/b/x/y}$ were consistently positive or negative regardless of the medium. However, the molecular weight range of the band was lower when isolates were grown in liquid. This may be because Lewis antigens are shed into the culture supernatant (Rasko *et al.* 2001, Rasko 2000).

Strains expressing Le^x or Le^y are significantly correlated with *H. pylori* colonization density and chronic gastritis (Heneghan *et al.* 2000). Over 80% of strains isolated from symptomatic patients express Le^x or Le^y (Wirth *et al.* 1997, Simoons-Smit *et al.* 1996, Wirth *et al.* 1996). Strains isolated from ulcer patients also express more Lewis antigens than dyspeptic patients (Monteiro *et al.* 1998). Consistently Le^x is associated with increasing severity of gastric inflammation and other symptomatic clinical manifestations (Rasko *et al.* 2001, Rasko 2000, Heneghan *et al.* 2000, Marshall *et al.* 1999, Heneghan *et al.* 1998). Conversely, NtHP isolates are primarily isolated from asymptomatic patients (Rasko *et al.* 2001, Heneghan *et al.* 2000). It would be interesting to use microarray analysis to determine the difference in the immune response induced by NtHP *H. pylori* compared to those expressing Le^x. *H. pylori* strains expressing Le^a and Le^b are relatively rare in Western countries, but are more prevalent in Asian countries (Monteiro *et al.* 2000b), and are also correlated with symptomatic patients (Rasko *et al.* 2001). Unlike other virulence factors, with one exception, there is no geographic trend in

Lewis antigen expression. Frequencies are relatively constant for Lewis antigen phenotypes of 94 isolates from North America, Australia, Europe, Africa and South America (Wirth *et al.* 1996). However, compared to other geographic locations. A larger percentage of isolates from symptomatic patients in China are NtHP (Simoons-Smit *et al.* 1996). The chemical structure of LPS from NtHP isolates in China should be further examined. It is possible that these strains actually express Lewis antigens, but that they are not recognized by conventionally used antibodies.

In total 51.7% of the pediatric isolates examined in this study were Le^x positive, 72.4% of isolates expressed Le^y, 48.2% expressed $Le^{x/y}$ and 20.7% were NtHP. These frequencies of occurrence were compared to data from Dr. David Rasko (Rasko 2000). This data set was chosen because it contained symptomatic and asymptomatic adults from the same population, the same types of Lewis antigens were examined, the methodology used was consistent and well explained and the isolates were North American.

One pediatric isolate in this study produced detectable levels of Le^a, but no Le^x. This is the second strain of *H. pylori* known to produce more type α 1,4 activity than α1,3 activity (Rabbani *et al.* 2005). It would be valuable to determine the substrate specificity and enzyme kinetics of the FucTs expressed by this strain.

The percentage of isolates from symptomatic pediatric patients expressing Le^x was not significantly different from the symptomatic or asymptomatic adults. Le^x expression is significantly different between asymptomatic adults and symptomatic adults, thus it might be expected that symptomatic pediatric patients would resemble the symptomatic adult population. The intermediate frequency of Le^x suggests that either

there are different selective pressures or the immune response to Lewis antigens is different in adults and children. However, the frequency of isolates expressing $Le^{x/y}$ resembled the symptomatic adults and was significantly different from asymptomatic adults. Le^{y} may play the same role in children and adults. The percentage of pediatric NtHP isolates was significantly different than found in isolates from symptomatic adults, but not significantly different from asymptomatic adults. This suggests that Lewis antigens may be more pathogenic in adults and that other virulence factors may be more important in children. These data do not support an immunomodulatory role for Lewis antigens because the majority of isolates from symptomatic adults and children express Lewis antigens.

None of the pediatric isolates were sialyl-Le^{x} positive. There is interest in finding FucTs capable of synthesizing sialyl-Le^{x} because synthetic sialyl-Le^{x} inhibits adhesion of lymphocytes to sites of inflammation (reviewed by Salo *et al.* 2005). Endothelial cells induced by inflammatory stimuli produce sulpho-sialyl-Le^{x} (Hemmerich *et al.* 1995) that is recognized by L-selectin on leukocytes (Hemmerich *et al.* 1995). This recruitment of lymphocytes can lead to rejection in organ transplants (reviewed by Salo *et al.* 2005). A strain of yeast has been genetically modified that expresses an α-1,3 FucT capable of producing sialyl-Le^{x}, but this is a multi-step process and reagents must be incubated with several compounds (Salo *et al.* 2005). A bacterial system for producing sialyl-Le^{x} may be more effective.

CagA was significantly correlated with isolates expressing Le^{x} and $Le^{x/y}$. Wirth *et al.* also found that strains expressing Le^{x}, Le^{y}, and $Le^{x/y}$ were significantly correlated with *cagA*+ (1996). Additionally, Le^{y} expression was reduced in an isogenic *cagA* mutant.

