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Immunoregulatory properties of *Helicobacter pylori* derived molecules

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Declaration

Unless otherwise acknowledged the work presented in this thesis is my own; no part has been submitted for another degree at the University of Nottingham or any other institute of learning.

Ekene Ogbodo

Dedication

This work is dedicated to Almighty God, whom His mercy had brought this far and to the blessed memory of my dad, John Ogbodo whom until his death had been my support system and to my Mum Florence Edna Ogbodo a woman of grace.

Abstract

Helicobacter pylori (*H. pylori*) is a micro-aerophilic, spiral-shaped Gram-negative bacterium which colonises the human stomach of approximately 50% of the population worldwide (1). The infection is asymptomatic in the vast majority of cases, however, about 10-15% result in peptic ulcer disease and 1-2% result in gastric adenocarcinoma. *H. pylori* prevalence in developed countries is decreasing as the incidence of asthma and allergy is increasing. *H. pylori* infections are usually established in early childhood when the immune system is developing and at a common age for asthma onset. The infection induces cellular immune responses of types known to inhibit those that drive allergy. Previously, we showed that *H. pylori* secreted component stimulated $CD4^{+}CD25^{hi}$ regulatory T cells (Tregs) expressing the anti-inflammatory cytokine interleukin-10 (IL-10) more strongly than whole cell lysate. SDS-PAGE was used to resolve the *H. pylori* and a whole-cell lysate to determine which proteins from the supernatant were enriched and Mass spectrometry (MALDI-TOF) was used to identify the major proteins by peptide mass fingerprinting (PMF). From the result of the MALDI-TOF, vacuolating cytotoxin A (VacA) catalase (KatA), γ- Glutamyl transpeptidase (GGT) and Peptidyl prolyl cis-trans isomerases (PPT) were selected based on the few pieces of evidence of their immunoregulatory abilities, their solubility and potential to be cloned and purified in the past.

The main aim of this study is to investigate candidate *H. pylori* protein factors that are involved in the induction of the immunoregulatory response and how these effects could be utilised in the treatment of allergy and autoimmune response. Four *H. pylori* candidate proteins (VacA, KatA, GGT and PPT), previously selected on the basis of their immunoregulatory potential, were used in *in vitro, in vivo* or *ex vivo* investigations.

Three of these candidate proteins factors, KatA, GGT and PPT were first investigated *in vivo*. To do this the genes for each *H. pylori* candidate protein were cloned and expressed in ClearColi® BL21(DE3) *E. coli* to minimise effects from LPS. The proteins were purified and characterised. LPS content in the recombinant proteins was assayed, using an E-TOXATE assay, and shown to be <0.1 EU/ml. Jurkat T-cells, THP-1 monocytic cells and AGS epithelial were incubated for 1 hour with 10, 25 and 50 μg/ml of the recombinant proteins, prior to activation with PMA/Ionomycin, LPS or TNF-. After 24 hours of treatment IL-2 (Jurkat cells), IL-6 (THP-1) and IL-8 concentrations were quantified by ELISA. All three proteins induced a dose-dependent reduction in cytokine production, compared to controls treated only with PMA/Ionomycin, LPS or TNF-. KatA most strongly suppressed IL-2 secretion by Jurkat cells (79.5% reduction with 50 μg/ml, $p<0.05$), whereas GGT was most effective in suppressing IL-6 from THP-1 cells (68.07% reduction with 50 μg/ml, $p<0.05$), there were no changes in the IL-8 production in AGS. There was no accompanying decrease in cell viability.

Likewise, VacA was investigated *in vivo* and *ex vivo* to compare the Treg response induced *in vivo,* by *H. pylori* mutants expressing different forms of VacA. Groups of 18 female C57BL/6 mice were infected orally with isogenic *H. pylori* SS1 mutants expressing the s1/i1 or s2/i2 form of VacA. A control group received plain Brucella broth as a placebo. Mice were killed at 3-, 6- and 9-weeks post-infection and their infection status confirmed. Spleen cells were isolated, stimulated with mitogens for 6 hours and stained with fluorochrome-conjugated antibodies. Treg populations were quantified by flow cytometry. Treg cells were purified and assayed for suppressive functional activity *in vitro.* Mitogen stimulation resulted in significantly increased frequencies of IL-10^{+} Tregs at all time-points and all groups ($p<0.001$). No statistically significant differences were found in frequencies of IL-10^{+} Tregs between the groups. There were also no differences in the functional suppressive activity of purified Tregs. Despite previously finding markedly increased Treg populations in peripheral blood from infected patients, we were unable to find increased Treg numbers in the spleen of infected mice. It is recommended that further investigation frequencies of Tregs in the gastric mucosa of the mice should be studied.

H. pylori infection exerts immunomodulation, through the Treg induction notwithstanding some of the protein factors such as GGT and KatA also have a direct immune regulatory effect on the immune and could be harnessed for the development of anti-inflammatory therapy.

Acknowledgement

My supervisors, Dr Karen Robsinson and Professor John Atherton for their proper guidance and support.

All the members of the *Helicobacter* research group.

Presentations

Oral presentations

Ogbodo, E., Kaneko, K., Reddiar. D. and Robinson, K.

Immunoregulatory properties of helicobacter pylori infection in vivo

School of Medicine Sue Watson Oral Presentation Event, November 2017

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In vivo investigation of immunoregulatory properties of Helicobacter pylori infection

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Poster presentations

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Ogbodo E, Rhead J, Letley D, Kaneko K, Winter J and Robinson K

Immunoregulatory properties of *Helicobacter pylori* Molecules

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List of Abbreviation Used

APC	Antigen Presenting Cell
ATCC	American Type Culture Collection identification number
B cells	B lymphocytes
BabA	Blood group Antigen-Binding antigen A
BFA	Brefeldin A
Bp	Base pairs
CagA	Cytotoxin-associated Antigen A
CagPAI	Cytotoxin-associated Antigen Pathogenicity Island
CASP1	caspase-1
CCL-	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CO_2	Carbon Dioxide
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
DupA	Duodenal ulcer promoting A
ECD	Ectodomain
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-Linked Immuno-Sorbent Assay
ERK	Extracellular signal-regulated kinases
FACS	Fluorescent and cell sorting
FBS	Fetal bovine serum
FCS	Fetal Calf Serum
FOXP3	Forkhead box P3
GERD	Gastroesophageal reflux disease
GGT	Gamma-glutamyl transpeptidase
HRP	Horse Radish Peroxidase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilised metal affinity chromatography
iNOS	inducible nitric oxide synthase
IPTG	Isopropyl β- d-1-thiogalactopyranoside
iTregs	Inducible regulatory T cells
ITP	Idiopathic thrombocytopenic purpura

KatA	Catalase
Kb	Kilobase
kDa	Kilo Daltons
Le^b	Lewis blood group B
LB	Luria Bertani
LPS	Lipopolysaccharide
MALT	Mucosa-Associated Lymphoid Tissue lymphoma
MAPK	mitogen-activated protein kinase
MHC	Major histocompatibility complex
Ml	Millilitres
mRNA	Messenger ribonucleic acid
MSK1	Mitogen- and stress-activated kinase 1
NFAT	Nuclear factor of activated T cells
NF- κB	Nuclear factor kappa light chain enhancer of activated B cells
NLRP3	NLR Family Pyrin Domain Containing 3
NO	Nitric oxide
NOD1	Nucleotide- bindin oligomerization domain 1

Ns	Not significant
NSAID	Non-steroidal anti- anti-inflammatory drugs
nTreg	Natural Regulatory T cell
OipA	Outer Inflammatory Protein A
OD	Optical density
P13K	Phosphatidylinositol 3-kinase
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PMA	Phorbol Myristate Acetate
PMN	Polymorphonuclear leukocytes
PPI	Proton Pump Inhibitor
PPT	Pathogen recognition receptor
PRR	Pattern Recognition Receptor
PUD	Peptic Ulcer Disease
PZ	Plasticity Zone

ROS	Reactive oxygen species
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
SabA	sialic acid-binding adhesion
SE	Size exclusion
SEM	Standard error of the mean
SOD	Superoxide dismutase
SS1	Sydney strain 1
T4SS	Type 4 secretion system
TCR	T cell receptor
Tc	Cytotoxic T cells (CD8+)
Tfh	T follicular helper
TGF-β	Transforming growth factor beta
Th	T helper cells (CD4+)
TLR	Toll like receptor
TNF-α	Tumour Necrosis Factor alpha
TPM	Tyrosine phosphorylation motifs

Tregs	Regulatory T cells
Tresp	Responder T cell
VacA	Vacuolating Cytotoxin A
UreA	Urease subunit alpha
UreB	Urease subunit beta

Table of Contents

Table of Figure

Chapter 1

General Introduction

1.1 General background

Helicobacter pylori (*H. pylori*) is a Gram-negative, spiral-shaped, flagellated, microaerophilic bacillus that inhabits the human gastric mucosa (Frydman et al., 2015, 2016a, Crowe, 2016, Wroblewski et al., 2010). Infection usually occurs during childhood but persists into adulthood when it frequently causes diseases (Blaser and Atherton, 2004). *H. pylori* have been implicated as the main causative agent in several gastro-duodenal diseases, including atrophic gastritis, peptic ulceration, and gastric cancer development (Cover, 2016b, Algood and Cover, 2006, Fox and Wang, 2007). This bacterium is also implicated for contributing to other disorders such as iron and vitamin B12 deficiency as well as growth retardation and idiopathic thrombocytopenic purpura (ITP)(Banatvala et al., 1993). The most remarkable feature of *H. pylori* infection is its ability to evade or skew the host immune response using several strategies, allowing the bacteria to successfully establish persistent colonization leading to chronic inflammation. Several virulence factors such as cytotoxin-associated gene A (CagA), Vacuolating cytotoxin A (VacA), HP0986, JHP0940, peptidyl propyl cis-trans isomerase (PPT), outer inflammatory protein (OipA), gamma-glutamyl transpeptidase (GGT), and duodenal ulcer promoting (dupA) play key roles in pathogenesis and also in the persistence of the bacteria. These proteins assist in the colonization of the host on a long-term basis including via subversion of the activation of innate and adaptive

immunity and achieving a balance between the pro-and anti-inflammatory response (Devi et al., 2015).

1.1.1 *H. pylori* and humans: An interesting relationship

The genus Helicobacter contains a number of species known to inhabit the gastrointestinal tract of mammals and birds. They are mostly host-specific showing possible coevolution of hosts with each infecting species (Falush et al., 2001).

Isolation of *H. pylori,* originally named *Campylobacter pyloridis,* from human stomach tissue sample was reported more than 30 years ago by Barry Marshall and Robin Warren as a certain spiral-shaped bacterium(Oleastro and Menard, 2013). It was shown to be a spiral-shaped bacillus of 2 to 4 µm long with a diameter of 0.5 to 1 µm with 3 to 5 polar flagella that made it efficiently motile (Weeks et al., 2000). Since the discovery of this curved, microaerophilic, urease, catalase, and oxidase-positive, Gram-negative bacterium; several studies have shown a worldwide spread of the infection, with more than 50% of the world population affected (Eusebi et al., 2014). Statistics report a higher prevalence rate of about 90% in developing countries and a lower rate of about 40% in most developed countries except for Japan (Garza-González et al., 2014). Despite this high incidence of the infection all over the world, only a minor fraction of infections results in the two most prominent diseases-peptic ulcer and gastric cancer. The reason for this phenomenon is yet to be answered fully (Atherton and Blaser, 2009). *H. pylori* are mostly acquired during childhood through the faecal-oral route, contamination coming mostly from

family members, and can persist for life if left untreated (Salama et al., 2013, Chen and Blaser, 2008). *H.pylori* association with humans has been dated have been occurring for tens of thousands of years, providing some evidence of co-evolution (Blaser and Atherton, 2004). Nonetheless, there has been a significant disappearance of the bacteria in recent years which has coincided with increase in other diseases notably, gastroesophageal reflux disease (GERD), allergic diseases such as asthma and type 2 diabetes (Atherton and Blaser, 2009). These evidence from epidemiological and experimental data suggests the relationship between *H. pylori* and immune-physiological imbalance and how it affects several disease outcomes.

In most cases, the immediate consequences of *H. pylori* infection include acute inflammation and gastritis which if the bacteria are not cleared can progress to chronic low-grade inflammation of the gastric mucosa(Buzás, 2014). The inflammation may be restricted to the antrum, angulus, or extended to the corporeal mucosa(Algood and Cover, 2006). This type of inflammation lacks the characteristics of classical inflammation since consequences such as pus production, abscess, granuloma, or sepsis are not seen(Buzás, 2014). At this stage, it is generally asymptomatic and persists for a lifetime in more than 85% of infected individuals despite the stimulation of a continuous immune response (Garza-González et al., 2014, Oleastro and Menard, 2013). Notwithstanding, about 10% of infected persons experience more severe consequences such as peptic ulcer disease, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma.

1.1.2 Peptic Ulcer Disease

Ulcers are deep lesions penetrating through the entire thickness of the gastrointestinal tract (g.i.t) mucosa and muscularis mucosa. A peptic ulcer is actually a broad term used in defining ulcers of the stomach or the duodenum(Kaur Amandeep, 2012). Peptic ulcer disease (PUD) is known to develop as a result of damage by gastric acid and pepsin of the protective cover offered by the mucus and bicarbonate secretion of the gastrointestinal mucosa. It is usually divided into a gastric ulcer, occurring in the stomach and a duodenal ulcer found mainly in the proximal duodenum (Sung et al., 2009) (Figure 1.1). Both are usually not easily differentiated by mere observation as they are generally characterized by epigastric pain, which is a feeling of abdominal discomfort and a burning sensation occurring shortly after a meal (Anand, 2016).

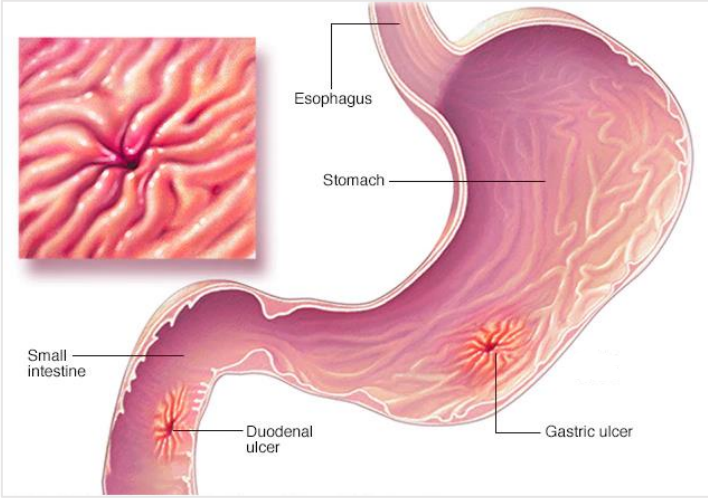


Figure 1.1 An injury in the stomach is known as a gastric ulcer while the development of an injury in the intestine is known as a duodenal ulcer. Together gastric ulcer and duodenal ulcers are called peptic ulcers (Mayo clinic).

H. pylori is present in more than 90% of duodenal ulcer patients and about 84% of those with gastric ulcers (Berstad and Berstad, 1993, Kuipers et al., 1995). Over the years, it has been shown that therapeutic eradication of *H. pylori* enhances healing of ulcers and also prevents their relapse, which supports a direct involvement of the bacterium in the disease condition (Laine et al., 1998, Rauws and Tytgat, 1990). It is still intriguing that among those with the infection only 10-15% develop PUD and the reasons for its occurrence in these individuals are not completely understood. Olbe *et al* (Olbe et al., 2000) and Dore and Graham(Dore and Graham, 2000) observed the important role of host factors, differences between strains, and bacterial load in the different outcomes of infection. Host immune products such as reactive oxygen species

and several other products released as a result of *H. pylori* contributes a great deal to the observed tissue damage (Nielsen and Andersen, 1992). Research has shown that bacterial characteristics such as cytotoxin-associated gene A (cagA)-positive and toxic type of vacuolating cytotoxin (*vac*A), i.e. strains signal sequences (s1), are associated with a higher tendency of leading to the development of PUD in an infected individual (Tham et al., 2001).

However, apart from the impact of *H. pylori* several other factors such as the use of nonsteroidal anti-inflammatory drugs (NSAIDs) low-dose aspirin, smoking, excessive alcohol use, emotional stress, and psychosocial factors have been observed to be important factors leading to the development of PUD (Peura et al., 1997) (Cryer and Kimmey, 1998).

1.1.3 Gastric Cancer

Russel (Russell, 1890) in the 1890s was the first to propose the possibility of bacteria being a carcinogen. Since then, Thomas Glover and others have shown this association between bacteria and some cancers by consistent isolation of the bacteria from the cancer tissue (Nath et al., 2010). Among these bacteria associated with cancer, the best studied is *Helicobacter pylori* which have been linked with MALT lymphoma and gastric adenocarcinoma (Marshall and Windsor, 2005). It was recognized as a type I carcinogen in 1994 and is seen as the commonest cancer-related etiologic agent(Wroblewski et al., 2010). Only 1-3% of *H. pylori* infections lead to gastric adenocarcinoma and 0.1% leads to MALT lymphoma(Peek and Crabtree, 2006), but together, both make up an alarming 5.5% of the total burden of

cancer worldwide and are the second most common cause of cancer-related death(Bozzetti et al., 1999, Parkin et al., 2005).

Eradication of *H. pylori* can completely cure MALT lymphoma at an early stage (Stolte et al., 2002). Eradication of *H. pylori* in laboratory animals can also significantly slow gastric cancer progression (Romero-Gallo et al., 2008, Wroblewski et al., 2010). Several independent case-control studies including Forman et *al* (Forman et al., 1991) and Parsonnet *et al* (Parsonnet et al., 1991) showed an increase in the risk of developing gastric cancer by 3 -5 times when infected with *H. pylori*. All these studies and more, substantiate the significance of *H. pylori* infection in gastric cancer and MALT lymphoma development.

1.1.4 Other diseases associated with *H. pylori*

Apart from the peptic ulcer disease and gastric cancer, there are a number of other serious illnesses that are frequently associated with *H. pylori* infection. Kaptan et al reported in their prospective cohort study that eradication of *H. pylori* infection effectively improved anaemia and vitamin B12 levels the infected patients (Kaptan et al., 2000). Similarly, studies were also able to link some cases of iron deficiency to *H. pylori* (Annibale et al., 2001). Interestingly, vitamin B12 and iron deficiencies are known to be common in gastric atrophy conditions which are a well-known condition linked-to *H. pylori* infection. Gastric atrophy usually results in the reduction of secretion of hydrochloric acid and a vitamin B12 binding protein termed intrinsic factor, which is essential for digestive efficiency (Cavalcoli et al., 2017).

1.1.5 *H. pylori* absence: The consequences.

As discussed earlier, *H. pylori* has coevolved with humans and this is believed to be since the human population started leaving Africa about 60,000 years ago (Atherton and Blaser, 2009). This "long-term relationship" may have resulted in human genetic modification to the presence of *H. pylori* which consequently created a permanent change in the physiology and immunology of the human host to enhance bacterial colonisation.

In recent years, the infection rate of *H. pylori* is on the decline as a result of improved hygiene and quality of life worldwide especially in industrialised countries (Yamaoka, 2009). This is believed to also have a ripple effect resulting in the drop in the prevalence of sequelae linked to *H. pylori* infection. One report showed a steady decline in the prevalence of *H. pylori* infection in Japan, from 59.1% in 1950 to 15.6 % in 1990 among individuals born within this period. It also went further to suggest a prevalence of less than 10 among individuals born after 1998 (Wang et al., 2017). Northern American average prevalence of *H. pylori* is down to 37.1% while in Oceania countries have an average prevalence of 24.4% (Hooi et al., 2017). However, as the overall in *H. pylori* prevalence is decreasing there is a concomitant increase in other diseases(Atherton and Blaser, 2009). The question arises, do *H. pylori* infection confer protection against certain diseases?

The most interesting association suggested that the absence of *H. pylori* is a significant contributor to the development of allergic diseases such as asthma. This association is supported by the hygiene hypothesis, which states

that exposure to childhood infection promotes the immune maturation necessary for protection against atopic, allergic, and autoimmune diseases (Gold and Wright, 2005). On the other hand, the hypothesis could also be explained in terms of disappearance of endogenous microbiota which have had a long term association with humans. In this case, *H. pylori* sufficiently fits the description of such an organism, having lived with humans for a long time, a childhood infection and currently, the prevalence is dropping (Blaser, 2008). Currently, a number of studies have reported a strong link between *H. pylori* and several atopic and allergic diseases (Matricardi et al., 2000, Chen and Blaser, 2007, Wang et al., 2013b, Correa and Piazuelo, 2012). To support this hypothesis, it has been shown that *H. pylori* strains with the *cagA* gene are more linked with asthma than strains without the *cagA* gene (Chen and Blaser, 2007).

Further support came in the light of the finding of differences in the gastric regulatory T- cell (Treg) levels in *H. pylori*-infected and *H. pylori* uninfected individuals. A higher level of Treg is associated with *H. pylori* colonisation which may be linked to allergic disease prevention as opposed to the uninfected individual (Robinson et al., 2008). Although these Tregs are induced in the gastric mucosa, there is evidence that the effect could be systemic, which explains the suppressive activities in the lungs (Arnold et al., 2011a). The Treg activity also has been linked to the tendency to develop peptic ulcer disease (PUD), as *H. pylori* infection tends to produce PUD individuals with low Treg as compared to the ones with higher Treg (Robinson et al., 2008). Again the presence of the *cagA* gene is known to influence

colonisation and therefore it is suggested to increase the level of Treg compare to the strains without *cagA (*Harris *et al.,* 2008*).* Another potential explanation for this phenomenon is the difference in the type of T-cells associated with *H. pylori* infection and allergic diseases; which are largely the Th1 and Th2 subtypes, respectively.

Similarly, there is a strong link between *H. pylori* eradication and obesity and/or obesity-related illnesses, especially in more industrialised countries. The mechanism is explained by the effect of bacterial colonisation on the two opposing hormones leptin and ghrelin. Leptin is a hormone that decreases appetite and hunger, thereby controlling the fat storage, on the contrary, ghrelin induces appetite and hunger, which could have a long-term effect on fat storage (Friedman and Halaas, 1998). *H. pylori* infection induces chronic inflammation in the stomach which impedes both leptin and ghrelin production. But overall leptin level remains the same in the body, this is because most of the leptin is produced else in the body other than the stomach. Contrastingly, the ghrelin level is affected since two-thirds of the hormone is produced in the gastric corpus on the stomach. Hence, the influence of *H. pylori* colonisation of the gastric corpus is observed on the ghrelin production rather than leptin which has been also confirmed by observed increases in serum ghrelin level as a result of *H. pylori* eradication (Nwokolo et al., 2003). Also, infection by *cagA*-positive strains decreases ghrelin levels compared to *cagA*-negative infections (Isomoto et al., 2005).

Another condition that could be negatively associated with *H. pylori* absence, is gastroesophageal reflux disease (GERD) including its complication at the lower oesophageal sphincter. Case studies have shown a difference in acid production in patients eradicated of *H. pylori* depends on the site of gastritis. Patients with corpus gastritis, when treated for the eradication of *H. pylori* experience a rise in acid production while the reverse is the case with patients with antral gastritis (Haruma et al., 1999, Atherton and Blaser, 2009). This finding supports the hypothesis that *H. pylori* presence in the stomach decreases the acid level at the corpus, thereby preventing acid reflux.

Finally, Atherton and Blaser.,2009 schematically summarise our relationship with *H. pylori* in a graph (Figure 1.2) in which they described the association as biphasic. They explained that the resultant of our coevolution entails that a *H. pylori*-free stomach, could be seen as an abnormality as it would dispose of an individual to one of the “modern diseases”. Hence, early childhood infection offers protection against a number of disease conditions such as allergy but not without a cost later in life such as ulcers.

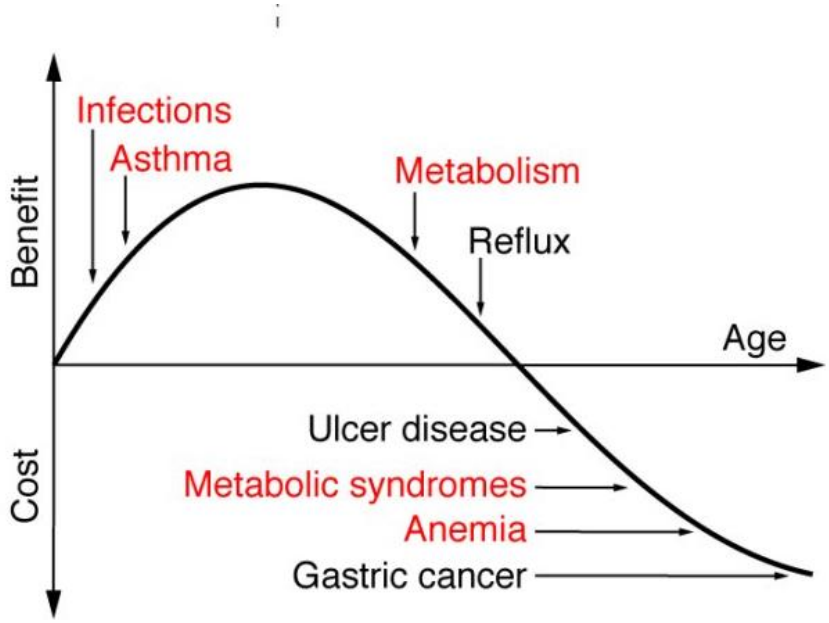


Figure 1.2 A graphical representation of the biphasic model relationship between H. pylori infection and human diseases. In which H. pylori is seen to protect from certain diseases while inducing others in a time-dependent manner.

1.1.6 Factors influencing the development of *H. pylori* diseases

There are variations in the outcome of an *H. pylori* infection, several factors which include the bacterial virulence factors, host genetic diversity, and environmental influences have been reported to play important roles in determining the extent of tissue damage due to *H. pylori* infection and also disease risk (Kodaman et al., 2014, Wroblewski et al., 2010).

1.1.6.1 Bacterial virulence factor

A number of virulence factors are known to play a role in determining the outcome of *H. pylori* infection. These include the cag pathogenicity island (*cag* PAI), vacuolating cytotoxin (vacA), adhesins, and outer membrane proteins (Wroblewski et al., 2010).

The *cag* PAI is a 40-kb DNA insertion element which is made up of 27 to 31 genes edged by 31-bp direct repeats. It encodes *Cytotoxin*-associated gene A (CagA) protein and its associated type IV secretion system (T4SS), encoded by about 18 *cag* genes and it is responsible for the transportation of CagA protein across the plasma membrane into the host cells (Cover, 2016a, Kodaman et al., 2014). *H. pylori* induces gastritis but strains with the cag PAI tend to cause severe gastritis and distal gastric cancer more common in contrast to those infected with strains without the pathogenicity island(Jung et al., 2012). CagA, a 120-to 140-kDA effector protein, primarily was strongly linked with peptic ulceration and serves as a marker for the presence of the whole cag PAI. Strains can be regarded as either *cag*A-positive or *cag*A-negative(Shimoda et al., 2016). Furthermore, polymorphisms in the CagA protein can also influence the risk of gastric pathology. One polymorphism is found in glutamate-proline-isoleucine-tyrosine-alanine (EPIYA) amino acid sequence motif within the carboxy-terminal of CagA which is also a site for CagA tyrosine phosphorylation. Four of these tyrosine phosphorylation motifs (TPMs) have been identified so far including EPIYA-A,-B,-C and D (Beltran-Anaya et al., 2014). The EPIYA-A and -B motifs are found in strains throughout

the whole world whilst the –C and –D motifs are present in strains from geographically distinct areas. EPIYA-D motifs as shown in Figure 1.2, are found in strains from Eastern Asian countries. It has been shown that the strains with the EPIYA-D motif have a tendency to induce higher amounts of interleukin-8 (IL-8) expression by gastric epithelial cells as opposed to those with EPIYA-C (Shimoda et al., 2016, Beltran-Anaya et al., 2014).

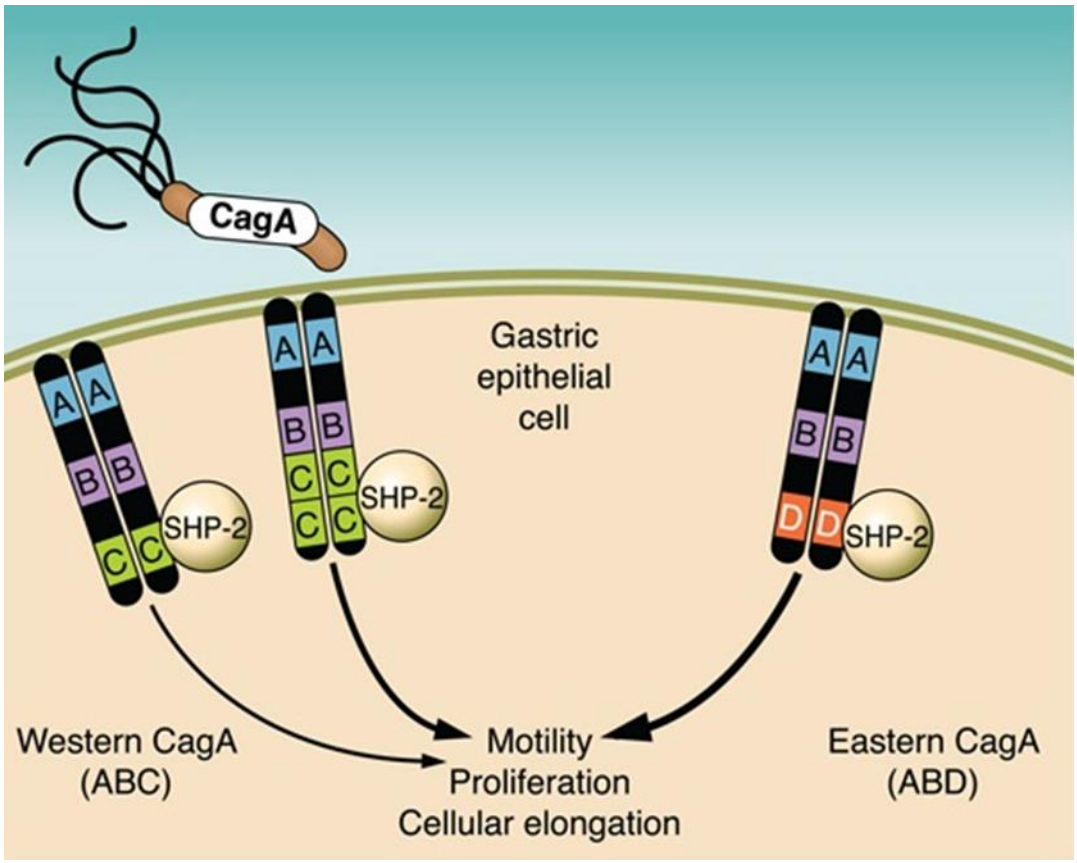


Figure 1.3 CagA phosphorylation motifs and cellular morphogenic alterations induced by intracellular CagA. A: tyrosine phosphorylation of EPIYA sites within the COOH terminus of CagA leads to alterations in host epithelial cells. Variation in the number and sequence of these sites determines the degree of CagA phosphorylation and the intensity of cellular changes. H. pylori

strain colonizing individuals in Western countries typically have Western-type CagA (C)

CagA phosphorylation motifs and cellular morphogenic alterations induced by intracellular CagA. *A*: tyrosine phosphorylation of EPIYA sites within the COOH terminus of CagA leads to alterations in host epithelial cells. Variation in the number and sequence of these sites determines the degree of CagA phosphorylation and the intensity of cellular changes. *H. pylori* strain colonizing individuals in Western countries typically have Western-type CagA (C) motifs, whereas those from East Asia have Eastern-Asian CagA (D) motifs (Peek et al., 2010a).

Apart from the CagA protein, the T4SS of the *H. pylori* also delivers soluble components of peptidoglycan into epithelial cells, this activates a pattern recognition receptor (PRR) Nod-1 that acts as a cytosolic ligand for peptidoglycan from Gram-negative bacteria. These interactions lead to activation of the transcription factor, NF-**κ**B, leading to the expression of proinflammatory cytokines such as IL-8 and β-defensin-2(Suarez et al., 2015). It has been suggested that other intracellular sensors could also interact with *H. pylori* peptidoglycan, leading to the induction of type I interferon production(Nagy et al., 2011).

VacA, an 87kDa protein was initially recognized for its ability to induce intracellular vacuolation in cultured cells(Sinnett et al., 2016). The protein is secreted by a type V autotransporter secretion system but gets into the

targeted via endocytosis and can possibly lead to cell death by apoptosis (Rassow and Meinecke, 2012). The *vacA* gene is present in almost all strains of *H. pylori,* and it is known to contribute to the long-term establishment of the infection by its ability to suppress the T-cell response(Utsch and Haas, 2016). There are considerable differences in the gene structure of *vacA* which affect the vacuolating activities of different strains. The gene structure includes the signal (s) region, the middle (m) region and between them is the intermediate (i) region. These three regions each have two subtypes, s1/s2, m1/m2 and i1/i2 respectively(Sinnett et al., 2016). It has been shown that s1/i1/m1 forms of VacA induce more vacuolation than the s2/i2/m2 equivalent(Wroblewski et al., 2010).

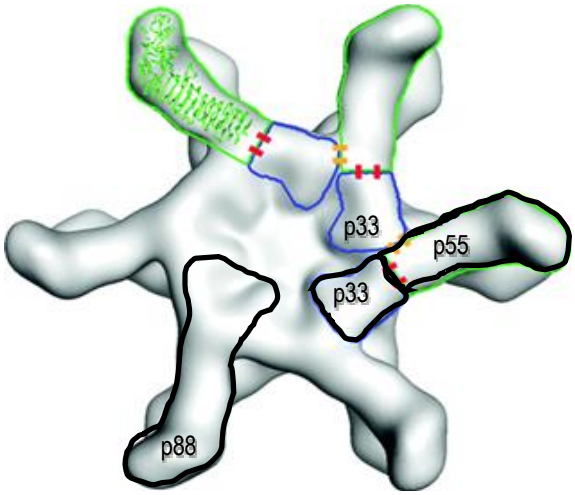


Figure 1.4 The proposed model multimeric structure of VacA showing the p33 and p55 subunits(Ivie et al., 2008).

There is a strong link between duodenal and gastric ulcer diseases and gastric cancer with the s1/m1 strains in the Western population, while no association

was established in East Asian where most strains are s1/m1 (Rhead et al., 2007, Doorn et al., 1999, Van Doorn et al., 1999). The presence of i-subtype may explain this as determines the outcome of the vacuolating activities of the chimera; s1/i1/m2s strains produce vacuolation while s1/i2/m2 strains would not. In other words, the i1 subtype is an important determinant of strain toxicity (Rhead et al., 2007).

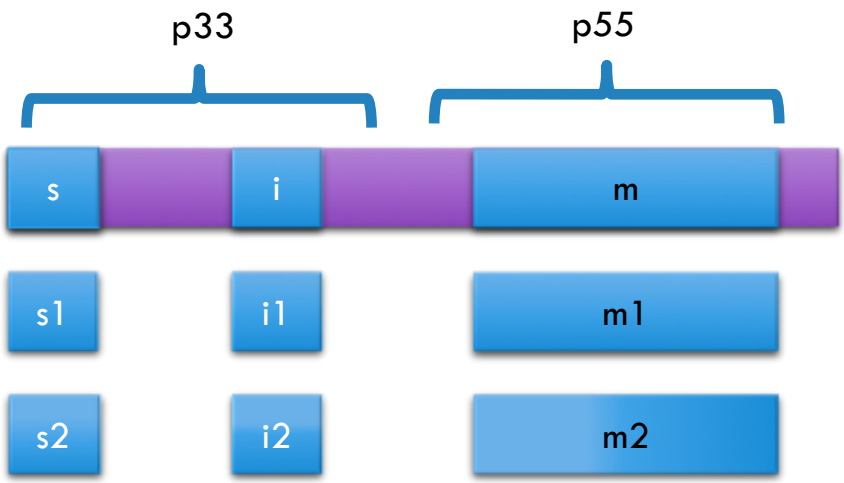


Figure 1.5 Schematic representation of the possible combinations of different varieties of VacA: s - signal region, i - intermediate region, and m - middle region. Classified into different genotypes; s1/i1/m1 type is strongly associated with disease and active toxin while s2/i2/m2 type is an inactive toxin.

Apart from s, m, and i–regions in the *vacA* gene, there is another determinant of toxicity, which is a deletion of about 61-to -81 bp, named d1 and d2 subtype for no deletion and deletion respectively. *vac*A d1 is associated with neutrophil infiltration and gastric mucosal atrophy(Ogiwara et al., 2009).

There is a number of important outer membrane protein (OMP) that work together to facilitate adherence of bacteria to the gastric epithelial cells leading to colonization and subsequent translocation of virulence factors via T4SS(Oleastro and Menard, 2013). One of the most researched *H. pylori* OMP is the blood group antigen-binding adhesin (BabA) which is encoded by a gene known as a *babA2* gene. This OMP bind to a fucosylated antigen (Le^b) found in the epithelial cells surfaces(Keilberg and Ottemann, 2016). The *babA2* gene has been strongly linked with gastric cancer, as well as duodenal ulceration and presence of *cagA* and *vacA* s1 alleles, increase the chances of severe disease development(Wroblewski et al., 2010).

Another *H. pylori* adhesin is the sialic acid-binding adhesion (SabA) which binds to the gastric epithelium via its sialyl-$Lewis^x$, a carbohydrate found on the surface of the host epithelial cells(Pinho and Reis, 2015). SabA is a phase variation regulated adhesin and is also strongly linked to an increased risk of gastric cancer but a reduced risk of duodenal ulcer (Yamaoka et al., 2006).

The Outer inflammation protein (OipA) was initially identified in relation to inflammation as a result of its tendency to induce proinflammatory cytokines such as IL-8, IL-1, TNF-α, and IL-17 which enhances neutrophil infiltration (Peek et al., 2010b). It has been shown that OipA is either present or absent in a strain and when present has a strongly associated with an increased chance of development of duodenal ulcers and gastric cancer(Cover, 2016b).

Duodenal ulcer promoting gene (*dupA*) is known for its tendency to increase the risk of duodenal ulceration in individuals infected with *dupA*-positive strains(Yamaoka and Graham, 2014). The *dupA* gene is found in the plasticity zone (PZ) of the bacterial genome and has a characteristic low G+C content(Wroblewski et al., 2010). It is believed to be associated with the virB4 gene and therefore linked with activities of the type IV secretion system (Grove et al., 2013). DupA increases the production of IL-8 in human gastric biopsy and is known to stimulate IL-12 from monocytes *in vitro* (Hussein et al., 2010). Like several other *H. pylori* virulence factor genes, *dupA* has two subtypes *dupA1* and *dupA2.* The *dupA1* allele has a sequence of about 1889-bp while dupA2 a shortened form with several mutation(Yamaoka and Graham, 2014).

1.1.6.2 Host Factors

Although the heterogeneity in the genetic makeup of *H. pylori* has been seen as a powerful tool of the bacterial infection and colonization(Honarmand-Jahromy et al., 2015), individuals colonized by a disease-causing strain type are still asymptomatic. This shows that bacterial factors acting alone are not sufficient to determine the outcome of *H. pylori* infection(Wroblewski et al., 2010). *H. pylori* infection affects the host in two main ways; by inducing gastric inflammation and causing changes in acid production in the stomach these, in turn, determine the progression of diseases(El-Omar, 2001).

Polymorphisms in the genes encoding a number of cytokines that are produced in response to *H. pylori,* such as IL-1β, IL-8, TNF-α, and IL-10, have been shown to strongly influence disease outcomes of the infection (El-Omar, 2001). The polymorphisms that promote increased expression of proinflammatory cytokines particularly, IL-1β, IL-8, and TNF-α, are associated with acid suppression and an increase in the risk of atrophic gastritis and gastric cancer (Rasmussen et al., 2012, Ohyauchi et al., 2005). Polymorphisms controlling *IL-10* gene expression which reduce expression of this anti-inflammatory cytokine increase the risk of gastric cancer development presumably by removing a limiting factor of gastric inflammation (Wroblewski et al., 2010).

1.1.6.3 Environmental Factors

In addition to the bacterial factors and host genetics, certain environmental factors contribute immensely to the development of *H. pylori*-associated diseases (Kao et al., 2016). One of the most important factors is a high intake of dietary salt (Gaddy et al., 2013, Monteiro C, 2016). A number of reports have shown a correlation between high intake of salt by *H. pylori*-infected individuals and enhanced colonization, as well as the increased risk of developing gastric diseases as compared to individuals with a lower salt intake(Shikata et al., 2008). This could be as a result of up-regulation of proinflammatory cytokines expressions such as IL-1, IL-6, and TNF-α(Juan Sun, 2016). High salt concentrations can also increase the expression of some virulence factors such as CagA (Loh et al., 2012). The suggested mechanisms

by which salt influences the risk of *H. pylori*-associated diseases include direct damage to the gastric epithelium, but the overall mechanism is still poorly understood(Wroblewski et al., 2010, Huang and Chiou, 2014).

A number of studies have shown an increase in the chances of *H. pylori* disease development due to cigarette smoking (Shikata et al., 2008). One study suggests that individuals infected with $CagA^+$ strains who are also cigarette smokers have an increased risk of gastric cancer development (Brenner et al., 2002). A handful of other studies showed that there is not a significant difference in gastric cancer risk between smokers and non-smokers but observed a trend for increased risk amongst current smokers(Wroblewski et al., 2010).

Some studies have shown that co-infection with *H. pylori* and helminths reduces the severity of *H. pylori*-associated gastritis, where T-helper (Th) response is directed towards Th2 with a decrease in Th1 cytokines (Fox et al., 2000). Whary *et a*l (Whary et al., 2014) observed increased frequencies of $FOXP3^+$ cells in the corpus and reduced *H. pylori*-associated gastric atrophy, dysplasia. Helminth co-infection also prevented *H. pylori*-induced changes in the gastric flora.

A couple of studies have shown that a diet high in antioxidants can offer some protection against the development of *H. pylori*-associated gastritis(Stanner et al., 2004). Researchers have suggested the importance of food supplements such as vitamin C and β-carotene in decreasing the chances of gastric cancer development in *H. pylori*-positive patients. Other studies have

shown that high vegetable consumption may provide protection against gastric cancer among individuals infected with *H. pylori* (Kim et al., 2013b, Mera et al., 2005).

1.1.6.4 Host Immune Response

H. pylori are generally regarded as a non-invasive bacterium and seldom cross the gastric mucosa but both surface and secreted bacterial proteins have been found in the lamina propria (Tan et al., 2015). The bacteria have the ability to induce epithelial damage, which then allows whole bacteria as well other bacterial components, delivered via outer membrane vesicles to reach the lamina propria where there are encounters with immune cells (Ito et al., 2008).

*H. pylor*i infection elicits a wide range of immune responses including innate and adaptive immunity and involving cell populations such as neutrophils, macrophages, dendritic cells (DC), T-cell, and B-cells. The combined activities of these immune responses to bacterial factors determine the extent of gastritis(Wroblewski et al., 2010).

Macrophages and monocytes are central to the immune response to *H. pylori* infection (Tan et al., 2015). Monocytes, circulating in the blood, are attracted to the gastric tissue where they differentiate into macrophages as a result of exposure to bacterial components and proinflammatory chemokines acting as chemoattractants (Tan et al., 2015). Macrophages are capable of infiltrating the mucosa as well as detecting the presence of *H. pylori* in the

lamina propria(Whitney et al., 2000). Detection of the bacterial pathogen-associated molecular patterns (PAMP) by its pattern recognition receptor (PRR), especially the Toll-like receptor families (TLR2, TLR4, and TLR5) and Nod1 (Fig 2) leads to the amplification of the inflammatory response and production of proinflammatory cytokines such as IL-1, TNF-α as well as IL-6(Peek et al., 2010a). These innate immune cells also participate in the activation of the adaptive immune system by producing IL-12 which activates Th1, the main secretor of IFN-ϒ (Wroblewski et al., 2010).

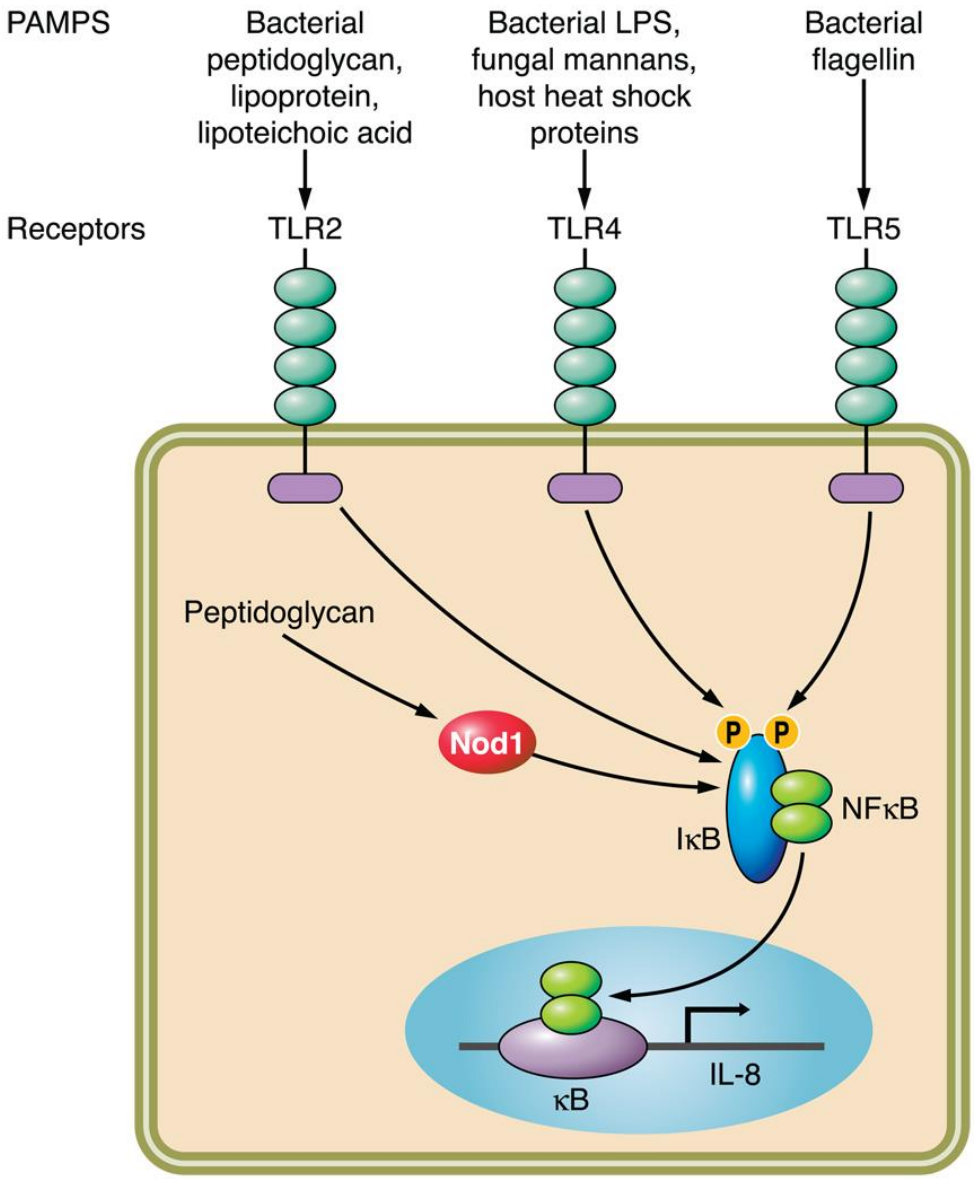


Figure 1.6 Toll-like receptors and pathogen recognition. Activation of Toll-like receptors (TLRs) and intracellular receptors (e.g., Nod1) by pathogen-associated molecular patterns (PAMPs) triggers multiple intracellular signaling pathways that culminate in NF-κB activation and subsequent production of inflammatory and immune effectors, such as interleukin-8. (Peek et al., 2010a)

Macrophages also produce nitric oxide (NO) which acts as an antimicrobial agent capable of eliminating *H. pylori* (Soleimani et al., 2016). This is elicited as a result of the upregulation of the enzyme inducible nitric oxide synthase (iNOS) by the *H. pylori* infection (Son et al., 2001).