However, Marshall *et al.* (1999) found no correlation between Lewis antigen phenotype and *cagA* genotype. Since there is evidence that both type II Lewis antigens and CagA are proinflammatory, it is not unexpected that they are corelated. It is not known why *cagA* would effect Le^y expression. One hypothesis is that CagA may upregulate other virulence factors including *futC*. Le^x was also correlated with *iceA1*. This is a novel finding. It has been previously hypothesized in this thesis that *iceA* is coregulated with other genes, but this data suggests it may also be coregulated with other virulence factors including *futA* and *futB*. Eighty percent of NtHP isolates are *babA*+, while less than 50% of all other phenotypes are *babA*+. This relationship is not statistically significant, but may be clinically significant. If Lewis antigens function as adhesins, BabA may play an increased role in adherence.

At this time virulence factors could not be correlated with clinical manifestations, but at present clinical data is only available for six patients. All six symptomatic patients were CagA positive, but only two of these patients had ulcers. With this minimal data set, CagA expression appears to increase the risk of severe pathology, but it does not appear to predict the clinical manifestation.

H. pylori has been suggested to be the most genetically diverse bacterial species studied (Cooke *et al.* 2005). This diversity may aide in immune evasion and be responsible for the variety of clinical manifestations associated with *H. pylori,* but it also makes studying *H. pylori* more difficult. It is essential to identify the host and microbial factors that are responsible for infection progressing towards a symptomatic state. The pathogen could be eradicated in these individuals, reducing their risk for the development of ulcers or gastric adenocarcinoma. Since young children display a

differential immune response a comparative approach could be adopted. Microarrays, which have been used successfully to study *H. pylori* (Guillemin *et al.* 2002), could be used to compare levels of host immune response genes and also microbial factors. The antibody response in pediatric patients should also be investigated and compared with adult populations. Since the initial antibody response in children appears to be directed towards smaller antigens (Mitchell *et al.* 1996), the antibody response in symptomatic and asymptomatic adults should be examined for differences. Research effort directed towards determining factors that allow young children to clear infection would also be informative.

Lewis antigens play a role in infection. Understanding this role would contribute to our knowledge of immunomodulation and may enable identification of key factors contributing to virulence. Isogenic mutants lacking known adhesins may elucidate what role Lewis antigens play in adherence and colonization. A mouse strain defective in fucosylated glycan expression (Smith *et al.* 2002) may also be modified for experimental use.

This study provides a good basis for further research. The significance of this study would be increased with the addition of pediatric samples from other parts of the world including Asia and South America, subtyping *vacA*, typing the *iceA2* allele, Western blotting for BabA and VacA, determining what percentage of strains translocate CagA and quantifying the number of CagA tyrosine phosphorylation motifs .

Isolates from symptomatic pediatric patients significantly differ from adult isolates. Although no correlations among genotyped virulence factors were discovered, the relative unreliability of PCR was emphasized. Clinical studies that rely on PCR

typing and not protein expression risk missing important associations in their data sets. The Lewis antigen phenotype of pediatric isolates differed significantly from asymptomatic and symptomatic adults. Lewis antigens may not play the same role in pediatric and adult infection. Furthermore, Le^x and $Le^{x/y}$ are significantly correlated with CagA expression

Chapter Four

Conclusion

4.1 Conclusion

H. pylori infects over 50% of the world's population and in approximately 10% of infected individuals, is associated with severe clinical outcomes (Ge and Taylor 1999, Gomez-Duarte *et al.* 1999). Unfortunately there has been little progress in several key areas of research. Although several risk factors for infection have been identified, universal virulence factors have not been identified. The majority of clinical research is focused on adults, but there are several differences between infection in adults and children. Childhood symptoms and virulence determinants need to be better understood. If there is indeed a link between specific *H. pylori* virulence factors and IDA or growth retardation, eradication therapy may be warranted in some cases as infection may result in irreversible health consequences.

Identification of novel virulence factors and a more complete characterization of known determinants would increase our understanding of *H. pylori* pathogenesis. As a result, several genes in the *cag*PAI and CagA deletion mutants were fused to adenylate cyclase in order to identify novel effector molecules translocated into host cells and the translocation domain of CagA. No novel effector molecules were identified, but a 371 bp region (amino acids 400-524) in the center of the CagA was identified as not being required for translocation. Unpublished work from Dr. Wolfgang Fischer's lab has determined that portions of the C-terminus and N-terminus are required for translocation (Wolfgang Fischer, personal communication). This is in agreement with our finding that the center of the protein is not required and that C-terminal and N-terminal mutants are not translocated. Future work should include creating CagA deletion mutants of increasing size centered around the region identified. Other means of identifying

translocated substrates could also be employed to elucidate the translocation domains as well as novel effector molecules.