H. pylori is also capable of evading nitric oxide-mediated destruction via the activities of its enzyme, arginase, this competes for the iNOS substrate L-arginine thereby suppressing the production of NO (Xiong et al., 2016). The bacterial enzyme metabolizes L-arginine to produce urea, this is the substrate for urease which is necessary for bacterial colonization via the synthesis of ammonia for neutralization of gastric acid (Wroblewski et al., 2010).

H. pylori also avoids other bactericidal functions of macrophage by evading phagocytosis (Lina et al., 2014). One of the ways it does this, is by ensuring that the phagosomes formed following phagocytosis fuse with each other to form a large phagosome known as a megasome. *Cag* PAI-positive and toxic *vacA* positive *H. pylori* can also prevent the fusion of lysosomes with the phagosome thereby ultimately preventing the killing of engulfed bacteria (Zheng and Jones, 2003).

Neutrophil infiltration of the mucosa is a very important hallmark of defence against *H. pylori* infection, which is followed by phagocytosis and subsequent elimination by oxygen-dependent and/or oxygen-independent killing mechanisms(Peek et al., 2010a). Neutrophils are activated by the interaction of PRR such as TLR2. interaction with the bacterial PAMPs especially the *H*. *pylori* neutrophil-activating protein (HP-NAP), to activates NF-

κB and subsequently upregulates expression of IL-12, IL-23, and TNF-α. Nevertheless, *H. pylori* have developed several mechanisms of evading the attack from neutrophils, which include avoidance of phagocytosis by preventing complement and antibody-mediated opsonization (Berstad et al., 1997, Rokita et al., 1998).

Dendritic cells (DCs) act as the bridge between innate and adaptive immunity, functionally capturing bacterial antigens, and presenting them to the T-cells (Novak et al., 2010). The DCs are thought to be capable of infiltrating the gastric epithelial apical-junction and interacting with the colonising bacterial cells and components (Wroblewski and Peek, 2011). The bacteria contain major ligands for PRRs on DCs leading to DCs activation and maturation (Fig 3). This leads to the activation of Th1, Th2, regulatory T cell (Treg), or Th17 responses directed by the expression of interleukin (IL)-12, IL-4, IL-10, or IL-23, respectively(Peek et al., 2010a)

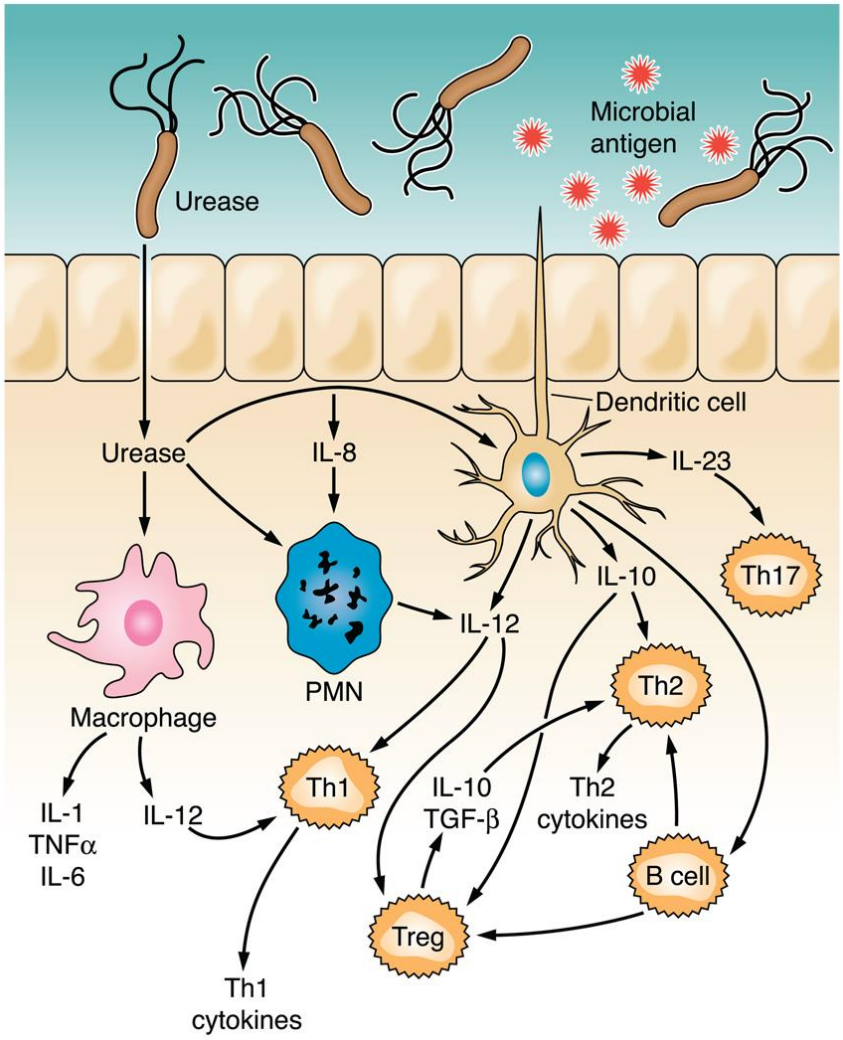


Figure 1.7 Schematic demonstrating how dendritic cells may bridge the innate and adaptive immune response directed against *H. pylori* within the gastric mucosa. Dendritic cells can penetrate the epithelial barrier in vivo and sample *H. pylori* antigens directly. Dendritic cells, in turn, activate T cells in different ways, being capable of inducing either a Th1, Th2/regulatory T cell (Treg), or a Th17 response by the generation of interleukin (IL)-12, IL-10, or IL-23, respectively. Direct interactions between *H. pylori* and gastric epithelial cells or *H. pylori* constituents such as urease can also activate polymorphonuclear (PMN) cells and/or macrophages, which further amplifies the T-cell response to this pathogen *(Peek et al., 2010b).*

T cells are broadly separated into helper T cells (Th) and cytotoxic T cells (Tc) also known as CD4 T cells and CD8 T cells respectively (2016b). The CD4 T cell specifically performs its function in the immune response by the production of cytokines and chemokines which activates targeted cells to perform specific actions and recruit other immune cells to the site of infection respectively(Charles A Janeway et al., 2001). The main function of CD8 T cells is to eliminate pathogen-infected and malignant cells, but are also capable of producing cytokines(Pennock et al., 2013).

An uncommitted T cell, known as a naïve T cell, is activated by engagement of its T cell receptor (TCR) by an antigen presented to it on the MHC (class I for CD8 and class II for CD4) of an antigen-presenting cell (APC)(Pennock et al., 2013).

The cytokines in the surrounding environment, produced as a result of interaction between innate immune cells and the pathogen, then strongly influence T cells differentiation into a particular type of T helper subsets such as Th1, Th2, Th9, T follicular helper (Tfh), Th17, and Treg (Baranovski et al., 2015). These types are conventionally divided based on cytokine differences and chronology (Th1 and Th2), cytokine profile (Th9 and Th17), and biological significance (Tfh) and (Treg)(Pennock et al., 2013).

Th1 is characterized by its production of IFN-ϒ and TNF-α, which act on macrophages and DCs to enhance their phagocytic and antigen-presentation function. Differentiation into Th1 of naïve T cells usually occurs in the presence of IFN-α/β and IL-12 cytokines generated by innate immune cells (Susanne J

Szabo, 2000). Th2 cells are characterised by the expression of IL-4, IL-5, and IL-13 and they are specialized in activating neighbouring eosinophils, mast cells, and basophils in addition to promoting the production of antibodies by B-cells. The differentiation is induced in the presence of IL-4 from innate immune cells (Fields et al., 2002). The Th9 subtype is similar to Th2, producing similar cytokines especially IL-4, IL-10, and IL-13 in addition to its signature cytokine IL-9. IL-9 supports CD4 T cell expansion and survival as well as having potent effects on mast cells. Th9 differentiation usually occurs in the presence of IL-4 and TGF-β (Dardalhon et al., 2008).

IL-17 together with IL-8 potently recruits neutrophil migration to the site of infection. IL-17 is mainly produced by the Th17. The differentiation into this subset usually occurs in an IL-6, IL-21, IL-23, and TGF-β rich environment (Mangan et al., 2006). Fazilleau *et al* (Fazilleau et al., 2009) described T-follicular helper cell (Tfh) as the class of effector T helper cells that regulate the step-wise development of antigen-specific B cell immunity. Naïve T cells differentiate into Tfh cells in the presence of IL-21 and IL-27 which induces the upregulation of the Bcl-6 transcription factor(Liu et al., 2012). Tfh cells are deployed to the B cell zones of lymphoid tissues where they usually have stable cognate interactions with B cells (Fazilleau et al., 2009).

The function of the Treg subset is to control the inflammatory response and as such maintain immune homeostasis (Pennock et al., 2013). They are quite unique in that some types called natural Tregs (nTreg) are produced directly in the thymus. Others known as induced Treg (iTreg) are differentiated

in the presence of TGF-β and retinoic acid(Pennock et al., 2013). Most Treg subtypes are $CD4^+$ and express high levels of CD25 (the IL-2 receptor) and have the expression of the FOXP3 transcription factor which mediates the immunosuppressive activities(Fontenot et al., 2003). Tregs can also be categorised on the basis of FOXP3 and CD45RA expression as resting ($FOXP3^{dim}$ $CD45RA^+$) or activated ($FOXP3^{high}$ $CD45RA^{-)}$ Tregs. In contrast, activated non-suppressive T cells are $FOXP3^{dim}$ $CD45RA^-$ (Miyara et al., 2009). Tregs may control immune responses by the production of anti-inflammatory cytokines, chief among them are IL-10(Pennock et al., 2013), TGF-β, and IL-35. Their function is to abrogate T-cell proliferation and cytokine production from other activated T cell subsets (Moore et al., 1990). Functionally, there are differences between human and mouse Tregs. In humans, only $CD4^+CD25^{hi}$ T cells can function as Tregs while in mouse, almost all $CD4^+CD25^+$ T cells function as Tregs (Lastovicka, 2013). Below (Table 1) are common extracellular and intracellular markers for Treg cells.

Table 1: Showing common surface markers for Treg

Name Marker	Common name	Status on Treg	Location	Function
FOXP3+	Forkhead box P3	+	Intracellular	$FoxP3^{+}$ is a transcription factor that is regarded to be a lineage molecule for Tregs. It is necessary for their thymic development, function, and phenotype and is responsible for controlling the expression of a number of genes including suppressive cytokines and Treg surface molecules.
Helios	Helios	+/-	Intracellular	An Ikaros family transcription factor originally used to differentiate induced Treg cells from natural Treg cells as nTregs (FoxP3+Helios+) and peripherally induced (FoxP3+Helios−) iTregs. Although presently CD304 is preferred as distinguishing factor
RUNX	Runt-related proteins	+	Intracellular	Binding of RUNX to Core-binding factor β (CBF-β) aids expression of FOXP3 in nTregs. It works together with CBF-β in maintaining the development of Treg. It is so important in that RUNX deficient mice to produce autoimmune disorders resembling those occurring in Foxp3-mutants, although the symptoms are less severe.
CD25	IL-2R –α	high	Surface	Deemed most important Treg marker, an IL-2 receptor thereby has the

				tendency of depriving other cells the needed IL-2 when present.
CD26	DPP-IV ectoenzyme	Low/-	Surface	CD26 is an extracellular serine protease with dipeptidyl peptidase IV (DPPIV) activity. Found always upregulated inactivated and memory T cells display a $CD26^{high}$ phenotype.
CD39	Ecto-nucleoside triphosphate diphosphohydrolase 1		Surface	50% to 90% of CD4**+**CD39**+** T lymphocytes are FoxP3**+,** but CD39 is also expressed on effector T cells upon activation. Also, a new subset of human CD4+CD39+FoxP3− T cells that produce IFN-gamma and IL-17 has been found
CD45RA		+/-	Surface	Tregs expressing CD45RA are resting /naive Tregs, while CD45RA negative are called effector or activated Tregs
CD45RO	alpha chain of VLA-4 integrin	-	Surface	CD45RO+ Tregs are so-called memory Tregs and exert enhanced suppressive effects on T cell proliferation and cytokine production
CD49d	VLA4α, Integrin α4	Low/-	Surface	Marker of effector T cells producing pro-inflammatory cytokines has been used for negative selection of Tregs, but in some degree expressed also on Tregs
CD127	IL7R- α	Low/-	Surface	They showed that the expression of CD127 inversely correlates with FoxP3 expression and suppressive activity of Tregs and has been used in combination with CD25 to isolate purified Treg.

1.1.6.5 T cells and *H. pylori*

Gastric inflammation is initiated by epithelial cells and innate immune cells following colonization by *H. pylori.* This is followed by the activation and attraction of lymphocytes, evident by the increase in CD8 and CD4 T cells ratio in the gastric mucosa (Portal-Celhay, 2006). A common observation is an increase in the IFN-ϒ producing T-lymphocytes in the lamina propria, which is characteristic of Th1 cells(Wroblewski et al., 2010). It has also been shown that in peptic ulcer patients, $CD4^+$ T-cell clones specific for *H. pylori* are mostly of the Th1 type, based on the cytokine profile(Fassi Fehri et al., 2010). Bamford *et al* observed that there are higher concentrations of IFN-γ and IL-2 than IL-5 and IL-4, which are the signature cytokines for Th1 and Th2 subsets respectively(Bamford et al., 1998). A number of studies have also shown that *H. pylori* infection preferentially induces production of IL-12 by DCs, which is necessary for Th1 differentiation (Haeberle et al., 1997, Fassi Fehri et al., 2010, Meyer et al., 2003).

Another cytokine that is increased at a high level in the infected gastric mucosa is IL-17, which is connected to the increase in the neutrophil level during *H. pylori* infection in mice and humans (Luzza et al., 2000). DeLyria *et al.* demonstrated that immunization of mice with a *H. pylori* lysate enhances IL-17 expression in the gastric mucosa. Also, $CD4^+$ T cells isolated from spleens and cocultured with *H. pylori*-pulsed DCs or macrophages showed increased gastric inflammation and attenuation of colonization (DeLyria et al., 2009). It is

observed that *H. pylori* infection induces a Th17 response in gastric mucosa which contributes significantly to the suppression of the infection (DeLyria et al., 2009).

1.1.6.6 The Treg Response to *H. pylori*

An inability to eliminate *H. pylori* by the immune response leads to chronic inflammation of the mucosa(Romero-Adrià n and Leal-Montiel, 2013). It has been shown that a number of the *H. pylori* components, such as CagA and TLR2 ligands were able to induce Tregs *in vitro* by skewing (Fig 4) the DC response towards the activation of IL-10 producing T cells phenotype. This was notably by a decrease in IL-12 production during the antigen presentation (Ya-Hui Wang, 2016, Xia Sun 2016). Serrano *et al*. (Serrano et al., 2013) showed that although there were no significant differences in the levels of colonization with *H. pylori* of the same genotype between children and adults, there was a significant reduction in the severity of gastric inflammation and neutrophil infiltration in children compared to the adults. This reduction in the gastric inflammatory response among children correlates with a reduction in Th17 and corresponding *IL-17* mRNA and an increase in IL-10 producing T cells, with increased IL-10 and *FOXP3* mRNA. The presence of elevated Th1 and Th17 cells in adults as opposed to FOXP3 Tregs and the associated regulatory cytokines (IL-10 and TGF-β) explains why children tend to be more susceptible to *H. pylori* colonization than the adults (Nader Bagheria, 2016). Investigations with a C57BL/6 (H2b) mouse model proved this observation to be correct, as FOXP3 Treg-depleted mice showed increased gastric inflammation, and immune cell

recruitment, with reduced bacterial colonization compared to non-Treg depleted *H. pylori*-infected mice(Rad et al., 2006). These findings show the ability of *H. pylori* to survive an inflammatory immune response by induction of the Treg response, thereby creating a state of equilibrium between the bacteria and the host.

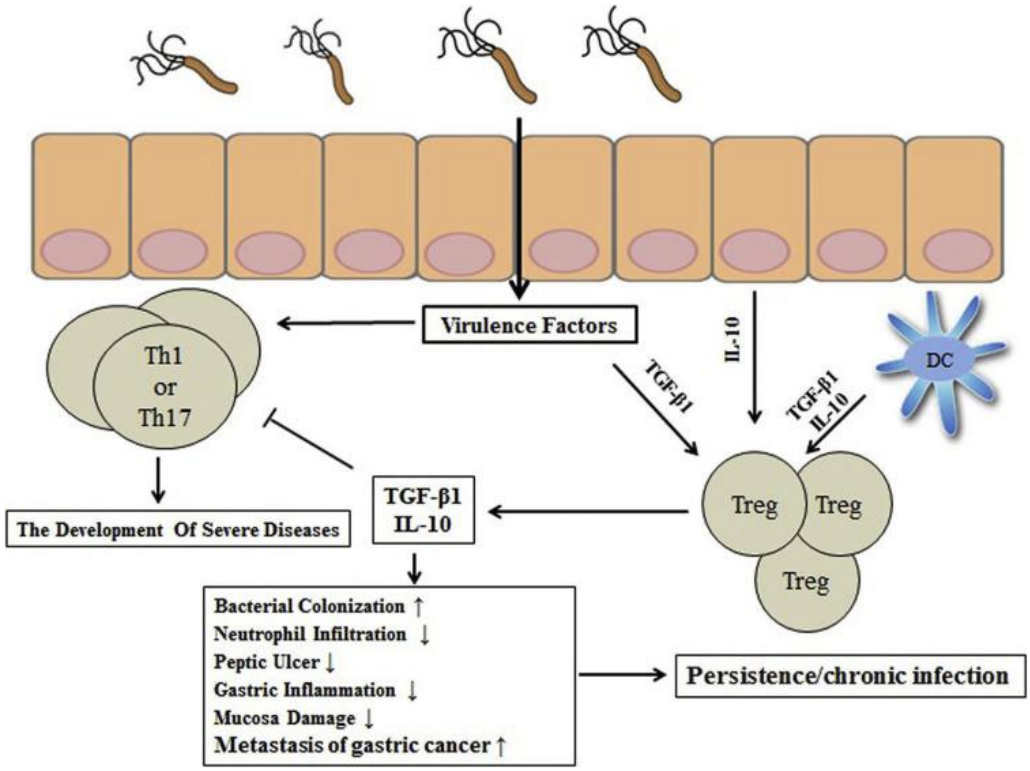


Figure 1.8. Role of Treg and *H. pylori* infection. *H. pylori*-induced gastritis is characterized by a predominant Th1/Th17 response that is regulated by Treg cells. The various T-cell lineages secrete different cytokines that modulate further the immune response that is a critical factor for the development of severe diseases such as a peptic ulcer or gastric cancer*(Nader Bagheria, 2016).*

It has also been shown that there is a reduction in IL-8 production in *H. pylori* primed-AGS cells when cocultured with Tregs as compared to those without(Nader Bagheria, 2016). Treg cells numbers have also been shown to correspond with an increase in the colonization of the gastric mucosa of *H. pylori*-infected individuals and tend to reduce together with the Treg cells

signature cytokines (IL-10 and TGF-β) following eradication therapy (Kindlund et al., 2009).

Furthermore, it was discovered that in peptic ulcer disease patients the CD4+CD25^{hi}IL10^{+} Treg response is significantly reduced compared to an infected asymptomatic patient and increased Th1 and Th2 responses are also present. This change in Treg numbers directly affects the response since reduced production of IL-10, in turn, could increase the severity of inflammation, leading to ulceration (Robinson et al., 2008). Robinson *et al.* also investigated the level of IL-10 production by *H. pylori* stimulated peripheral blood mononuclear cells (PBMCs) and showed that there was reduced IL-10 production by PBMCs from peptic ulcer patients compared to asymptomatic patients (Robinson et al., 2008). This result partly explains how an imbalance in the Treg/Th cells leads to ulcer disease.

In contrast to PUD, there is a remarkable increase in the Treg response and its corresponding cytokine expression in gastric cancer tissue with a parallel decrease in Th1/Th2 signature cytokines (Shen et al., 2009, Nader Bagheria, 2016). Investigations showed that Treg differentiation is enhanced in response to a CagA^{+} strain of *H. pylori,* which permits chronic colonization and inflammation (Jang, 2010). Even higher populations of FOXP3^{+} CD4^{+}CD25^{+}CD127^{low} Tregs are present in gastric cancer patients (Nader Bagheria, 2016).

Chronic inflammation resulting from *H. pylori* infection has a large impact on the gastric acid secretion by parietal cells of the stomach, which could lead to atrophic gastritis (Crowe, 2015). The overall clinical outcome is determined by a combination of several host and bacterial factors together with influence from the environment and others still to be discovered factors(Crowe, 2015). Bacterial factors such as Catalase, CagA, VacA,γ-glutamyltranspeptidase (GGT), urease, and peptidoglycan have been shown to exact substantial influence on the magnitude and nature of the gastric mucosal inflammatory and immune response (Nader Bagheria, 2016).

1.1.6.7 Catalase

H. pylori catalase (KatA) is one of the highly expressed proteins in the bacteria accounting for approximately 1% of the total protein (Hazell et al., 1991). The enzyme is responsible for the notable effervescence reaction observed when the bacteria is mixed with hydrogen peroxide and this is used in the biochemical identification of the bacteria (Ayala et al., 2014). KatA is an ~59 kDa heam-containing tetrameric protein known to be a very soluble ubiquitous enzyme. KatA has been identified to be expressed in the cytoplasm, and periplasm, and is released in outer membrane vesicles and adsorbed onto the cell surface of the bacteria (Hazell et al., 1991).

During *H. pylori* infection and corresponding immune response, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are released

by innate immune cells; polymorphonuclear leukocytes (PMNs), and monocytes (Cadamuro et al., 2014). Nevertheless, *H. pylori* a survive these hostile environments owing to its ability to counteract the harmful effect of these bactericidal chemicals. The mechanism through which *H. pylori* does this include; conversion of the O_2 into H_2O_2 by its superoxide dismutase (SOD) and subsequent removal of the still harmful H_2O_2 by the catalase (Harris et al., 2002) (Figure 1.9). KatA has been shown to be essential for the persistence of *H. pylori in vitro* and *in vivo* and resistance to a high concentration of hydrogen peroxide is eliminated in mutants lacking KatA(Richter et al., 2016).

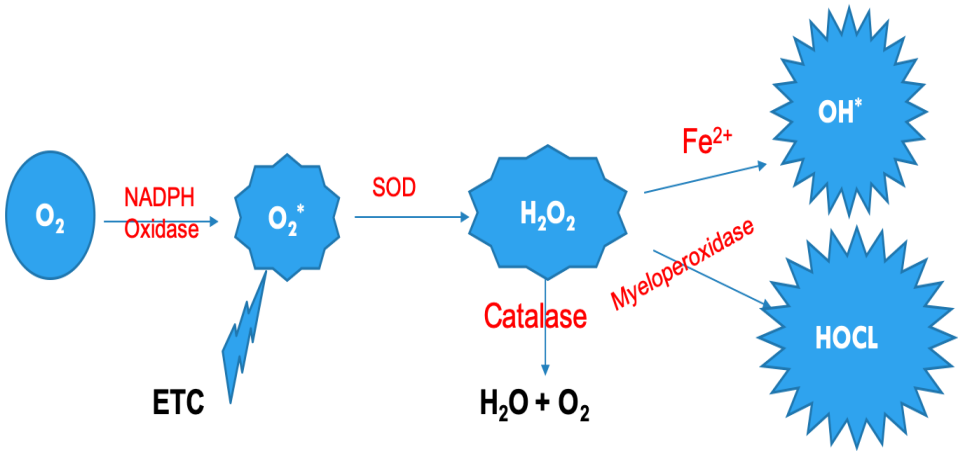


Figure 1.9 Catalase catalyses the breakdown of harmful H2O2 formed during inflammation to harmless H_2O and O_2.

Apart from the potency of hydrogen peroxide to react with reduced metallic oxides to produce toxic hydroxyl radical which is capable of causing DNA damage, hydrogen peroxide is also known to influence NF-kB activation leading to the production of cytokines such as TNFα and IL-1β (Seo et al., 2002). Consequently, by catalase potential to remove hydrogen peroxide from the

system, it could also be said to have an indirect influence on the immune response in an infected patient. Furthermore, a recent report by Richter *et al.* (Richter et al., 2016) provided some evidence of the mechanism through which *H. pylori* utilizes the interaction between KatA and vitronectin (Vn), plasma component, in preventing complement activation, a very important innate immune response.

Antibodies against *H. pylori* KatA are known to be detected in serum but the protective role of these antibodies is still questionable, even though it gave the idea of the involvement of KatA in adaptive immunity(Kotiw et al., 2012).

The *H. pylori* catalase amino acid sequence is unique when compared with other prokaryotic or eukaryotic catalase, in that it possesses a tetra-lysine motif on the C-terminus of the protein (Harris and Hazell, 2017). The importance of this unique sequence is yet to be discovered.

1.1.6.8 Cag Pathogenicity Island

Cag pathogenicity island (CagPAI) contains 27-31 genes that encode a type IV secretion system (T4SS). This is a channel system for the translocation of CagA protein, another protein encoded by the island into the cytoplasm of the host cells (Cook et al., 2014). When delivered, CagA is phosphorylated at the EPIYA sites by host tyrosine kinases such as SRC and ABL, depending on the CagA subtype (Beltran-Anaya et al., 2014, Kodaman et al., 2014). Apart from

the well-studied translocation into epithelial cells, CagA has been discovered to be injected into B lymphoid cells as well as dendritic cells (Nader Bagheria, 2016).

When delivered into the dendritic cells Tanaka *et al*. demonstrated that following CagA phosphorylation, the CagA protein negatively regulates the functions of DCs by causing a decrease in the IL-12p40 production and an increased in IL-10 secretion. *Cag*-PAI-Positive strains, therefore, induce regulatory immune responses enhancing chronic colonization by the bacteria (Tanaka et al., 2010). Again, there is a high expression of the receptor CCR6, the receptor for the chemokine CCL20, on the Treg (Kitamura et al., 2010). Studies have shown that there is an increase in both the concentration of CCL20 and migration of Treg into the gastric mucosa of a host infected with *cag*-PAI-positive strains(Cook et al., 2014).

1.1.6.9 Vacuolating cytotoxin A (VacA)

A pore-forming protein that is encoded by the *vacA* gene which is present in all strains of *H. pylori* (Sinnett et al., 2016). Because of the ability to damage the epithelial cells and disrupt the tight junctions, VacA has clear access to interact with the T-cells in the lamina propria (Müller et al., 2011b). Takeshima *et al*. showed that VacA prompts gastric inflammation by activating NF-κB in a classical rather than alternative manner. The process leads to the initiation of IL-8 production in epithelial with contrasting interference with T-cell activation in a way of inhibiting IL-2 secretion, thereby having a contradictory effect (Takeshima et al., 2009). In another instance, VacA has

also been shown to promote inflammation by disruption of autophagy which aids infection (Raju et al., 2012). Oertli *et al.* investigated the effect of two factors VacA and GGT and suggested that isogenic *H. pylori* mutants lacking either VacA or GGT are capable of inducing stronger Th1 and Th17 responses in mice. The presence of both factors induces a Treg response in a manner yet to be properly understood in humans (Oertli et al., 2013a). They also showed an increase in TGF-β1 in the gastric mucosa, which has been shown to induce suppression in T-cells, induction of IL-10 production in VacA exposed DC, as well as promotion of Treg differentiation. Mammalian microRNAs have been suggested to play a key role in regulation of the immune response and *H. pylori* infection was shown to elicit the expression of miR-155, a microRNA, both *in vivo* and *in vitro*, miR-155 inhibits protein kinase A inhibitor α (PKIα), a regulator of the feedback mechanism for cyclic adenosine monophosphate (cAMP) activities, thereby allowing continuous expression of cAMP which induces upregulation of FOXP3 transcription factor in T-cells (Fassi Fehri et al., 2010).

1.1.6.10 γ-glutamyltranspeptidase (GGT)

All strains of *H. pylori* constitutively express γ-glutamyltranspeptidase (GGT) and it has been demonstrated to be very important in colonization of the mouse gastric mucosa by the bacteria(Chevalier et al., 1999). GGT is known to have direct immunosuppression effects on both T- cells and DCs. On T-cells it induces cycle arrest at the G1 phase by a mechanism described by Schmees *et al.* and disruption of a Ras-dependent signal pathway (Schmees et al., 2007).

The mechanism by which GGT induces polarisation of DCs and subsequent induction of a Treg response is still not yet well understood. But it has been shown that GGT exposed DCs produce IL-10 and induce the FOXP3 and contact-dependent differentiation of T-cells into CD4+CD25+FOXP3+ regulatory T-cells while simultaneously preventing T helper 1 (Th1) and Th17 differentiation (Oertli et al., 2013a).

1.1.6.11 Urease

H. pylori urease is made up of two subunits, UreA and UreB. Among the two, UreB is the immunogenic subunit. The protein makes up to 5% of total *H. pylori* protein and is capable of eliciting Th17 responses both *in vivo* and *in vitro* (Jin-Yu Zhanga, 2011). *H. pylori* infection stimulates caspase-1 (CASP1) in macrophages and DCs which leads to activation of ASC- and NLRP3-containing inflammasome and subsequent IL-1β and IL-18 production (Hitzler et al., 2012). IL-1β promotes Th1 and Th17 activation which is proinflammatory leading to gastric immunopathology (Kim et al., 2013a), whilst IL-18 are protective against immunopathology by promoting Treg differentiation (Oertli et al., 2012, Kim et al., 2013a). Koch *et al.* showed that activation of NLRP3 and TLR2 by *H. pylori* is required for stimulation of the inflammasome in DCs. *H. pylori* LPS elicits expression of pro- IL-1β while the UreB subunit of the *H. pylori* urease is essential for NLRP3 inflammasome activities by activating TLR2. A *ureB* mutant was unable to activate caspase-1 and failed to induce IL-1β and IL-18, and as a result, was unable to promote Treg differentiation. This shows the importance of the TLR2/NLRP3/CASP1/IL-18 axis in immunoregulation (Koch et al., 2015).

Chapter 2

Helicobacter pylori molecules with potential Immunomodulatory properties

2.1 Introduction

2.1.1 Immunomodulatory properties of *H. pylori* molecules

A genetic predisposition to developing allergic diseases does not always culminate in the appearance of the disease, and this explains the interaction between genetics and the environment in the manifestation of allergy (Eder et al., 2006). The role of environmental factors is epitomised in the overall increase in the prevalence of asthma and other allergic diseases among children in recent times (Eder et al., 2006). The "hygiene hypothesis," suggests that cleaner living conditions are associated with the development of allergies (Strachan, 1989). That is to say that sufficient exposure to factors in the environment is necessary for normal maturation of the immune system. This led to a slight modification of the hypothesis to focus on the ancestral microorganisms and parasites which have coexisted with humans for years and are capable of bringing about a balance in the immunity (Gold and Wright, 2005). Therefore, it was concluded that reduced contact with these ancestral microbiotas due to hygiene and the use of antibiotics during childhood is one of the main reasons for the increase in allergy, especially in western countries (Blaser and Falkow, 2009, Blaser and Atherton, 2004).

Helicobacter pylori is a component of this ancestral microbiota, as the bacteria coexisted with humans for over 60,000 years, but the prevalence of the infection has continually decreased over recent decades (Correa and Piazuelo, 2012, Linz et al., 2007). *H. pylori* is known to colonise around 50% of

the world's human population, and it has evolved a strategy to persist life-long within its host. Therefore it is considered to be the most effective gastric colonizer, causing conditions such as gastritis, peptic ulceration, and gastric cancer (Parsonnet et al., 1991, Cover and Blaser, 2009). There is evidence to show that disease outcome is not only a result of the *H. pylori* virulence factors but also due to the activities of the immune response (Robinson et al., 2008). Nevertheless, there has been overwhelming evidence of inverse associations between childhood *H. pylori* infections and allergic diseases (Bodner et al., 2000, Matricardi et al., 2000, Tsang et al., 2000, Wang et al., 2013b, Chen and Blaser, 2008, Konturek et al., 2008).

There are a number of regulatory mechanisms that block the debilitating effect of immune response and one of the crucial mechanisms is through the $CD4^{+}CD25^{+}$ Treg, which could be natural Tregs (thymus developed) or induced Tregs (in the periphery as a result of a response to antigens). Studies have shown that the tolerogenic ability of *H. pylori* infection is facilitated by dendritic cells (DCs) which are skewed towards a tolerogenic phenotype, and IL-10 producing $CD^{+}CD25^{+}$ Treg cells (Arnold et al., 2011a, Ray et al., 2010, Wang et al., 2013b).

Two *H. pylori* virulence factors, vacuolating cytotoxin A (VacA) and γ-Glutamyl transpeptidase (GGT) have been shown to aid *H. pylori* colonisation since mutant strains lacking these factors failed to colonise mice (Chevalier et al., 1999, Salama et al., 2001a). Both factors were also shown to inhibit T-cell proliferation (Gebert et al., 2003, Schmees et al., 2007), (Oertli et al., 2013a)

showed that VacA and GGT are independently capable of reprogramming DC leading to persistent colonisation and induced immune tolerance in mice since DCs exert a strong influence on the adaptive immune response. They further showed that *vacA* and *ggt* null *H. pylori* mutants induced more effector T cell responses, had a reduced ability to inhibit DC maturation, failed to induce $CD4^+CD25^+$ $Foxp3^+$ Tregs, and infections were cleared relatively easily compared to the wild type.

The *Helicobacter* research group has previously shown reported an increase in IL-10 producing Tregs in gastric biopsy samples of *H. pylori*-infected patients, compared to uninfected patients. They also found that peripheral blood Tregs from infected patients (but not uninfected patients) responded to stimulation with *H. pylori* antigens by secreting IL-10. Components in the secreted fraction of an *H. pylori* culture were markedly more effective in stimulating an IL-10 response.

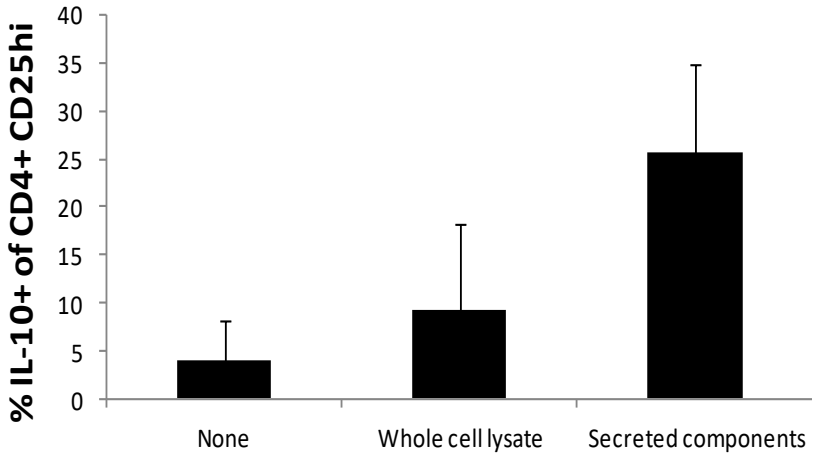


Figure 2.1 IL10^{+} producing CD4^{+}CD25^{hi} cell frequencies in the PBMCs stimulated with the secreted component, whole-cell lysate, and culture medium. 25 µg/ml of total protein SS1 *H. pylori* whole cell lysate, 25 µg/ml total protein from culture supernatant (secreted components) or Hams F12 medium (control) alone were cultured with peripheral blood mononuclear cells (PBMCs) from 9 *Hp* infected patients for 16 hours and Treg stimulation (proportion of CD4+CD25hi cells expressing IL-10) was analyzed by flow cytometry (Dr Karen Robinson, unpublished data)

Dr Jody Winter, another member of the group, identified the major constituent proteins in the *H. pylori* secretome (unpublished data). To do this, equal amounts of protein from culture supernatants of the SS1 *H. pylori* strain and whole-cell lysates were resolved by SDS-PAGE. The result showed enrichment of certain proteins in the culture supernatant compared to the whole cell lysate. Next, using MALDI-TOF mass spectrometry peptide mass fingerprinting (PMF), the major constituent proteins were identified (Figure 2.2).

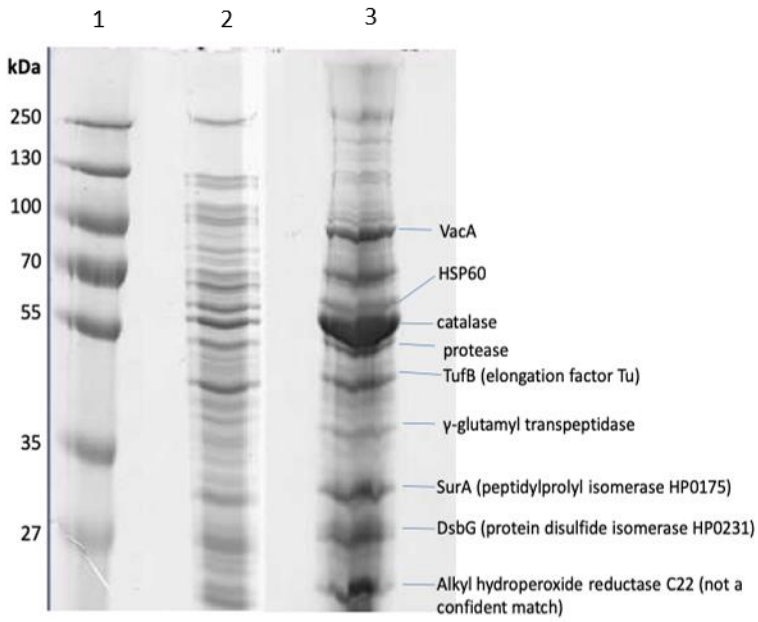


Figure 2.2 The SDS-PAGE analysis of the protein constituents of **H. pylori** SS1 whole cell lysate and its culture supernatant. Lane 1, prestained protein standard; Lane 2, H. pylori SS1 whole cell lysate; Lane 3, H. pylori SS1 culture supernatant containing the secreted components. From the peptide mass fingerprinting (MALDI-TOF), proteins were identified as shown above.

Three of the most enriched proteins were selected for the study. Catalase (KatA, HP0875), γ- Glutamyl transpeptidase (GGT, HP1118), and Peptidyl prolyl cis-trans isomerase (PPT, HP1075) were selected for an initial investigation of their potential immunomodulatory properties.

2.1.2 Catalase

Catalase is one of the most conserved *H. pylori* factors with the approximately 98-100% amino acid sequence similarity among different strains of the bacteria species (Zamocky and Koller, 1999). Catalase gene is 1515 bp

which makes a haem-containing enzyme of 505 amino acids (Loewen et al., 2004). In conditions of oxidation stress especially during *H. pylori* infection, KatA enzymes have been shown to be overexpressed account for 1% of the total *H. pylori* protein (Huang and Chiou, 2011, Wang et al., 2006, Alyamani et al., 2007). This ubiquitous enzyme is essential in the gastric colonisation of the bacteria (Hazell et al., 1991). Interesting, the catalase enzyme retains its activity at a lower pH of 3 compare to urease, another protective enzyme found in *H. pylori,* which its activity is deactivated at pH of 5 (Bauerfeind et al., 1997). Therefore, catalase is believed to be more essential to the survival of *H. pylori* in the lower pH environment such as the human gut (Ramarao et al., 2000).

Schriner et al showed that the maximum life span of transgenic mice overexpressing human catalase in the peroxisome, the nucleus or mitochondria were maximal increased (Schriner et al., 2005). Similarly, other literature has shown that acatalasemia, an inherited deficiency of catalase activity which was originally thought to be an asymptomatic disorder, could be responsible for a number age-associated disease conditions such as diabetes mellitus, hypertension, anaemia, vitiligo, Alzheimer's disease, Parkinson's disease, bipolar disorder (Goth, 2001, Goth and Nagy, 2012, Nandi et al., 2019). During *H. pylori* infection, inflammatory responses by the immune cells produce reactive oxygen species in the gastric mucosa, which could be detrimental by triggering DNA damage and may lead cancer (Robinson et al., 2007, Acheson and Luccioli, 2004). Hydrogen peroxide has been shown the act as a first messenger able to transmit cell-to-cell proinflammatory signals such as nitric oxide and TNF-α (Gunawardena et al., 2019). Siwale et al

demonstrated that incubation of human endothelial cells with mammalian catalase and then stimulating the cells with LPS, pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 were reduced by 90%, 21% and 42%, respectively (Siwale et al., 2009).

H. pylori catalase (KatA) share about 50% amino acid sequence similarities with that of human and since mammalian catalase have been shown to have direct inhibition of proinflammatory cytokine. Consequently, there could be a possibility of catalase produced during the immune response against *H. pylori* infection directly playing anti-inflammatory function.

2.1.3 γ-glutamyltranspeptidase (GGT)

All strains of *H. pylori* constitutively express γ-glutamyltranspeptidase (GGT) and it has been demonstrated to be very important in colonization of the mouse gastric mucosa by the bacteria (Chevalier et al., 1999). The active enzyme is made up of two subunits, the 37 kDa large subunit and 20 kDa small subunit, processed from the originally synthesised 60 kDa pro-enzyme. Catalytically, GGT is known to catalyse the reactions involving the transfer of γ-glutamyl moiety from γ-glutamyl compounds such as glutathione to amino acids or water (Chevalier et al., 1999). As in other organisms, the human version of this enzyme is known and it is routinely utilised in tests as an indicator of hepatic and biliary tract-associated diseases (Hanigan and Ricketts, 1993) Just as *H. pylori* GGT is known to generate H_2O_2 which could cause (Gong

et al., 2010), it is also known to generate ammonia from the hydrolysis of glutamine (Shibayama et al., 2007). Likewise, *H. pylori* GGT activity has been shown to independently control the growth of the bacteria in vitro, as isolate with higher GGT activities grows more profusely as against the ones with lower GGT activity (Gong and Ho, 2004). Although there have been mixed reports on the importance of *H. pylori* GGT in gastric pathogenesis of the bacteria, one report by (Chevalier et al., 1999), argued that it is essential for the colonisation of the gastric mucosa of mice. While using gnotobiotic piglets and C57BL/6 mice (McGovern et al., 2001) reported a significant reduction in colonisation among the *ggt*-isogenic mutant infected animals as compared to the wild-type rather than total clearance of the mutant. Largely, both pieces of evidence project the importance of GGT in the *H. pylori* colonisation. GGT is found in a wide range of organisms from bacteria to mammals and performs a similar function of catalysing the transfer of a γ-glutamyl moiety from γ-glutamyl compounds to molecules such as amino acids and water (Boanca et al., 2006). However, analysis of the *H. pylor*i GGT amino acid sequence shows a remarkable difference from its counterpart in other bacterial species such as *Bacillus subtilis, E. coli* and *Pseudomonas aeruginosa* with only 38%, 52.5% and 47.7 % sequence identity, respectively (Chevalier et al., 1999).

An investigation has shown the mixed role of GGT in the immune response to *H. pylor*i infection, with the protein being reported as being both pro-inflammatory and anti-inflammatory. As pro-inflammatory, *H. pylor*i GGT is known to induce IL-8 secretion in AGS and gastric epithelial cells through the NF-κB pathway (Gong et al., 2010). Hence, GGT synthesis could be the major

reason for the rise in the chemokine, IL-8, during *H. pylor*i infection(Fan et al., 1995). Another report suggested that during *H. pylor*i infections there is an increase in expression of cyclooxygenase-2 (COX-2) and corresponding prostaglandin E_2, both of which are vital in proinflammatory response.

As an anti-inflammatory molecule, GGT is known to have direct immunosuppression effects on both T- cells and DCs. On T-cells it induces cycle arrest at the G1 phase by a mechanism described by Schmees et al. and disruption of Ras-dependent signal pathway (Schmees et al., 2007). The mechanism by which GGT induces polarisation of DCs and subsequent induction of a Treg response is still not yet well understood. But it has been shown that GGT exposed DCs produce IL-10 and induce the FOXP3 and contact-dependent differentiation of T-cells into $CD4^+CD25^{hi}FOXP3^+$ regulatory T-cells while simultaneously preventing T helper 1 (Th1) and Th17 differentiation (Oertli et al., 2013a).

2.1.4 Peptidyl prolyl cis-trans isomerase

Peptidyl prolyl cis-trans isomerase (PPT) is another enzyme family that is evolutionary conserved and are expressed in a wide range of organisms ranging from bacteria to mammal. Their basic role is to catalyse slow the cis-trans isomerization of proline imidic peptide bonds which is important in several biological processes such as protein folding, biomolecular assembly and molecular switch (Shaw, 2002). Due to the likelihood of the cis and trans conformation in the PPT backbone, the enzyme possesses a wide range of catalytic ability and importance(Michnick et al., 1991) thus making it a target

for immunosuppressive agents such as cyclosporine A (CsA), rapamycin and FK506 which are to known to bind immunophilins, a cytoplasmic receptor and member of PPT superfamily(Kim et al., 2010, Bertrand et al., 1999). There are three classes of PPTs, Cyclophilins, FKBP and parvilins. The first PPT member was isolated in 1984 from the porcine kidney cortex and is an 18 kDa cytosolic receptor known as cyclophilin and later an FKBP12, a 12 kDa FKBP class (Fischer et al., 1984, Rahfeld et al., 1994, Harding et al., 1989). In bacteria PPT was discovered first in *E. coli,* a 10.1 kDa PPT class, named parvulin (Rahfeld et al., 1994) which is similar to Par14 another member parvulin which is found in a lot of human tissues with PPT activities.

Tomb et al complete genome sequencing of *H. pylori,* described as a gene which code for the protein designated HP0175 (Tomb et al., 1997). This protein is a ~30 kDa secretory protein with peptidyl-prolyl isomerase activity (McAtee et al., 1998). Since then several investigations to ascertain other activities of this protein have shown a tendency to induce the proinflammatory cytokines, apoptosis and autophagy (Halder et al., 2015, Basak et al., 2005). It is associated with severity gastritis, initiation gastric pathologic lesions which may progress to peptic ulcers or gastric cancer (Zhu et al., 2015). Some other reports inferred that the HP-PPT induces Th17 signature pro-inflammatory cytokines such as IL-17 and IL-21 by lymphocyte infiltration in the tumour from cancer patients (Amedei et al., 2014). With the knowledge of the role of PPT in the cellular proliferation, concluded that it is the imbalance between the apoptosis and the cellular proliferation that could be promoting carcinogenesis (Zhu et al., 2015, Oghalaie et al., 2016), asserts that it could be the imbalance

between induced apoptosis and induced cellular proliferation that promotes carcinogenesis.

To this end Basak et al, 2005 went on to show that in AGS cells, there is a direct and specific interaction between TLR4 and PPT and likewise that the signalling pathway activated by PPT is downstream of TLR4. This is in agreement with other studies which depict the upregulation of TLR4 and MD-2 in the gastric mucosa in cases of *H. pylori* infection (Ishihara et al., 2004). Similarly, PPT was also demonstrated to interact with TLR 4 in a macrophage-monocyte model, THP-1, and PBMC binding directly binding to the ectodomain (ECD) of the TLR4. Following this interaction, MAPK ERK and p38 MAPK signalling pathways are activated in P13K/Ras, Rac1-dependent manner and subsequently, activation of MSK1, which is important in NF-κB activation (Pathak et al., 2006).

It is also known to readily induce antigen-specific serum antibodies in an infected patient, making it a good marker for a non-invasive serology assay (Atanassov et al., 2002). Which also shows that PPT interacts with antigen-presenting cell and adaptive immunity.

Although the interactions of PPT with TLR4 are well-established, it's not known whether PPT produces other effects on the immune cells order than its ability to trigger the caspase pathways leading to apoptosis in epithelial cells (Basak et al., 2005) and supposed IL-6 induction in macrophages (Pathak et al., 2006). We hypothesis that *H. pylori* PPT might have a direct anti-inflammatory influence on some immune cells.