Unfortunately there were many problems associated with using adenylate cyclase as a reporter gene for translocation by *H. pylori*. Increasing the MOI between *H. pylori* and tissue culture cells resulted in an exponential cAMP increase for constructs that had high levels of protein expression. This may be the result of AGS cell lysis. Not all adenylate cyclase fusions were well expressed, and since the wild-type *cag* genes were not knocked out, the fusions proteins had to compete with wildtype proteins. Using isogenic mutants with the gene of interest knocked out would increase the significance of the assay. In addition, expressing the fusion protein from a multi-copy plasmid would also be advantageous. No *E. coli*/*H. pylori* strain G27 shuttle vectors have been designed. Engineering a shuttle vector for G27 could be attempted. Alternatively, a different strain of *H. pylori* could be used.

Fifty-six isolates from symptomatic pediatric patients were genotyped for several virulence factors and phenotyped for Lewis antigen expression. 57.1% of isolates were *babA* positive, 62.5% were *cagA* positive, 76.8% were positive for *iceA* and 67.9% were positive for *vacA*. There were no significant correlations among the aforementioned virulence factors. In total 44.6% of isolates were type I, which falls below the North American average of 60-90% (Prinz *et al.* 2001). Type II isolates did not appear to grow as well under laboratory conditions as type I isolates.

The *cagA* PCR typing results differed from the CagA Western blotting results. Western blotting identified four isolates that were false negatives in the PCR typing. Nilsson *et al.* (2003) also determined that PCR typing of the *cag*PAI resulted in several

false negatives. One isolate, 2176, was *cagA+*, but did not express CagA. These results suggest that PCR typing is indicative of phenotype, but should not be used in studies examining correlations between virulence factors and clinical manifestations. Future studies should also determine what percentage of isolates that express CagA are capable of translocating it into host cells. A correction factor could then be applied to clinical studies that only determine CagA expression.

The Lewis antigen phenotype of fifty-eight of the pediatric isolates was also determined. Two isolates were Le^a positive, 3 were Le^b positive, 30 were Le^x positive, 42 were Le^y positive, and 12 were NtHP. No isolates produced sialyl-Le^x. Of interest, one isolate W3471, produced Le^a and not Le^x. This is the second known strain of *H. pylori* known to possess more type α 1,4 activity than α1,3 activity (Rabbani *et al.* 2005). The next step is to determine the substrate specificity and enzyme kinetics of the W3471 FucTs.

Lewis antigen expression in the pediatric isolates differed significantly from symptomatic and asymptomatic adults. Fewer pediatric isolates expressed Le^x and Le^y than asymptomatic adults ($p<0.05$). Furthermore, the ratio of NtHP was significantly higher in isolates from symptomatic pediatric patients than symptomatic adult patients ($p<0.001$). This suggests that Lewis antigens may play a different role in pediatric infection. A better understanding of the role of Lewis antigens in *H. pylori* infection is necessary to further explain these results.

CagA was significantly correlated with isolates expressing Le^x and $Le^{x/y}$. Wirth *et al.* (1996) also found that strains expressing Le^x, Le^y, and $Le^{x/y}$ were significantly correlated with *cagA+*. *iceA1* was also correlated with Le^x. It is possible that there is a

universal upregulation of several virulence factors. The promoter of the methyltransferase M.*Hpy*I is located in the *iceA* gene (Xu and Blaser 2001). *cagA* and *futA* or *futB* may be regulated by M.*Hpy*I, which may in turn be dependent on the *iceA* allele present. In the future, *H. pylori* microarray data from several strains and their isogenic ΔM.*Hpy*I mutants could be compared.

Microarrays could also be employed to compare isolates from symptomatic children, asymptomatic children, symptomatic adults and asymptomatic adults. The data set could be examined for determinants that differ among symptomatic and asymptomatic isolates. The immune response to symptomatic and asymptomatic isolates could also be compared.

When clinical data is obtained for all of the pediatric isolates used in these studies further statistical analysis will be completed. For each clinical manifestation every possible combination of virulence factors will be examined for correlation using a ROC curve. A principle components analysis will also be undertaken to determine if the isolates can be subgrouped. The relationship between age and sex and *cagA, babA, iceA1, vacA* and Lewis antigens will also be examined.

H. pylori is a model system for chronic bacterial infections (Monack *et al.* 2004). A better characterization of *H. pylori* virulence determinants will facilitate our understanding of its immunomodulatory activities. Identification of virulence determinants or combinations of virulence factors that are universally correlated with clinical outcome would allow identification of individuals likely to develop severe manifestations. These individual's infections could be targeted for eradication.

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Appendix 1- IG9 Promoter Sequence

CNTTCCAAAAATATAANNTTTTNAGCCTCTTCGCCCTCATAAAATAAAATACT
ATTTTTTTGAATTTTTTTAATTTTCCTACACTAGATAAAAGTTCTAAATAATCT
TTCATTATATACATTTTCCATTCTTAACTTATGTTAAATTTAATTTATCTTATTT
TTGCTATATTAACGCCATAAAATTAACATTTAAGAAAGGCT