2.2 Hypothesis

Data from humans and mouse models have shown that stronger Treg responses are linked to higher levels of *H. pylori* colonisation, and these cells also contribute to the persistence of the infection (Robinson et al., 2008, Robinson et al., 2005). Work from our group showed that *H. pylori* factors very potently stimulated human peripheral blood Tregs *in vitro* to express the anti-inflammatory cytokine IL-10. However, IL-10 mediated immunoregulation is not the only path to regulation of immune response. Gebert et al reported that VacA, a *H. Pylori* factor, abolished the nuclear translocation of nuclear factor of activated T cells (NFAT) thereby downregulating IL-2 production in T cells (Gebert et al., 2003). It was hypothesised that some other bacterial factors could exert direct suppressive effects on proinflammatory cytokine production by immune and epithelial cells. Aims of the research were therefore

- To express *katA, ggt* and *ppt* genes in ClearColi® BL21 (DE3) competent cells to avoid LPS effects, and purify the recombinant proteins.
- To examine the LPS contamination of recombinant protein preparations
- To characterise the recombinant proteins and assess the catalase activity of rKatA.
- To determine the ability of the recombinant proteins to suppress the production of cytokines by activated human T cells (IL-2), monocytes (IL-6) and gastric epithelial cells (IL-8).

2.3 Materials and Methods

2.3.1 Preparation of the Recombinant Proteins

2.3.1.1 Gene cloning (carried out by Dr Jody Winter)

The genes encoding the proteins Catalase (KatA), Gamma Glutamyl Transpeptidase (GGT) and Peptidyl Prolyl Transferase (PPT) were cloned into pET -41a (+) plasmid (Novagen) (Appendix Figure A) expression vector that has a 6 x His- tag which is needed for the immobilized metal affinity chromatography protein purification. Polymerase chain reaction (PCR) was used to amplify the genes of *katA, ggt* and *ppt* (Appendix Table 1) according to the genomic DNA of *H. pylori* strain 26695 (complete genome (Tomb et al., 1997).

To confirm the amplification of the genes, the amplified genes were separated on a by 1 % agarose TAE gel with ethidium bromide which stains the DNA. Then using the QIAquick gel extraction kit (Qiagen), the DNA products were extracted, before ligation into the plasmid pJET1.2 vector, containing Ampicillin resistant gene, the ends of the purified DNA products were blunted using a blunting enzyme (Fermentas, CloneJet kit). Using heat shock, the resultant plasmids were transformed into a chemically competent *E. coli* (NEB 5α) which is then plated on to Luria Bertani (LB) agar plates containing 100 μg/ml Ampicillin as a plasmid selecting agent. To confirm the gene insertion a colony PCR was performed and then the positive colonies were transferred to

the 5 ml LB broth containing 100 μg/ml Ampicillin. After overnight incubation at 37 °C in a shaker, the cells were pelleted by centrifugation and the plasmids were purified using a GenElute™ Plasmid Miniprep Kit (Sigma). To ensure there was no introduction of mutations by the PCR, sequencing was performed on the plasmid. NdeI and KpnI restriction enzymes were used to subclone the genes into a pET-41a expression vector (Novagen), containing the kanamycin-resistant gene. The presence of the subcloned genes was established by restriction digestion analysis and using heat shock (Kushner, 1978), the resultant plasmids were transformed into chemically competent *E. coli* B834 (DE3). Although this *E. coli* strain is easy to manipulate for high protein expression and yield, it has the problem of purification as the purified proteins from the strain usually contains lipopolysaccharide (LPS) bound strongly to the protein. The LPS bound to the protein could easily trigger an endotoxic response in cell culture of human origin.

2.3.1.2 Transformation into ClearColi®

ClearColi® BL21 (DE3) competent cells are a commercially available genetically modified *E. coli* BL21 (DE3) strain which does not trigger the endotoxic response in human cells. This was created by deletion of two of the secondary acyl chains in a wild type hexa-acylated LPS thereby abolishing its endotoxicity in human cells. NF-κB is activated when the six chains of acyl group of the LPS activate the Toll-like receptor 4(TLR4)-myeloid differentiation factor 2 (MD-2) complex leading to the production of the proinflammatory

complex. But the deletion of 2 out these 6 acyl chains results in Lipid IV_A which does not activate the formation of TLR4/MD-2 complex thereby not induce the endotoxic response. ClearColi BL21 (DE3) is most suitable for several applications since the plasmids or proteins purified from ClearColi does not LPS contain and the presence of the LPS in form of Lipid IV_A does not elicit an endotoxic response in eukaryotic cells (Lucigen, 2019)

E. coli B834 (DE3) transformed previously in section 2.4.1.2 were cultured in 5 ml LB broth supplemented with 30 μg/ml kanamycin sulfate and grown overnight. The cells where harvested and the plasmids purified using a GenElute™ Plasmid Miniprep kit (Sigma). The *katA*-pET-41a, *GGT*-pET41a and *PPT*-pET41a recombinant plasmids were transformed into ClearColi® BL21 (DE3) electrocompetent cells by electroporation as follows:

The electroporation cuvettes (0.1 cm gap) and microcentrifuge tubes were placed on ice prior to the transformation. Aliquots of the *E. coli* strain BL21 (DE3) with modified non-stimulatory LPS (Lucigen) were removed from the -80 °C freezer and thawed completely on wet ice and 25 µl was pipetted into the microcentrifuge tubes. 1 µl of the extracted plasmid was added and the mixture stirred with the pipette tip. To the chilled cuvette, 25 µl of the cell/DNA mixture was added and electroporated at 25 µFD Capacitance, 200 Ω and 2.5 volts using a Gene Pulser (BioRad) and Pulse Controller (BioRad). Within a few seconds of the pulse, 975 µl of expression recovery medium (Lucigen) was added to the cuvette and pipetted up and down to mix. The cells in the cuvette were transferred to 15 ml culture tubes and incubated in a

shaking incubator for 1 hour at 37 °C. 100 µl was then spread onto LB plates containing 30 µg/ml kanamycin, which were incubated for 40 hours at 37 °C. The transformed and antibiotic selected colonies were picked and subcultured onto fresh LB plates with 30 µg/ml kanamycin.

2.3.1.3 Confirmation of Transformation via Polymerase Chain Reaction

To confirm the presence of the plasmid in the transformed cells, colonies from original LB plates as well as the subcultured plates were tested for the presence of the *katA* genes. The PCRs was carried out in 20 µl volumes and consisted of the appropriate volume of PCR buffer, dNTPs (0.2mM concentration each), 0.5U of a Taq green DNA polymerase mix (9:1) and 10-100ng of the template DNA. The KatA primers used are shown in Table 3. The reaction was carried out in a PCR Express thermal cycler (Thermo Hybaid) using the following parameters: 95 °C for 10 min initial denaturation, followed by 95 °C for 1 min denaturation, 50 °C for 30 seconds annealing and 72 °C for 2 min extension. A final extension period of 10 min at 72 °C was performed. The bacterial colonies shown to contain the plasmid were then frozen and stored in 75% glycerol in -80 °C.

2.3.1.4 Protein Expression

A single colony of transformed ClearColi® BL21 (DE3) was inoculated into 40 ml Luria Bertani (LB) broth containing 30 μg/ml kanamycin sulfate and supplemented with 0.5% (w/v) glucose and incubated at 37 °C in a shaker incubator. Following overnight incubation, the 40 ml culture was inoculated into 1 litre LB broth supplemented with 30 μg/ml kanamycin sulfate and incubated with shaking at 37 °C until an OD_{600} of 0.6-0.8. Subsequently, a final concentration of 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) Melford, Darmstadt, Germany) was used to induce a conformational change in the repressor to give access to T7 RNA polymerase together with other protein synthesis machinery in the cell for overexpression of the intended recombinant proteins. The cultures were placed in a shaker at 30 °C for 4 hours and placed on ice for 10 minutes then the cells were harvested by centrifugation at 5,000 x g for 10 minutes at 4 °C. The pellets were resuspended in 20 ml binding buffer (20 mM Na_3PO_4, 0.5 NaCl, 10 mM imidazole pH 7.4) (GE Healthcare, life sciences) and stored in -20 °C freezer.

2.3.1.5 Protein Purification-First step purification

The induced frozen cell suspensions were allowed to thaw completely, then a complete EDTA free protease inhibitor tablet (Roche) was added to 10 ml of culture then vortexed to dissolve completely. Afterwards, the cells were lysed on ice using Soniprep (Sanyo) at 10 seconds burst x 4 with 30 seconds

break after each burst to avoid overheating and destruction of the recombinant proteins. The lysed ClearColi® BL21 (DE3) suspension was centrifuged on 9,000 x g at 4 °C for 20 minutes, then the supernatants portion which contains the soluble proteins were loaded into 10 ml His GraviTrap™ Talon© (GE Healthcare, life sciences) prepacked gravity flow column pre-equilibrated with 10 ml of the binding buffer (GE Healthcare life sciences) for an immobilised metal affinity chromatography. The flow-through contain the unbound constituent from the supernatants and column was afterwards washed with 3 x 10 ml binding buffer (GE Healthcare, life sciences) which washes off the weakly unbound materials. Lastly, the his-tagged recombinant proteins still left bound to the column resins were eluted using the 3 ml Elution buffer (20 mM Na_3PO_4, 0.5 NaCl, 500 mM imidazole pH 7.4) (GE Healthcare, life sciences).

2.3.1.6 Further protein purification-Second step purification

The eluted products were further purified to obtain a purer product and exchange the imidazole containing buffer. To do this, the GraviTrap Talon elution was first filtered using 0.2 μm Minisart® NML Syringe Filters (Sartorius) and loaded onto a Hiload 26/60 s200, size exclusion chromatography column (GE Healthcare) pre-equilibrated with 50 mM Tris base pH 8.8, 200 mM NaCl. The system was run using an AKTA prime plus (GE Healthcare) at 0.5 ml/min and 5 ml fractions were collected. Then the fractions containing the recombinant protein were concentrated using Vivaspin 20, 50,000 MWCO PES (sartorius) and the concentration of purified recombinant proteins were

determined using Pierce™ BCA protein assay kit (Thermo Scientific) following the manufacturer's instructions. The proteins were then stored in the -80 °C until required for various assays.

2.3.1.7 Characterisation of the purified protein

2.3.1.7.1 SDS-PAGE and PageBlue™ Protein Staining

Following the method established by (Laemmli, 1970), the protein samples were resolved by denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Initially, the protein samples were heated to the temperature of 100 °C for 5 minutes in a 1 x reducing SDS sample buffer (50 mM Tris-Cl, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and then 10 μl of each of the samples loaded into the wells of the gel together with 3 μl of Precision Plus Protein™ Prestained Standards (Bio-rad, UK). The prestained standard was used in assessing the approximate size of the proteins after separation by electrophoresis.

The SDS-PAGE gel included the resolving gel which contained 15% polyacrylamide and the stacking gel which contained 3% polyacrylamide, the gel was cast in the Invitrogen™ Empty Gel Cassettes, mini, 1.5 mm (ThermoFisher). Table 2.1 below indicates the components of the gel.

Table 2.1 Composition of the Resolving gel and Stacking gel preparation for the SDS-PAGE.

Resolving Gel (7.5 ml) for one gel	**Stacking gel (5 ml) for one gel**
- 2.5 ml of 30 % Bis-acrylamide solution: bis37:1 (Severn Biotech Ltd, UK)	- 0.65 ml of 30 % Bis-acrylamide solution (37:1 (Severn Biotech Ltd, UK)
- 1.875 ml 1.5 M Tris HCL pH 8.8	- 1.25 ml 0.5 M Tris HCL pH 6.8
- 3.125 ml distilled H_20	- 3.05 ml distilled H_20
- 0.1 μl 10% (w/v) SDS	- 50 μl 10% (w/v) SDS
- 25 μl of 10% Ammonium persulphate (APS) (SIGMA)	- 25 μl of 10% Ammonium persulphate (APS) (SIGMA)
- 5 μl of TEMED (Melford)	- 5 μl of TEMED (Melford)

The BioRad mini Protean II gel system (BioRad PowerPac 200/2.0) containing electrode buffer (5 x stock: 0.125 Tris, 0.95M glycine 0.4% SDS pH 8.3) ran at 180 V constant voltage, was used to separate the proteins. At the end of the electrophoresis, the cassette was opened, and the gel transferred to a clean tray containing 100 ml of distilled water and heated gently to 65 °C for 3 minutes. The water was removed and 20 ml of PageBlue™ Protein Staining Solution (Thermo Scientific) was added to visualise the protein bands and developed for 4 hours. The dye was decanted, and the gel washed twice

with distilled water, then the gel was destained with 10 % acetic acid for 2 hours, Molecular Imager® Gel Doc™ XR+ Imaging system (BIO-RAD) was used to scan the gel.

2.3.1.7.2 Western Blot analysis

After the SDS-PAGE, the gel for the western blot was equilibrated in the blotting buffer (5.8g glycine, 11.6 g tris base, 7.4ml 10% SDS, 400ml methanol and 2L distilled water) for 10 minutes prior to transblotting. The nitrocellulose paper and a couple of Whatman filter papers cut to the same size as the gel (8.5 cm x 6.5 cm) were soaked in the blotting buffer. Together with the Scotch-Brite pad, the gel, nitrocellulose paper and filter paper were assembled in a sandwich manner on a mini blotter cassette (BioRad) taking care no air bubble is trapped by rolling a pipette gently over the sandwich and the top of the gel align with the nitrocellulose. The cassette was placed in the electrode assembly with the nitrocellulose facing the anode and frozen ice-pack inserted as the cooling unit all in the buffer. The cell was connected to the power pack (Bio-Rad) through the lid and ran at a constant current of 150mA for 60 minutes. Proper transfer of the prestained molecular weight marker was used to adjudge the efficiency of the transfer.

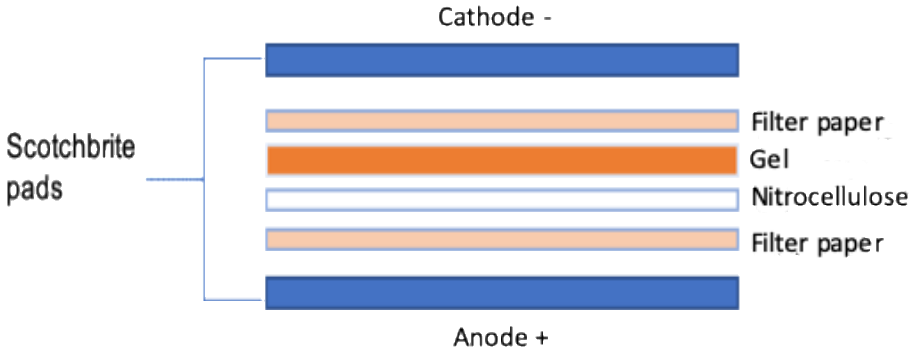


Figure 2.3 Gel nitrocellulose assembly for electrophoretic transfer cells.

The blot was blocked for 1 hour on a shaker at room temperature with 2% skimmed milk powder (Marvel) in PBS-Tween (500 µl $Tween_{20,}$ 1X PBS tablet in 1L distilled water) (Blocking solution). Following blocking, the primary antibody (6x-His Tag monoclonal antibody) (ThermoFisher Scientific) was added at the dilution of 1:20000 and incubated overnight at 4 °C on the shaker. After incubation, the blot was washed in PBS-Tween 3 x with 5 minutes shaking incubation in between each wash. The blot was placed in the 1:50000 dilution of the secondary antibody Anti-rabbit IgG-horseradish peroxide conjugate (Sigma), and incubated for 1 hour. Subsequently, the blot was washed 5x for 5 minutes in PBS-Tween and 1 x in PBS.

The chemiluminescent readout was accomplished using the enhanced chemiluminescence (ECL) substrate kit (ECL Western blotting detection reagent: GE Healthcare). The blot was incubated in the ECL for 1 minute and then placed in the autoradiography cassette. Working in the dark, the autoradiography film as used to detect the bands for 1 minute and then

developed using the developer and replenisher (Carestream GBX) and fixer and replenisher (Carestream GBX). The film was washed with distilled water and allowed to dry.

2.3.1.7.3 Catalase Assays

2.3.1.7.3.1 Catalase activity

Catalase activity was measured using Amplex® Red Catalase Assay Kit (ThermoFisher Scientific) following the manufacturer's protocol described briefly as follows; The standard curve of 0 to 4.0 U/mL was prepared from the 1000 U/mL catalase stock solution and 25 μL of each of the standard as well as the sample (diluted in the 1X reaction buffer) were added to 96-well microplate with the negative control as 1X reaction buffer. 25 μL of 40 μM H_2O_2 was also added to each of the wells of the standards, samples and control and incubated at room temperature for 30 minutes. Following incubation, 50 μL of Amplex Red/HRP (containing 0.4 U/mL and 100 μM Amplex red reagent in 1X Reaction buffer) was added to each of the wells of standard, samples and control and incubated for 30 minutes at 37 °C away from light. The absorbance was measured in a microplate reader at 595 nm. The absorbance was reported by substracting the sample from the control without any catalase and catalase activities were interpolated from the standard.

2.3.1.7.3.2 Catalase biochemical test

To carry out the test, 5 μL of the sample (rKatA) was dropped on a glass slide while 5 μL rGGT was on another slide as a negative control. Then 5 μL of

3 % H_2O_2 was added to each of the sample and the control. A positive result was considered by the rapid evolution of oxygen in the form of effervescence.

2.3.2 Jurkat T Cell Suppression Assays

The Jurkat T cell line was maintained in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS) and 100 μg/ml and 100 U/ml of penicillin/streptomycin antibiotics and incubated at 37 °C in an atmosphere 5 % CO_2. The 96 microwells flat bottom tissue culture plate (Thermo scientific) were pipetted with 200 μl of the cell suspension at the density of 1 x 10^6 cells/ml in triplicate. Then the cells were stimulated with increasing concentration (10, 25 and 50 μg/ml in Tris buffer) of each of the purified recombinant KatA, GGT and PPT and incubated for 1 hour at 37 °C. Then the cells were further stimulated with 50 ng/ml phorbol myristate acetate (PMA) and 1 μM ionomycin, and incubated for an additional 24 hours. The supernatants were harvested after centrifugation to remove the cells. Finally, the resulting supernatants were stored in a – 80°C freezer until assayed for the presence of IL-2 by ELISA as per the manufacturer's instruction (ThermoFisher scientific).

2.3.3 THP-1 Cell Suppression Assays

THP-1 monocytic cells were maintained in culture media, RPMI 1640, supplemented with 10 % fetal bovine serum (FBS) and 100 μg/ml and 100 U/ml penicillin/streptomycin antibiotics incubated at 37 °C in an atmosphere of 5 % CO_2. The 96 microwells flat bottom tissue culture plate (Thermo scientific)

were pipetted with 200 μl of the cell suspension at the density of 1 x 10^6 cells/ml in triplicate. Then the cells were stimulated with increasing concentration of each of recombinant KatA, GGT and PPT (10, 25 and 50 μg/ml) and incubated for 1 hour at 37 °C. Then the cells were further stimulated with 40 ng/ml LPS (mitogen) and incubated for an additional 24 hours. The supernatants were harvested by centrifuge to remove the cells. Finally, the resulting supernatants were stored away in the – 80 °C freezer and assayed for the presence of IL-6 by ELISA as per the manufacturer's instruction (ThermoFisher scientific).

2.3.4 AGS Cell Suppression Assays

AGS cells, a human adenocarcinoma cell line, were maintained in culture media, F-12 Ham, supplemented with 10 % fetal bovine serum (FBS), L-glutamine and 100 μg/ml and 100 U/ml incubated at 37 °C in an atmosphere 5 % CO_2. The 24-well flat-bottom tissue culture plate (Thermo scientific) were pipetted with 200 μl of the cell suspension at the density of 5 x 10^5 cells/ml in triplicate. Then the cells were stimulated with increasing concentration of each of recombinant KatA, GGT and PPT (10, 25 and 50 μg/ml) and incubated for 1 hour at 37 °C. Then the cells were further stimulated with 40 ng/ml TNF-α (mitogen) and incubated for an additional 24 hours. The supernatants were harvested after centrifugation to remove the cells. Finally, the resulting supernatants were stored away in the – 80 °C freezer and assayed for the presence of IL-8 by ELISA as per the manufacturer's instruction (ThermoFisher scientific).

2.3.5 Cytokine ELISA Assays

Human IL-2, IL-8 and IL-6 concentrations were measured in the supernatant from cell culture induction assays of Jurkat, AGS and THP-1 cells respectively, using Human IL-2, IL-8 and IL-6 ELISA kits (Invitrogen) following the manufacturer's protocol, with a detection limit <2 pg/ml. The optical densities of the 96 microtiter plate wells were measured using a plate reader (LabSystems iEMS reader MF) at dual wavelengths of 450 nm and 620 nm, and the data were analysed using Gen5 Data Analysis Software (BioTek). To calculate the cytokine concentration in each of the samples, a standard curve on each of the assay microplates (positive control) was used as the reference. Four control wells containing no cytokines were used to determine the assay sensitivity, calculated as the mean plus 3 times the standard deviation.

2.3.6 Statistical analysis

Graphs were prepared and statistical analysis was carried out using GraphPad Prism version 8.3 software. Data and error bars indicate the mean ±SEM. Statistical significance was determined using a one-tailed Student T-test for data with a non-Gaussian distribution. Differences were considered significant at $p<0.05$*, $p<0.01$** and $p<0.001$***.

2.4 Results

2.4.1 Expression and purification of rKatA, rGGT and rPPT

2.4.1.1 Cloning and Colony PCR of the *katA, ppt* and *ggt* genes to conform the transformation into ClearColi cells

The recombinant plasmids containing the *katA, ggt* and *ppt* genes, made by Dr Jody Winter (Section 2.4.1.1) were purified and subject to transformation by electroporation into ClearColi®. Transformed cells were plated on selective medium (LB agar containing 30 μg/ml). After incubation, the resulting colonies were tested using colony PCR analysis, picking 6-7 colonies from each plate. PCR products were analysed on an agarose gel. Gels were checked for the bands corresponding base pairs for each of the genes (*katA, ggt* and *ppt* genes, in Figure 2.4 A; *ppt* in Figure 2.4 B; and *ggt* in Figure 2.4 C)

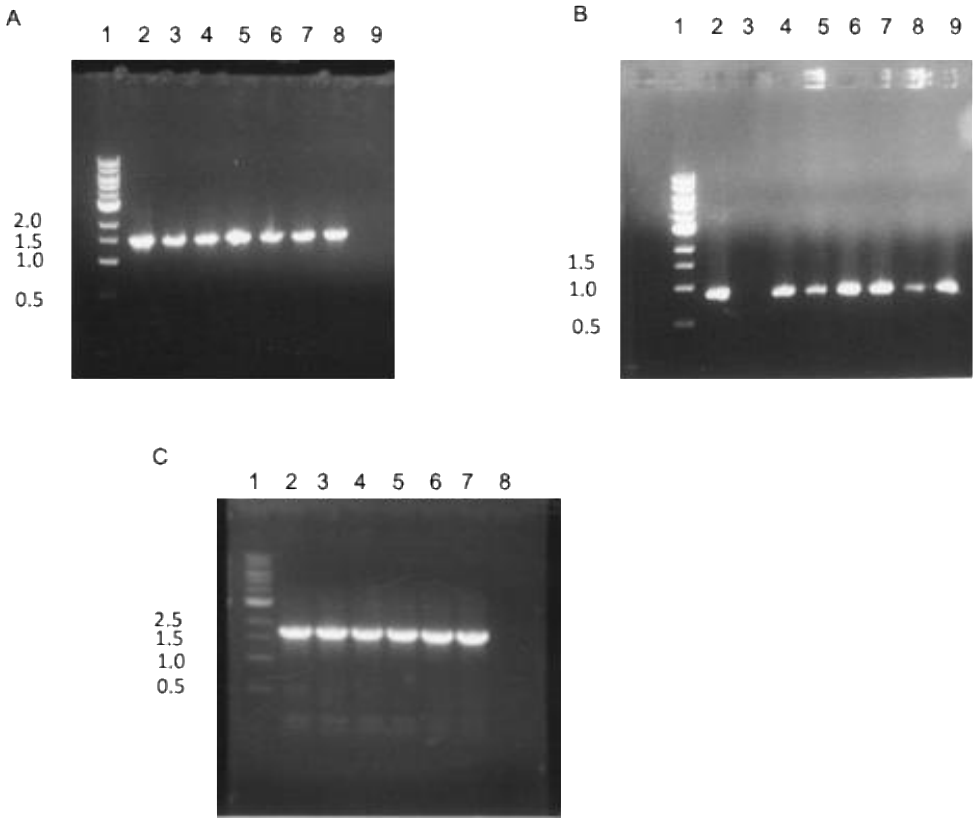
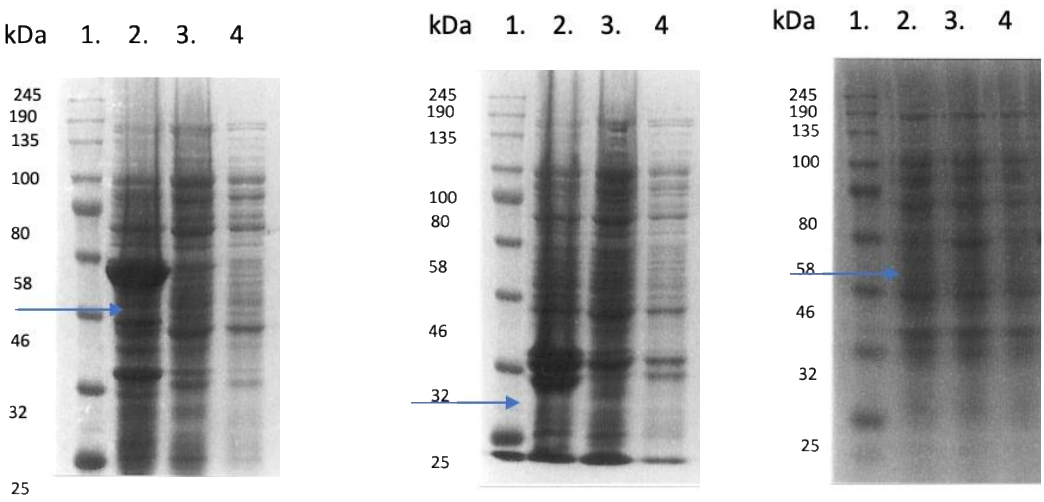


Figure 2.4 Confirmation of the transformation of Clearcoli competent cells with the recombinant plasmids. A. 0.8 % (w/v) TAE agarose gel stained with ethidium bromide: Lane 1, 1kb DNA ladder; Lane 2, the purified katA DNA template as the positive control; Lane 3 -8 are 6 different colonies from the transformed LB plate; Lane 9, is the PCR prep without any DNA as the negative control. B. 0.8 % (w/v) TAE agarose gel stained with ethidium bromide: Lane 1, 1kb DNA ladder; Lane 2, the purified ppt DNA template as the positive control; Lane 3; is the PCR prep without any DNA as the negative control; Lane 4-9 are 6 different colonies from the transformed LB plate. C. Lane 1, 1kb DNA ladder; Lane 2, the purified ggt DNA template as the positive control; Lane 3 -7 are 5 different colonies from the transformed LB plate; Lane 8, is the PCR prep without any DNA template as the negative control.

2.4.1.2 Overexpression of the recombinant protein and purification by Immobilized metal affinity chromatography (IMAC)

The expression of the three proteins in ClearColi® was confirmed by analysing the pre and post-induction whole-cell samples by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The corresponding bands to the molecular weight for each of the recombinant proteins were visualised using PageBlue™ Protein Staining Solution. (rKatA in Figure 2.5 A; rPPT in Figure 2.5 B; and rGGT in Figure 2.5 C)



C. rKatA 59kDa B. rPPT 34kDa A. rGGT 61kDa

Figure 2.5 A typical SDS-PAGE showing the expression of the recombinant proteins rKatA, rPPT and rGGT. The ClearColi transduced with the his-tagged recombinant DNA were grown to the OD_{600} of 0.6-08, then induced with 1 mM IPTG and further incubated for 4 hours at 30 °C in a shaker incubator. The cells were lysed by sonication on ice, then clarified by centrifuge to harvest the soluble fraction. Lane 1, prestained protein standard; Lane 2 Induced soluble lysate; Lane 3, the whole cells of IPTG induced ClearColi; Lane 4, pre-induction ClearColi whole cells.

To aid purification of the proteins using immobilised metal affinity chromatography, the proteins were expressed in the ClearColi® as a hexahistidine-tagged protein at the N-terminal. Then sonication was used to lyse the cells on ice in a binding buffer containing 20 mM Na_3PO_4, 0.5 NaCl, 10 mM imidazole pH 7.4 with Protease Inhibitor Cocktail Tablets added. Afterwards, the lysates were separated by centrifugation and a sample of the supernatants expected to contain the soluble proteins were resolved by SDS-

PAGE and visualised using PageBlue™ Protein Staining Solution (rKatA in Figure 2.5 A lane 2; rPPT in Figure 2.5 B lane 2; and rGGT in Figure 2.5 C lane 2).

The supernatants containing the soluble proteins were then loaded onto 10 ml His GraviTrap™ Talon© columns for the first step purification and washed three times with the binding buffer containing 10mM imidazole to ensure the weakly bound impurities were washed off. Subsequently, the desired recombinant proteins are eluted in the elution buffer containing 500mM imidazole. The purified eluted fractions were resolved by SDS-PAGE to ascertain the purity of the eluted purified product (rKatA in Figure 2.6 A; rPPT in Figure 2.6 B; and rGGT in Figure 2.6 C)

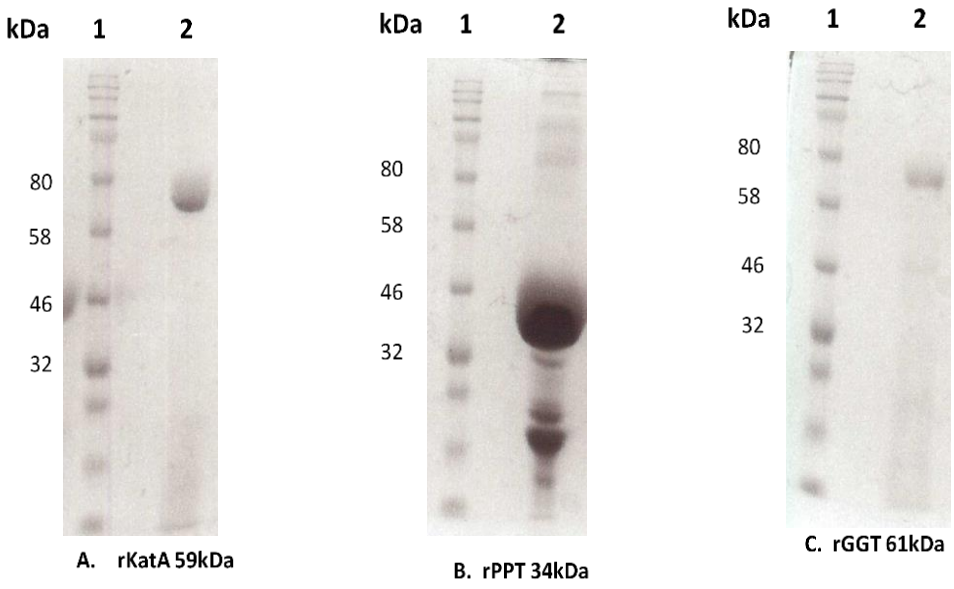


Figure 2.6 A typical SDS-PAGE analysis from the purification of rKatA, rPPT and rGGT. The induced ClearColi containing overexpressed recombinant proteins were lysed by sonication and centrifugation was used to separate the soluble fraction which was then applied to a prepacked His GraviTrap 1 ml TALON superflow chromatography. Lane 1, prestained protein standard; Lane 2, Final wash of the Talon; Lane 3; Final Talon elution.

2.4.1.3 Further Purification of the Proteins by Size-exclusion Chromatography

Size exclusion chromatography (SEC) also known as gel filtration, works with the principles of separating molecules based on the differences in the size following their movement through a column packed resin. This was employed to further purify the proteins after the metal affinity chromatography and to also carry out buffer exchange to remove the 500mM imidazole in the first stage elution buffer. This second stage purification helped for removal of

contaminating proteins from the ClearColi® host cells. To ensure that the purified proteins were collected at the correct molecular weight fraction, proteins standards were first to run in the SEC column and the resulting peaks from the calibration curve were used as a marker (Figure 2.7).

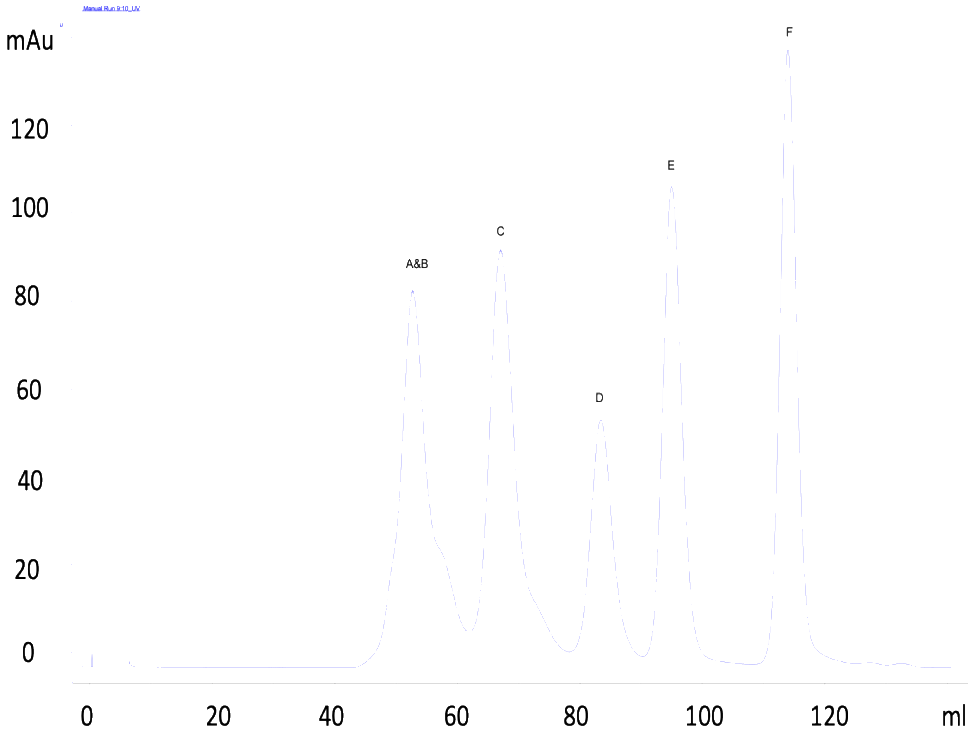


Figure 2.7 Standard calibration for size exclusion columns with peaks.

A; Protein aggregate (void peak), B, Thyroglobulin 670 kDa-49 ml; C; γ-globulin 158 kDa-63.8 ml; D; Ovalbumin 44 kDa – 80.45 ml; E; Myoglobin 17 kDa – 92.4 ml; F; Vitamin B_{12} 1.35 kDa -112.14 ml.

2.4.1.4 rPPT purification by Size-exclusion chromatography

The filtered rPPT samples from the first step of His GraviTrap™ Talon© purification were loaded on to the SEC column and eluted into 5 ml fractions. Each of the fractions was resolved on 12% SDS-PAGE to confirm the presence of the desired protein in the eluted fraction, comparing the expected molecular weight from the calibration curve of the standard and the peaks from the purification run. The chromatogram below (Figure 2.8 A) shows asymmetric rPPT elution, indicating a homogenous elution of rPPT.

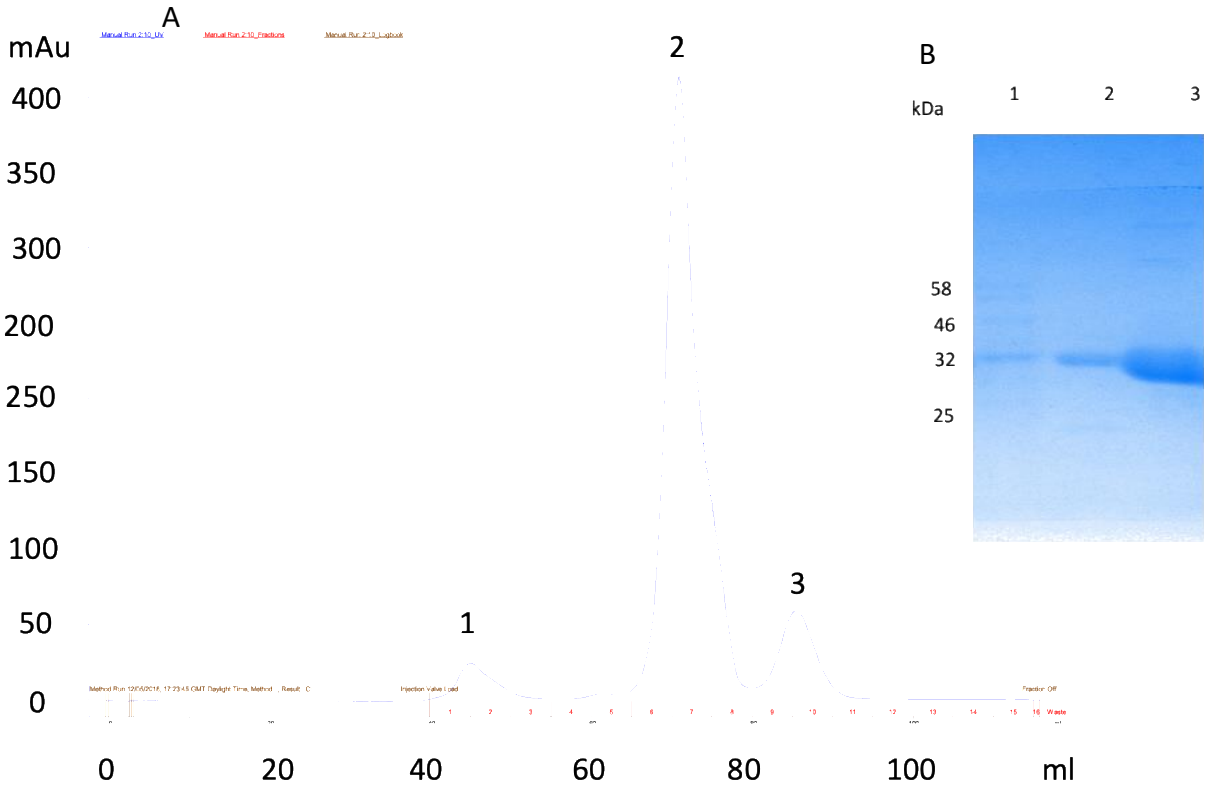


Figure 2.8 A typical size exclusion (SE)/gel filtration chromatogram/ trace for rPPT purification. A. The vertical / X-axis indicates the level of absorbance downstream of the column measured by the UV detector in miliabsorbance units at 280 nm. The horizontal/Y-axis indicates the volumes separated by the system and collection in fractions of 5 ml. The fractions are indicated by rows of red lines with each peak representing the volume contain the proteins. The peak number 1 represents the larger aggregate of proteins also known as a void peak. Peak 2 represents the target protein rPPT and peak 3 represent the smaller molecular weight imidazole. B. A typical SDS-PAGE analysis of the fractions from the size exclusion purification of rPPT to confirm the presence of the target protein. Lane 1, prestained protein standard. Lane 2, a pool of fractions 4, 5,6, 7 and 8, Lane 3 is the concentrated fraction protein using a vivaspin concentrator.

The SDS-PAGE analysis of the fractions showed that the recombinant protein was present in fractions 4, 5, 6, 7 and 8 according to the expected molecular weight of rPPT. The weak band at 34 kDa was due to dilution of the fraction from the SEC elution (Figure 2.8 B Lane 2). The protein in the pooled fractions was further concentrated (Figure 2.8 B lane 3) and the total protein concentration was measured using BCA assay. The SDS-PAGE analysis indicated an approximate 90% purity. The concentrated protein was aliquoted, subjected to a quick freeze in liquid nitrogen and stored in -80 °C until needed.

2.4.1.5 rKatA purification by Size-exclusion chromatography

The chromatogram of rKatA samples subjected to purification by SEC column and collected in of 5 ml fractions (Figure 2.9 A). Each of the fractions was resolved on 10% SDS-PAGE gel to confirm the presence of the desired protein. Eluted fraction 6, 7 and 8, comparing the expected molecular weight from the calibration curve of the standard and the peaks from the purification run. The SDS-PAGE (Figure 2.9 B) shows asymmetric rKatA elution, indicating a homogenous elution of rKatA of an approximate 90% purity.

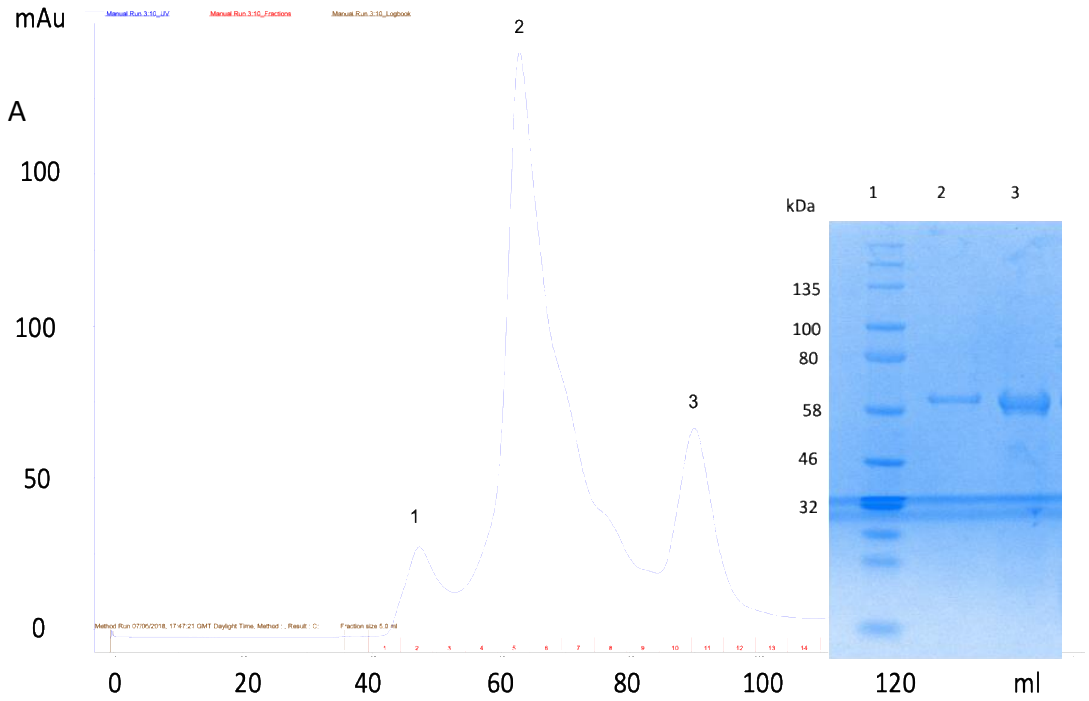


Figure 2.9 A typical size exclusion (SE)/gel filtration chromatogram/ trace for rKatA purification. A. The vertical / X-axis indicates the level of absorbance downstream of the column measured by the UV detector in miliabsorbance units at 280 nm. The horizontal/Y-axis indicates the volumes separated by the system and collection in fractions of 5 ml. The fractions are indicated by rows of red lines with each peak representing the volume contain the proteins. The peak number 1 represents the larger aggregate of proteins also known as the void peak. Peak 2 represents the target protein rKatA and peak 3 represent the imidazole which is quit a smaller molecular weight. B. A typical SDS-PAGE analysis of the fractions from the size exclusion purification of rKatA to confirm the presence of the target protein. Lane 1, prestained protein standard, Lane 2, pooled fractions 6, 7 and 8, Lane 3 is the concentrated fraction protein using a vivaspin concentrator.

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2.4.1.6 rGGT purification by Size-exclusion chromatography

The chromatogram of rGGT samples subjected to purification by SEC column and collected in of 5 ml fractions (Figure 2.10 A). Each of the fractions was resolved on 10% SDS-PAGE gel confirm the presence of the desired protein. Eluted fraction 6, 7 and 8, comparing the expected molecular weight from the calibration curve of the standard and the peaks from the purification run. The SDS-PAGE (Figure 2.10 B) shows asymmetric rGGT elution, indicating a homogenous elution of rGGT at about 90% purity.

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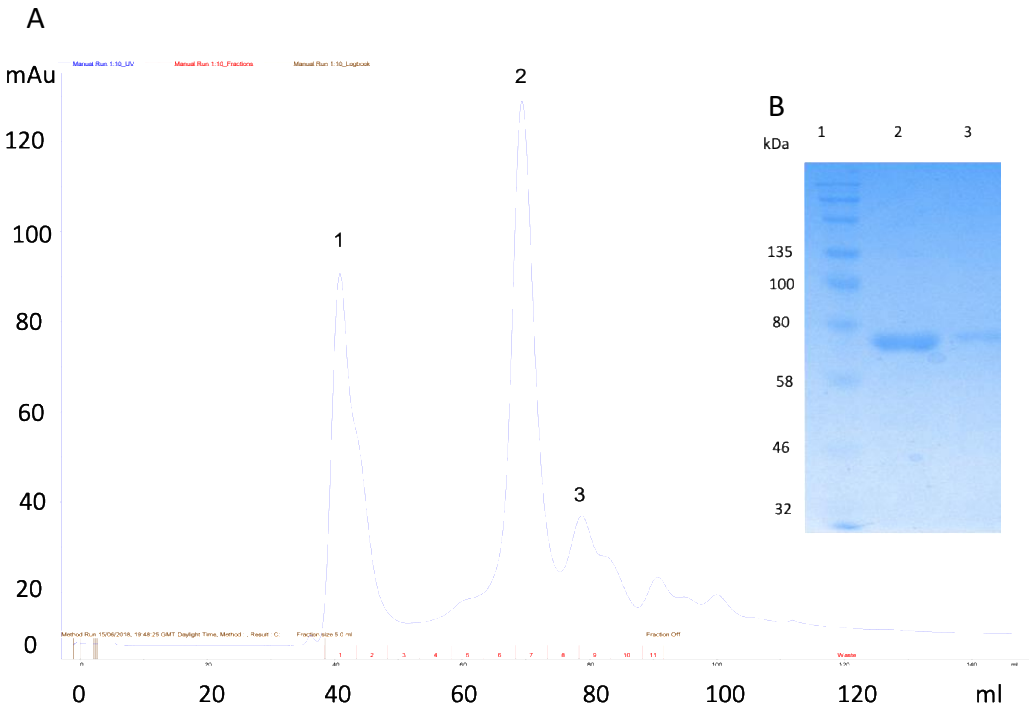

Figure 2.10. A typical size exclusion (SE)/gel filtration chromatogram/ trace for rGGT purification. A. The vertical / X-axis indicates the level of absorbance downstream of the column measured by the UV detector in miliabsorbance units at 280 nm. The horizontal/Y-axis indicates the volumes separated by the system and collection in fractions of 5 ml. The fractions are indicated by rows of red lines with each peak representing the volume contain the proteins. The peak number 1 represents the larger aggregate of proteins also known as the void peak. Peak 2 represents the target protein rGGT and peak 3 represent the imidazole which is the smaller molecular weight. B. A typical SDS-PAGE analysis of the fractions from the size exclusion purification of rGGT to confirm the presence of the target protein. Lane 1, prestained protein standard, Lane 3, a pooled fraction of 6 and 7, 8, Lane 2, is the concentrated the fraction containing the protein using vivaspin concentrator.

2.4.2 Western Blot analysis of Size exclusion chromatography purified recombinant proteins.

To confirm the identity of the three purified proteins (Section 2.4.1), each of the protein products from the second step of the purification process was resolved by SDS-PAGE and transferred on to a nitrocellulose filter and probed with an anti-hexahistidine-tagged antibody. The bands were visualised using a chemiluminescent substrate and autoradiography film (Figure 2.11). The sizes of the bands were compared with a prestained protein marker.

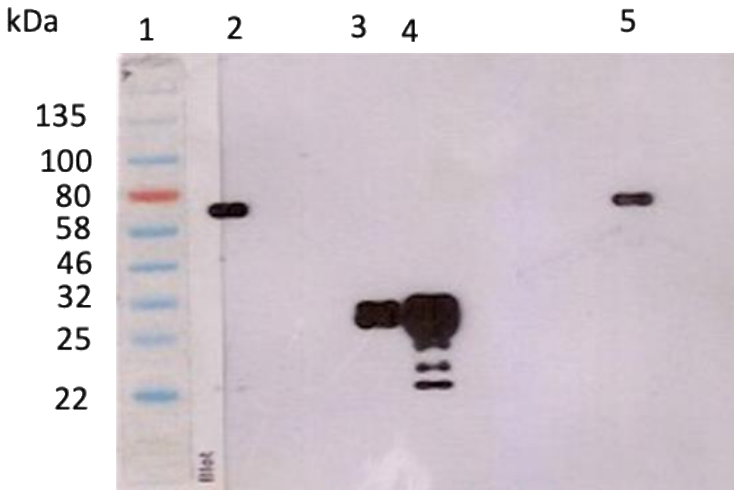

Figure 2.11 His tag Immunoblot confirmation of purified of rKatA, rPPT and rGGT. ClearColi® BL21(DE3) cells containing overexpressed recombinant proteins were lysed by sonication and the soluble fraction were subject to metal affinity purification on His Gravitrap Talon (GE Healthcare). Afterwards, the recombinant proteins were concentrated using a vivaspin concentrator and further purified by size exclusion chromatography. Lane 1, prestained protein standard; lane 2, rKatA; lane 3, rPPT; lane 4, metal affinity-purified rPPT (positive control); lane 5, rGGT.

2.4.3 Catalase Assay

2.4.3.1 Qualitative Catalase biochemical test

Catalase is a very active enzyme that can degrade in the removal of peroxides such as hydrogen peroxide (H_2O_2). Catalase acts on H_2O_2 to releases oxygen bubbles in the form of effervescence. Therefore, the catalase produced and purified above (Section 2.4.1.6) was subjected to this simple test, by

adding 2µl of 3% H_2O_2 to the 2µl of Catalase solution on a clean glass slide. Effervescence was produced on the catalase slide depicting catalase activity, while no effervescence was observed on the control containing the rGGT produced above (Section 2.4.1.6). Therefore, the purified recombinant KatA (catalase) retains its activity throughout the purification process.

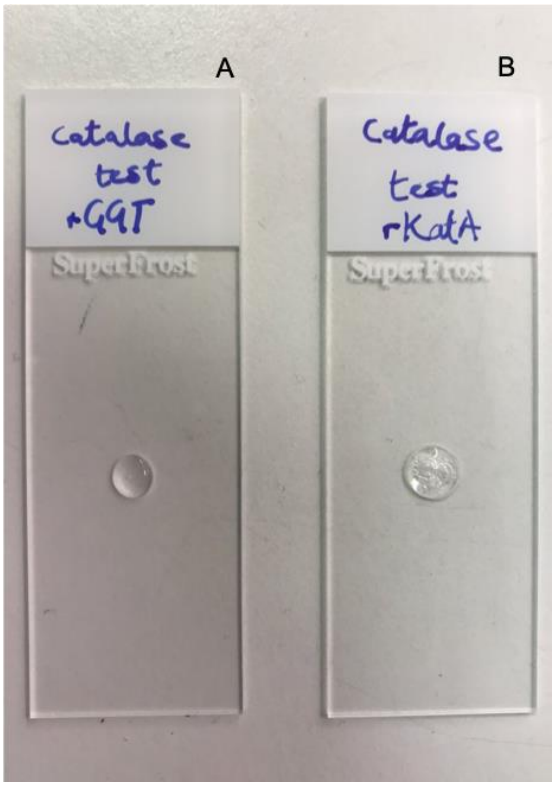

Figure 2.12 Qualitative Catalase biochemical test. The rKatA purified was placed on a glass slide (5 µL) with 5 µL of rGGT on another slide as control, 5 µL of 3 % H_2O_2 was added to each of the slides and prompt effervescence indicates presence and activity of the catalase. A. Contain the purified rGGT and produce no effervescence. B. Contain the rKatA and shows the presence of effervescence.

2.4.3.2 Catalase activity

To quantitatively measure the activity of the recombinant purified *H. pylori* catalase (KatA) (Section 2.4.1.6), a commercially available Amplex® Red Catalase A standard curve was used to determine the level of activity in the sample. (Table 2.1) The assays determined that the purified recombinant catalase protein at the concentration of 317.849 μg/ml had an enzymatic activity of 3.572 U/μg.

Table 2.1.Catalase activity

Protein concentration (μg/mL)	**Catalase activity (U/mL)**
317.849 (Neat)	3.572

2.4.4 Investigation of the role of KatA, PPT and GGT in Immunomodulation

2.4.4.1 Analysis of the ability of rKatA, rGGT and rPPT to inhibit IL-2 production by activated Jurkat T cells

To investigate the ability of the purified recombinant proteins to inhibit activation of T cells, a Jurkat T cell suppression assay, previously used by Gebert et al.(Gebert et al., 2003) was employed with a readout of IL-2 production.

In a dose-dependent manner 10 μg/ml, 25 μg/ml and 50 μg/ml of rKatA were used to treat Jurkat cell for 1 hour. To ensure the effectiveness of the Tris buffer (50 mM Tris base pH 8.8, 200 mM NaCl) in which the protein is stored did not affect the outcome of the investigation, control cells were treated with the buffer only. Subsequently, the cells were stimulated with 20 ng/ml PMA/ 1 μM ionomycin for 24 hours. The supernatants were harvested by centrifugation and the levels of IL-2 expression were measured by ELISA.

The buffer alone was found not to have a significant detrimental effect on the cells (presented below in Figure 2.14). The results showed dose-dependent reductions in IL-2 production, however, significant differences from the buffer control were found only with the highest concentration of rKatA (50 μg/ml) ($P<0.05$) (Figure 2.14A) and with only 50 μg/ml rGGT ($P<0.05$) (Figure 2.14B). There was no significant difference for all concentrations of rPPT although there were observable changes with 25 - 50 μg/ml.

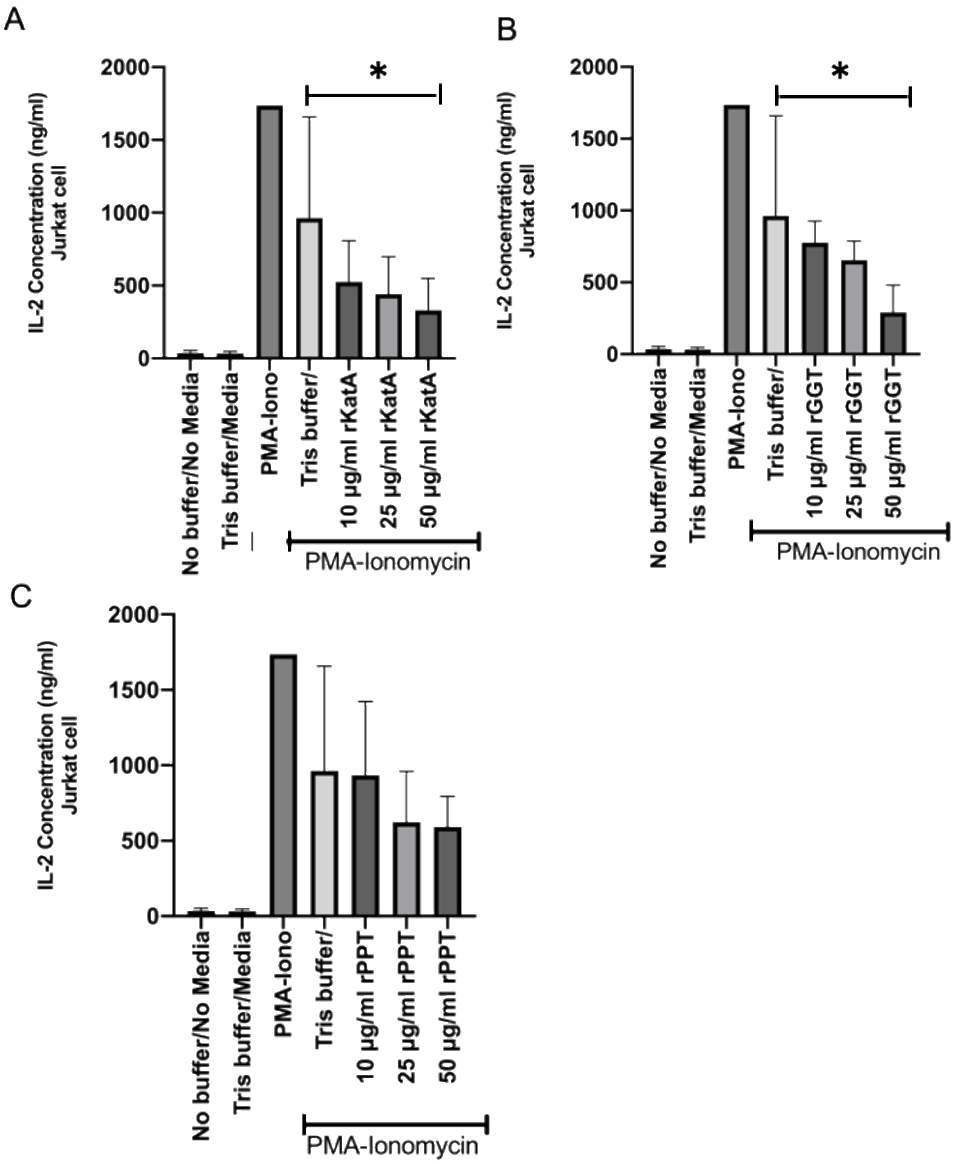

Figure 2.13 Effects of purified KatA, GGT and PPT on IL-2 secretion by Jurkat T cells. Effects of purified KatA, GGT and PPT on IL-2 secretion by Jurkat T cells. Jurkat cells were seeded in 96-well plates at 1 x 10^6 per well and were pretreated with purified rKatA, rGGT and rPPT at the indicated concentrations* for 1 hour at 37°C and 5% CO_2. The cells were then stimulated with phorbol* myristate acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml). After 24 hours the cells were pelleted and the concentration of IL-2 in the supernatant was analysed by ELISA. Bars represent the mean and standard deviation from three

independent experiments. (A) Only the highest concentration of rKatA (50 μg/ml) significantly reduced the IL-2 response ($P<0.05$). (B) Also, with only 50 μg/ml rGGT significantly suppressed IL-2 secretion ($P<0.05$). (C) rPPT did not have immunomodulatory activity on Jurkat cells.

2.4.4.2 Analysis of the ability of rKatA, rGGT and rPPT in the inhibition of IL-6 production in monocytic THP-1 cells.

Similarly, the ability of the *H. pylori* factors under investigation to suppress IL-6 production in Monocyte-macrophage cell model was also investigated.

As shown in the Figure 2.15 A, B, C with all the concentration of proteins, rKatA, rGGT and rPPT, 10, 25 and 50 μg/ml there was no significant suppression of IL-6 compared to the Tris buffer control. There were trends for changes in the level IL-6 with an increase in the concentration of the proteins, especially with rKatA and rGGT.

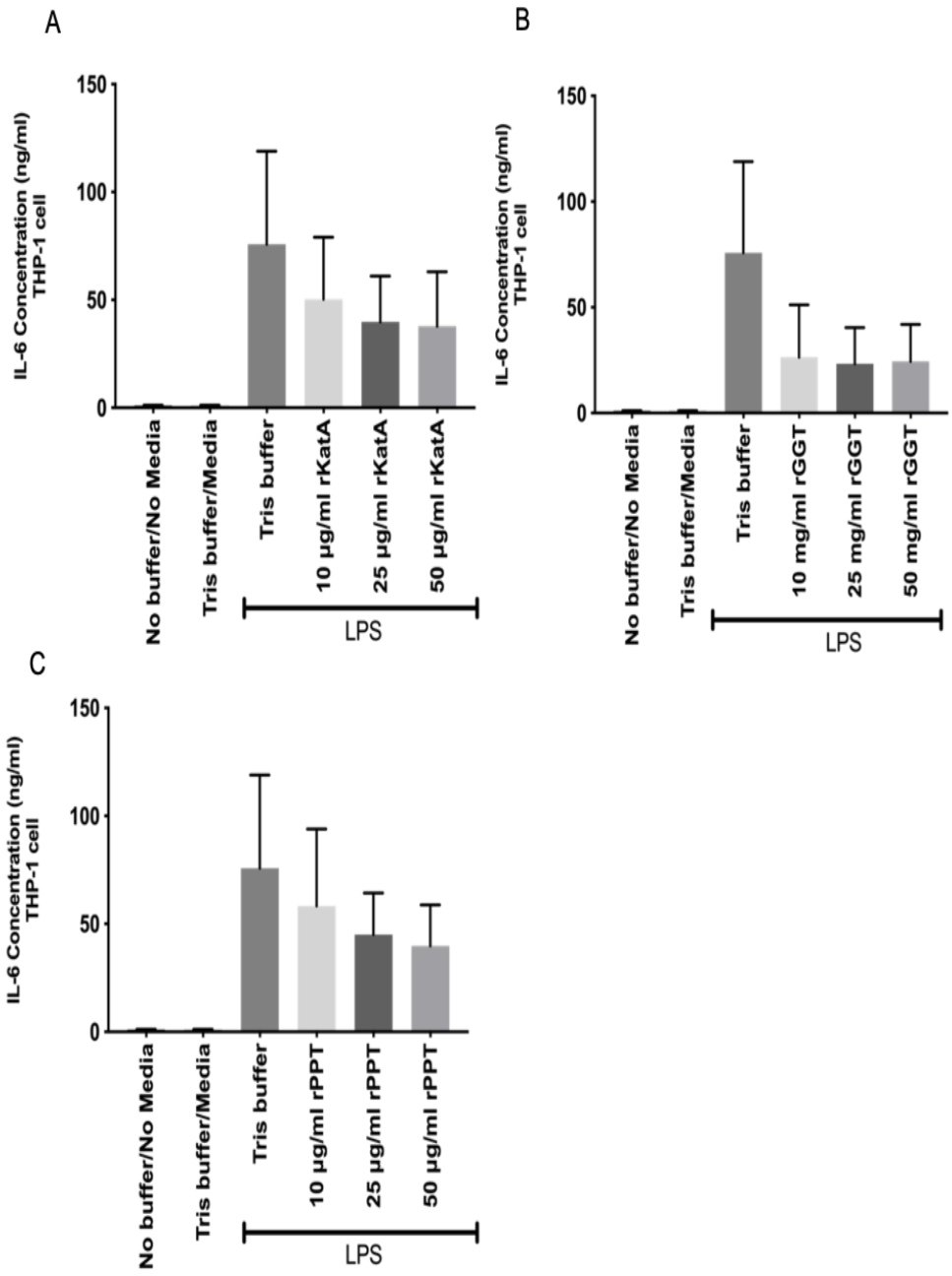

Figure 2.14 Effects of purified KatA, GGT and PPT on IL-6 secretion by THP-1 cells. Effects of purified KatA, GGT and PPT on IL-6 secretion by THP-1 cells (monocyte-macrophage cell model). THP-1 cells were seeded in 96-well plates at 1 x 10^6 per well and were pretreated with purified rKatA, rGGT and rPPT at the indicated concentrations for 1 hour at 37°C and 5% CO_2. The cells were then stimulated with LPS. After 24 hours the cells were pelleted and the

concentration of IL-6 in the supernatant was analysed by ELISA. Bars represent the means and standard deviations of three independent experiments. There was an inhibition of IL-6 with rKatA, rGGT and rPPT, but the difference were not statistically significant.

2.4.4.3 Analysis of the ability of rKatA, rGGT and rPPT in the inhibition of IL-8 production in AGS cells

Following a similar technique described in section---- used in for Jurkat cells IL-2 suppression assay, the ability of the proteins- rKatA, rGGT and rPPT to suppress IL-8 in epithelial cells were tested.

In all the concentrations (10, 25 and 50 μg/ml) of the recombinant proteins, rGGT and rPPT, there was no suppression of IL-8 secretion compared to the tris buffer (Figure 2.16 B and C). The plots rather showed an increase in IL-8. rKatA (Figure 2.16 A) at concentrations 10 and 25 μg/ml exhibited no suppression of IL-8 secretion compared to the Tris buffer but only 50 μg/ml concentration showed suppression of the cytokine but still significant to conclude on the effect.

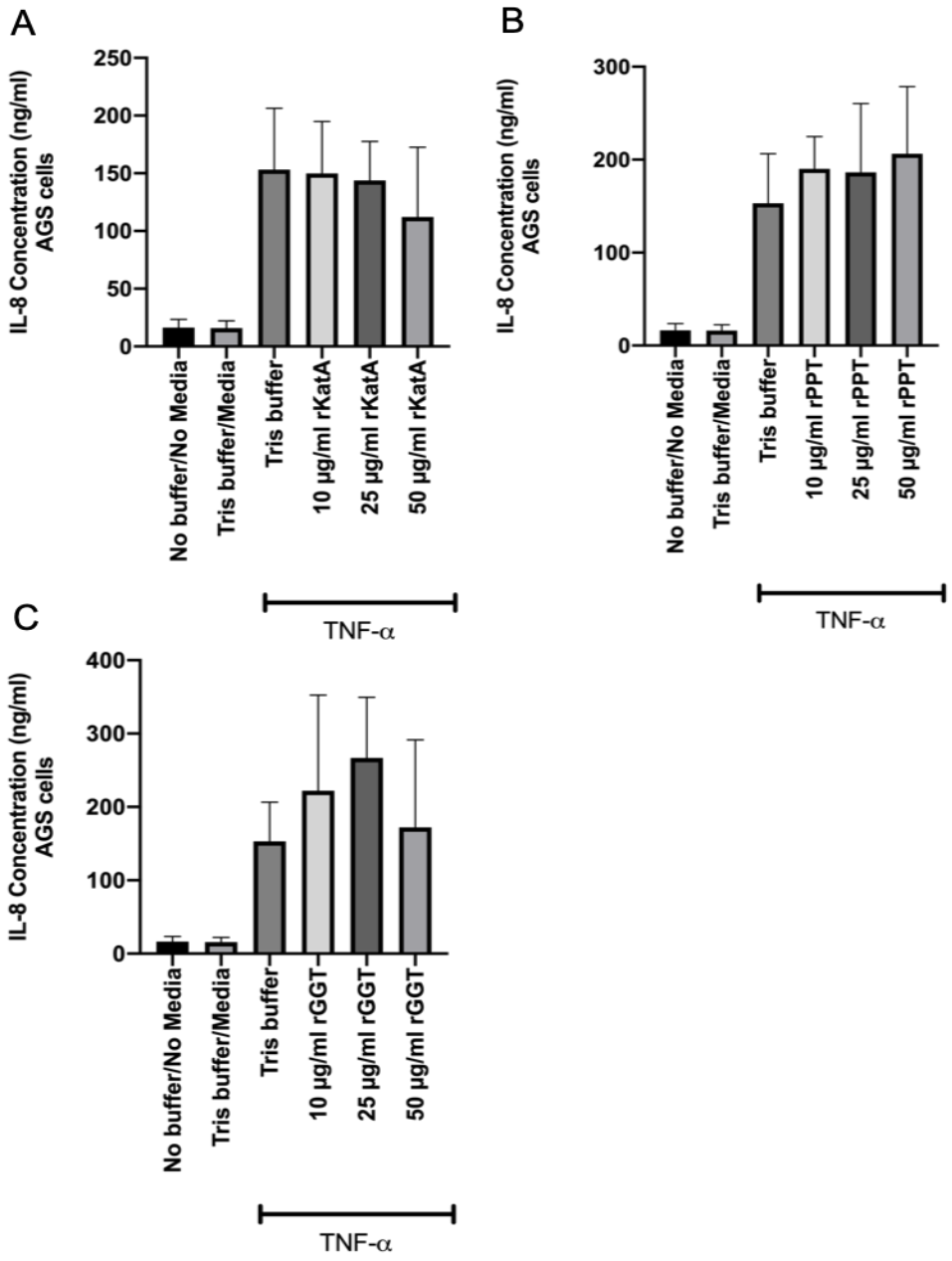

Figure 2.15 Effects of purified KatA, GGT and PPT on IL-6 secretion by AGS cells**.** Effects of purified KatA, GGT and PPT on IL-8 secretion by AGS cells monocyte-macrophage. THP-1 cells were seeded in 24-well plates at 1 x 10^6 per well and were pretreated with purified rKatA, rGGT and rPPT at the indicated concentrations for 1 hour at 37°C. The cells were then stimulated with TNf-α. After 24 hours the cells were pelleted and the concentration of IL-8 in the supernatant was analysed by ELISA. Bars represent the means and standard deviations of three independent experiments. There was an inhibition of IL-8 with rKatA, rGGT and rPPT, but the difference were not statistically significant. Bars represent the means and standard deviations of three

independent experiments. There was an inhibition of IL-8 with rKatA, rGGT and rPPT, but the differences were not statistically significant

2.5 Discussion

The ultimate aim of this study was to investigate the ability *H. pylori* factors that suppress inflammation by interacting with a variety of cell types. The main conclusion from the present results was that KatA and GGT showed significant immunoregulatory activities by inhibiting IL-2 production T cell model, similarly KatA, GGT and PPT showed a trend in in reduction of IL-6 production in macrophage-monocyte cell model with increase in concentration of the proteins. Present results point towards possible alternative ways utilised by the bacteria to down-regulate inflammation in the gastric mucosa.

2.5.1 Recombinant proteins expression and purification

Fitting to the purpose for which the proteins were to be tested, the recombinant proteins KatA, GGT and PPT were successfully expressed in ClearColi®BL21 (DE3) electrocompetent cells but not without problem. These all were used to avoid contamination of the protein by LPS. Unfortunately, ClearColi (DE3) is known to grow 50% slower than po most *E. coli* strains such as BL21 (DE3) (Lucigen, 2019)and B834. Thus, longer growth time is needed to reach the required optical density (OD) prior to the protein induction and as such protein yield at optimal exponential growth phase were low compared to the wild type. To overcome the low yield, several batches of the induced proteins are needed to obtain a concentration that could be purified using gel filtration, especially with GGT.

The recombinant protein was purified in a two-step purification process according to the features of the proteins, which are the hexahistidine at the N-terminal and the sizes. This is deemed necessary as the purified product of metal immobilised affinity chromatography (the first step) could be contaminated by other *E. coli* proteins such as SlyD (29kDa). SlyD has (Parsy et al., 2007). Hence, the second step, size exclusion chromatography (SEC) was used to ensure that purer proteins were obtained, this technique separates the proteins, based on the sizes of the constituent molecules. Regardless, there could still be the probability of having a contaminant of rich histidine domain, which might show affinity and the same size as the targeted recombinant proteins. For rKatA, a combination of quantitative and qualitative catalase enzyme assays helped to prove its biological activities. The anti-his-tagged Western Blot assay, which probes for the hexahistidine sequence at the N-terminal of each of the protein using a monoclonal antibody provides extra assurance of the purity of the recombinant proteins.

The use of the ClearColi® BL21(DE3) electrocompetent cells, commercially available cells, as the host for the recombinant plasmids, reduced the chances of common LPS contamination of a recombinant protein preparation from the host *E. coli* (Heumann and Roger, 2002, Mamat et al., 2015). More than half of recombinant proteins in biopharmaceutical and research industries are produced in *E. coli* and stand a chance of being contaminated with the immunostimulatory molecule LPS(Huang et al., 2012). Unfortunately, no known post expression method has been successfully used

to entirely eliminate LPS from recombinant protein products (Wakelin et al., 2006). Some of the few post expression LPS removal methods such as Triton X separation, adsorption on activated carbon, ultrafiltration, anion-exchange chromatography, IMAC and polymyxin B treatment are known to be inefficient and poor selectivity (Petsch and Anspach, 2000). LPS is a known agonist of TLR4, CD14 and MD-2 receptor complex which is present in several cells of the innate immune system (Triantafilou and Triantafilou, 2002). The recognition of the receptor complex enables the phosphorylation and recruitment of the cascade of intracellular adaptor kinases leading to NF-κB activation and subsequent pro-inflammatory cytokine secretion (Palsson-McDermott and O'Neill, 2004, Rossol et al., 2011). In the light of these, instead of post-processing removal of LPS, the LPS was genetically modified to lipid IV_A in a BL21 (DE3) background. This bacteria cell line was created to process uncontaminated recombinant proteins. This was achieved by seven genetic deletions (ΔgutQ ΔkdsD ΔlpxL ΔlpxM ΔpagP ΔlpxP ΔeptA) which alter the LPS to Lipid IV_A and one additional mutation to preserve the viability of the cells with Lipid IV_A. This newly created ClearColi® cells lack the agonists for TLR4/MD-2 activation, therefore the signal from the ClearColi are reported to be far lower in magnitude compared to the wild-type. Similarly, recombinant proteins prepared from ClearColi® are practically endotoxin-free with minimal purification (Lucigen, 2019, Mamat et al., 2015). To avoid extraneous contamination of preparations, all water sources used were commercially available endotoxin free, heat treat depyrogenated glassware were employed, brand new purification column was

purchased, disposable pipette tips were used, and good laboratory sterile cleaning technique were the precautionary measures employed.

Although using ClearColi® BL21 (DE3) afford purity and immunological advantages especially in that the proteins are free from LPS, there are a number of drawbacks in using this strain. ClearColi® BL21 (DE3) has a slow growth rate compared to wildtype and this, in turn, affects the overexpression of the protein.

2.5.2 Investigation of the mechanism through which KatA, GGT and PPT contribute to immune regulation

In this study the suppression assay was used to investigate any possible mechanism of KatA, GGT and PPT mediated regulation. The assay was suppression of IL-2 in T-cell model (Jurkat cells), IL-6 in the monocyte-macrophage model (THP-1 cells) and epithelial cell model (AGS cells). With the exception of IL-8 secretion in AGS, GGT and KatA at high concentration suppressed significantly the expression of the IL-2 and IL-6 in Jurkat and THP-1 cells. The result offers a substantial optimism for the mechanistic study of secretion process of the protein *in vivo* and the mechanism of their interaction downstream of immune cells.

2.5.2.1 IL-2 suppression in Jurkat cells

T cells has been shown to be vital in eradiction of bacterial infection (Ermak et al., 1998). However, *H. pylori* VacA protein was shown to block T cell proliferation and activation by arresting G_1/S cycle following stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin (Gebert et al., 2003, Torres et al., 2007). The report explained that VacA interferes with the T cell receptor/interleukin-2 signalling pathway by inhibiting the transcription factor NFAT leading to downregulation of IL-2 transcription. PMA is an analogue of diacylglycerol that stimulates Jurkat cell by diffusing into the cell and activating protein kinase C(PKC). It works synergistically with Ionomycin, a calcium ionophore, to elicits calcium release (Chatila et al., 1989).

The result of this experiment showed that a high concentration of (50 μg/ml) KatA and GGT strongly suppressed IL-2 secretion by Jurkat cells (79.5% reduction with 50 μg/ml, $p<0.05$), while PPT did not suppress IL-2 production in Jurkat cell. The potential and the mechanism through which GGT inhibits IL-2 production could be associated to its capacity to block T-cell proliferation by induction of cell cycle arrest from G1 via suppression of G1 cyclin-dependent kinase activity(Gerhard et al., 2005) this is in similar fashion as the mechanism utilised by VacA (Sundrud et al., 2004, Sewald et al., 2011). This is in contrast to *H. pylori* KatA activity on which has no known direct immune-regulatory attribute on T cell prior to this research.

In the past, most of KatA immunoregulatory properties have been in an indirect manner and mainly linked to its activities as an antioxidant molecule

(Seo et al., 2002). But with the result of this experiment showing that IL-2 is suppressed at the highest concentration of KatA, it is only logical to assume that KatA suppression of IL-2 is concentration-dependent and it is only at 50 μg/ml that KatA is enriched enough to exact immunosuppression. The argument is deemed strong when compared to VacA, another *H. pylori* factor that is secreted via a type V auto-transport mechanism (Palframan et al., 2012) and therefore easily accessible to immune cells in enriched level sufficient to exact immunosuppression. On the other hand, KatA is a cytoplasmic protein with no known signal-protein sequence for translocation across the cell envelope. Although reports have shown the presence of this enzyme both in the periplasmic space and on the bacterial cell surface (Harris et al., 2003, Phadnis et al., 1996), a number of recent researches have reported the presence of KatA in the outer membrane vesicles (OMV) that are constitutively shed by *H. pylori* (Wang et al., 2006, Mullaney et al., 2009). Surprisingly, till date, no other means of translocation of this protein is known, yet it is constantly found in abundance in the environment of *H. pylori* both *in vivo* and *in vitro.* Furthermore, more than 7% of the OMV was shown to be KatA alone (Mullaney et al., 2009). Lekmeechai et al in their recent work showed that KatA is enriched in the OMV the compared with the outer-membrane of an intact bacterial cell, up to seven-fold higher (Lekmeechai et al., 2018).

With this knowledge, it could be speculated that the KatA immunosuppressive activity would be more pronounced *in vivo* where it is concentrated to a significantly higher through the aid of OMV.

Additionally, as there has been no evidence of any known KatA receptor on any cell surface, it is suspected that KatA could probably be binding to an internal receptor or molecule. Past studies have shown that the key mechanism bacterial OMV mediated inflammation is in its ability to penetrate non-phagocytic cells and induce production of cytokines (Kaparakis et al., 2010, Bomberger et al., 2009, Chatterjee and Chaudhuri, 2011). While *H. pylori*, is known to be non invasive, nevertheless the bacteria, still could deliver factors into the host cells (Parker et al., 2010).

Hence, we conclude that at high concentration, which is evidently possible *in vivo*, there is a possibility that KatA plays a role in the immunoregulatory function of *H. pylori*.

2.5.2.2 IL-6 suppression in THP-1

During almost every inflammation condition cytokines such as IL-1, TNF-α and IL-6 are usually elicited and hence are been of the prominent targets for anti-inflammatory therapy (Scheller et al., 2011). IL-6 is a 21-28 kDa glycosylated protein which binds to the IL-6 receptor, IL-6R, (gp80) and type I transmembrane signal transducer protein (gp130). To activate the JAK/STAT, ERK and P13K pathways, IL-6 binds to the IL-6R to form a complex which in turn binds to the two molecules of gp130, leading to signal transduction and this is called classical signalling (Oberg et al., 2006). In macrophages, neutrophils, some T cells and hepatocytes express IL-6R on the surface while gp130 are

present on most cells (Rose-John et al., 2007, J and S, 2006). But to complement the absence of IL-6R on most the cells surfaces there is soluble IL-6 receptor (sIL-6R) in the plasma, which complex with IL-6 to agonistically bind to gp130-expressing cells (Rose-John and Heinrich, 1994, Rose-John et al., 2007). This activation by sIL-6R/IL-6 complex of cells expressing gp130 only, called trans signalling, is very important in inflammation especially for lymphocyte attraction into the area of inflammation and promotion of T cell proliferation (Chalaris et al., 2007, Chen et al., 2006). Consequently, turning off production IL-6 in THP-1 step using the rKatA, rGGT and rPPT will be steps toward therapeutic blockade of IL-6 effect.

The result did not show a significant reduction in IL-6 production in THP-1 cell stimulated with LPS. interestingly to see that there is a noticeable change in IL-6 level as the concentration of each factor increases. GGT was most effective in suppressing IL-6 from THP-1 cells (68.07% reduction with 50 μg/ml).

2.5.2.3 IL-8 suppression in AGS cell

IL-8, a member of the CXC chemokine family was formerly known as a neutrophil chemoattractant (Schroder et al., 1987, Walz et al., 1987). It is also known as CXCL8 and is known to play an important role in inflammation and defence mechanisms (Zlotnik and Yoshie, 2000). Further research has shown that IL-8 could perform other functions apart from being a chemoattractant for neutrophils in acute and chronic inflammation in the gastrointestinal tract

(GIT). The pool of IL-8 in the GIT is generally from both local cells such as epithelial cells, endothelial, fibroblasts, and migratory cells, such as monocyte and neutrophils. IL-8 expression can be acute, as in the case of microbial infection and reports have shown continuous IL-8 production in cancerous gastric epithelial cells. Dysregulation of IL-8 is hypothesized to play a part in the pathophysiology of the development of several diseases, and IL-8 could, therefore, be a target for therapeutic use as a potential biomarker (Cotton et al., 2015). *H. pylori* is known to induce IL-8 cytokine secretion in the gastric epithelial cell line *in vitro* in a sequence of events that culminates in activation tyrosine kinase activation and NF-κB pathway (Sharma et al., 1995, Sharma et al., 1998). Consequently most studies have focused on the induction of IL-8 by *H. pylori* in the mucosal especially in relation to *cag* PAI (Peek et al., 1995, Yamaoka et al., 2006, Crabtree JE, 1994, Yamaoka, 2010).

An experiment was set up to investigate immunosuppression of one more pro-inflammatory cytokine, IL-8, by the *H. pylori* factors, GGT, KatA and PPT in AGS cells stimulated with TNF-α. Interestingly, the GGT results showed the opposite, raising the question of whether the molecules could be playing a role in proinflammation induction. KatA showed a noticeable trend in the reduction in IL-8 production at a concentration of 50 µg/ml which is not statistically significant, similar to result obtained with PPT. Conversely, GGT tends to show in the concentration of IL-8 with the increase in the concentration of the protein. Two studies reported the abrogation of IL-8 induction in epithelial when there is a loss of function of genes close to the *cag*

PAI (Li et al., 1999, Sharma et al., 1998). Their results showed that IL-8 induction in AGS is mainly abrogated by molecules exhibiting protein tyrosine kinase inhibitors. While Segal et al using kinase inhibitors, staurosporine and genistein, reported that besides of tyrosine phosphorylation of the cellular 145 kDa protein with inhibition in IL-8, there could be other ways of IL-8 inhibition which is independent of tyrosine phosphorylation (Segal et al., 1997).

In several studies, IL-8 expression down-regulation has been widely reported in epithelial and endothelial cells in a redox responsive transcriptional manner (DeForge et al., 1993, Roebuck, 1999, Lakshminarayanan et al., 1997, Lakshminarayanan et al., 1998, Vlahopoulos et al., 1999). But it was Lekstrom-Himes et al that showed that catalase specifically has the ability to significantly suppress IL-8 expression by suppression the IL-8 mRNA transcription (Lekstrom-Himes et al., 2005). They reported that catalase performs this function by acting as hydrogen peroxide scavenger. Hydrogen peroxide is to chemically inhibit the activities NADPHnoxidase activity thereby stimulating the increase in IL-8 mRNA production in cells. Nevertheless, the result of this experiment failed to establish any of this with H. pylori KatA.

2.6 Conclusion

- KatA and GGT at high concentration of 50 µg/ml significantly inhibit IL-2 in T cells model and could be deemed to have had direct immunosuppressive activities.
- PPT are not immunosuppressive in T cell model

- The three *H. pylori* factors do significantly suppress IL-6 production in a monocyte-macrophage cell model, although there were trends on decrease cytokine production with the increase in the concentration of the proteins.
- IL-8 production in epithelial cell model was not significantly suppressed by any of the factors but KatA and PPT showed a trend in reduction of the cytokine production. Conversely, GGT was shown to increase the production of IL-8 in AGS cells

Chapter 3

In vivo investigation of immunoregulatory properties of *Helicobacter pylori* infection VacA

3.1 Introduction

Originally, the virulence factor, VacA was primarily known by its vacuolating activity in the eukaryotic cytoplasm (de Bernard et al., 1997). In recent decades, VacA researches have revealed so many other functions of the protein, one of which is immunomodulatory activities. This activity of VacA on the immune cells could be perceived to be a very important step towards the understanding of the colonisation ability of the bacteria. In mouse models, Salama et al reported that the wild type strains expressing VacA readily outcompete the mutant strains lacking VacA in a mixed infection of the mouse stomach: showed that a VacA plays a role in the establishment of infection hence, in a single infection, the ID_{50} of the wild type was two times higher than the mutant (Salama et al., 2001a). Oertli et al working on VacA and GGT established a similar result of the inefficiency of *vacA* mutant to colonise compare to the wild type (Oertli et al., 2013a). It has been revealed also that most of the human isolates are positive for *vacA*-positive, although there is variation in the alleles and expression level (Xiang et al., 1995). A study by Winter *et al* on the allelic differences revealed that *H. pylori* producing the less active type s2i2 form of VacA promote colonisation the mice gastric efficiently than the VacA deficient isotype or the more active s1i1 form. On the other hand, the more active form of VacA was implicated in the study for induction of extensive metaplasia and inflammation in mouse compared to the less active form (Winter et al., 2014). Other studies were also in agreement with finding, additionally, they also reported the association between s1i1 strains with

gastric cancer and premalignant lesions (Rhead et al., 2007, Basso et al., 2008). The inference from these reports answers the question of why *H. pylori* strains would actively be producing a supposedly "not needed molecule" – the nonactive toxin? Which could go on to explain that it largely performs other functions different from the toxin activities. Hence, cytotoxic activities and immunological activities are divergent and unrelated.

The immunomodulation effect of VacA has been well studied to shed light on a number mechanism through which this is achieved. These include interference with phagosome maturation and intracellular trafficking in macrophages (Allen et al., 2000), interference with T-cell receptor/interleukin-2 signalling pathway through the Ca^{2+}/calmodulin-dependent phosphatase calcineurin which culminates in VacA inhibition of T-cell proliferation and finally, promotion of differentiation of regulatory T-cell by hijacking the development of dendritic cells (DC)(Djekic and Müller, 2016).

3.2 Hypothesis

One of the profound effects *H. pylori* on the immune system, both in vivo and in vitro, is its ability to reprogram of the DC towards tolerogenic phenotype, and consequently the bacteria colonisation (Wang et al., 2010). Downstream, the reprogrammed DC is skewed towar5ds priming the regulatory T-cell instead of the effector T-cells (Th1 and TH17) thereby favouring an anti-inflammatory immune response over a pro-inflammatory response (Oertli et al., 2012). A study by Oertli et al on the immunomodulatory effect of VacA prove similar effect as the *H. pylori* itself. They demonstrated that DCs isolated and purified immunomagnetically from mice infected with wild-type (VacA-proficient) *H. pylori* strains and those from the uninfected mice or VacA mutant infection exhibit a remarkable difference in the type of T-cell response. They showed induction of regulatory T-cell response by DCs from the wild type as opposed to the DCs from either uninfected or VacA mutant (s1i1) infected mice, which exhibited proinflammatory responses characterised by a higher number of Th1, Th17, their associate cytokine; IFN-γ and IL-17 as well as low numbers of $Foxp3^{+}$ $CD25^{+}$ regulatory T-cells. In addition to these, they also confirmed the lower colonisation level among the VacA mutant compared to the wild type(s2i2) (Oertli et al., 2013a).

This study and many other similar studies failed to provide information on the difference in regulatory T-cell induction by various VacA variants. But since it has already been proven that less vacuolating cytotoxic form promotes colonisation more efficiently compared to the more toxic form (Winter et al.,

2014), we hypothesis that the non-cytotoxic VacA type, s2i2 promotes the induction of $CD4^{+}CD25^{hi}Foxp3^{+}$ Treg more efficiently than the toxic VacA type. Therefore, the following objectives were set out.

- To compare the $CD4^{+}$ T lymphocyte frequencies in the spleens of uninfected and *H. pylori*-infected mice at different time intervals of 3, 6 and 9 weeks.
- To quantify the fraction of this $CD4^{+}$ T lymphocyte exhibiting Treg properties of $CD4^{+}CD25^{hi}Foxp3^{+}$ and signature cytokine IL-10.
- To compare the difference in $CD4^{+}CD25^{hi}Foxp3^{+}$ Treg frequencies in mice infected with different forms of VacA expressing H. pylori
- To compare the suppressive potential of Treg from the spleen of uninfected mice and VacA-proficient *H. pylori*-infected mice

3.3 Materials and Methods

3.3.1 Study team and organisation

This experiment was carried out as part of multidisciplinary researches in the *Helicobacter pylori* research group of the Nottingham Digestive Disease Centre. This comprises of the Molecular microbiology team, Immunology team and PhD students lead by Dr Karen Robinson, Dr Darren Lentley, Dr Kazuyo Kaneko, Mrs Jo Rhead, Dona Reddiar and yours sincerely. The experimental procedures were carried out under Home Office project licence number 30/3298, with ethics approval and in line with institutional guidelines on animal welfare.

3.3.2 Infection of mice with *H. pylori*

The 6 weeks old female C57BL/6 mice were obtained from Charles River. They were housed and feed appropriately and allowed to acclimatise one week before the experiment began.

Groups of 18 female 6-week-old C57BL/6 mice were infected with *H. pylori* by administering oral doses of 100 µl of Brucella broth (Difco Laboratories) containing 1 x 10^9 bacteria of the SS1 strain (either the wild type expressing the s2/i2 form of VacA or a mutant strain expressing the s1/i1 form of VacA). Doses were administered on three alternate days. A further 18 mice received oral doses of plain Brucella broth as a placebo control. At 3 weeks, 6 weeks and 9 weeks post-infection one-third of each treatment group was

terminated. Colonization densities and histological parameters were assessed from stomach tissue and spleens were removed for comparative analyses of the immune response.

3.3.3 Splenocyte preparation

Spleens were removed and rubbed through a 40 µm cell strainer (BD Biosciences Oxford, UK) using a sterile disposable 1 ml syringe**.** The splenocyte suspensions were washed and a sample was removed and mixed with 3% acetic acid with methylene blue (Stemcell Research) for counting using a haemocytometer. The cell concentration was then adjusted to 1 x 10^7 per ml, suspending the cells in culture medium (RPMI with 10% foetal calf serum (FCS)/100U/ penicillin G/100 µg/ml streptomycin sulphate).

3.3.4 Quantifying Tregs amongst splenocytes

1ml of each splenocyte suspension (containing 1 x 10^7 cells) was centrifuged at 300 x g for 3 minutes and resuspended in 1 ml red blood cell lysing buffer (Sigma Aldrich). After 1 minute 10 ml of culture medium was added and the cells were washed twice, spinning the tubes at 300 x g for 3 minutes. Finally, the cells were resuspended in 10 ml of culture medium.

130 µl of phorbol myristate acetate (PMA) / lipopolysaccharide (LPS)/ionomycin stimulation cocktail was added to 1 ml of the cell suspensions in sterile tubes and incubated at 37°C in an atmosphere with 5% carbon dioxide

for 1 hour. 10 µl Brefeldin A Solution (BFA) was added to the tubes to give a final concentration of 1 x 10^6 per ml and incubated at 37°C for a further 5 hours.

Unstimulated controls were prepared from each cell suspension. These were stored in the cold room at 4°C.

3.3.5 Cell staining and flow cytometry

Following incubation, the cells were pelleted at 300 x g for 5 minutes before staining with the appropriate combination of fluorochrome-conjugated monoclonal antibodies against extracellular markers CD4 and CD25 (see Table 3.1

TABLE 3.1 Flow cytometry antibodies for quantification of Treg cells.

Marker	Antibody-fluorochrome conjugate	Manufacturer	Catalogue	Volume per tube	Clone
Extracellular antibodies added directly to the cell pellet					
CD4	PE/Dazzle Anti-mouse CD4	Biolegend	100566	1 µl	RM4-5
CD25	BV421 Anti-mouse CD25	Biolegend	102043	0.5 µl	PC61
Intracellular antibodies added to cells					
Foxp3	FITC Anti-mouse FOXP3	eBioscience	11-5773-82	1 µl	FJK-16s
IL-10	AF647 Anti-mouse IL-10	Biolegend	505016	0.5 µl	JESS-16E3
Isotype controls					
Iso-CD25	BV421 anti-mouse IgG1	Biolegend	401911	0.5 µl	GO114F7
Iso-Foxp3	FITC anti-mouse IgG2a	Biolegend	400505	1 µl	RTK2758
Iso-IL-10	AF647 anti-mouse IgG2b	Biolegend	400626	0.5 µl	RTK4530

NB: PE, phycoerythrin;

FITC, fluorescein isothiocyanate;

BV421, BD Horizon; Alexa Fluor 647

Antibodies against CD4 and CD25 were added and the tubes were incubated on ice for 30 min. The cells were then washed twice with 1 ml PBA buffer (phosphate-buffered saline (Oxoid) containing 2% bovine serum albumin (BSA) (Sigma) and 0.1% sodium azide (Sigma)), before fixing in 0.5 ml fixation buffer (Biolegend) at room temperature for 20 mins in the dark.

Cells were then washed with 1 ml PBA and permeabilized by washing first with 1 ml PBA-saponin (PBA with 0.1% saponin (Sigma), and then with 1 ml PBA-saponin/10% FCS. The cell pellet was then stained with the IL-10 antibody conjugate according to a previously described protocol (Biolegend), incubating for 2 hours at 4^{o}C with periodic gentle mixing. At the end of the incubation, the cells were washed with 1 ml PBA-saponin and then with 1 ml 1x Perm buffer (Biolegend), before adding 1 ml 1x Perm buffer and incubating at room temperature for 10 min in the dark. The cells were pelleted, and Foxp3 antibody conjugate was added directly to the pellet and incubated at room temperature for a further 30 min in the dark. After the incubation, the cells were washed twice in 1 ml PBA and finally resuspended in 0.5 ml PBA.

Data on 200,000 events were acquired using a MoFlo Astros EQ flow cytometer (Beckman Coulter). Controls consisted of unstained cells, cells stained with a single fluorochrome-conjugated antibody and the staining antibody combinations minus one. These were included to optimise compensation settings. Data analysis was performed using Kaluza Analysis 1.5a (Beckman Coulter).

3.3.6 T cell isolation

$CD4^+$ $CD25^+$ T regulatory (Treg) cells and $CD4^+$ $CD25^-$ T effector cells (Teff) were purified from the splenocyte suspensions from the treatment groups using a magnetic bead separation kit (Miltenyi Biotec). Because of the requirement for large numbers of cells for this process, 5 x 10^7 cells from each of the two samples were combined. Each group of six splenocyte suspensions, therefore, resulted in three purifications per group. $CD4^+$ $CD25^-$ splenocytes from a further two untreated animals were also purified in the same way to generate a single pool of $CD4^+$ $CD25^-$ T responder (Tresp) cells for functional suppression assays.

Each spleen cell suspension was passed through a pre-separation filter (Miltenyi Biotec, cat. 130-041-407) to remove clumps and then centrifuged for 3 minutes at 200 x g. The supernatant was removed completely, and the cells were suspended in 400 µl of MACS buffer (2.5 ml MACS BSA Stock Solution and 50 ml MACS rinsing solution, Miltenyi Biotec). Then 100 µl $CD4^+CD25^+$ regulatory T cell biotin-antibody cocktail was added, and the tubes were incubated for 10 minutes at 4°C.

The cells suspension was then applied to an LD column in a midiMACS separator (Miltenyi Biotec) which negatively separated the $CD4^+$ cells. These cells, which were not retained on the column, were spun down and re-suspended in 900 µl of MACS buffer (Miltenyi Biotec). Then 100 µl anti-PE microbead cocktail (a mixture of 300 µl MACS buffer + 200 µl anti-biotin microbeads + 100 µl CD25-PE antibody) was added to the cells and incubated for 15 minutes at 4°C.

At the end of the incubation, the cell suspension was applied to an MS column in a miniMACS separator (Miltenyi Biotec), for positive selection of $CD4^+CD25^+$ cells on the column whilst $CD4^+CD25^-$ cells flowed through. The $CD4^+CD25^+$ cells were collected by removing the column from the magnetic separator, applying 1 ml of MACS buffer and using the column plunger to expel the cells. To ensure high purity of $CD4^+CD25^+$ cells the collected cells underwent a further purification cycle, applying the suspension to a new MS column.

The purified $CD4^+CD25^-$ Tresp and Teff cells and $CD4^+CD25^+$ Treg cells were spun down at 200 x g for 3 minutes and re-suspended in a culture medium, counted and adjusted to 1 x 10^6 cells/ml.

3.3.7 Carboxyfluorescein succinimidyl ester (CFSE) labelling of Tresp cells

To investigate the Tregs functional suppression assay, the $CD4^+CD25^-$ Tresp cells isolated from uninfected mice were spun down at 200 x g for 3 minutes. The pellet was resuspended in 4.5 ml of CFSE (10 µl of 5mM CFSE stock solution in 5 ml 0.1% BSA/PBS) and incubated at 37°C for 10 minutes. At the end of the incubation, 20 ml of culture medium was added, and the cells were retained on ice for 5 minutes. The cells were then washed three times and finally re-suspended in 4.5 ml culture medium (1 x10^6 per ml).

3.3.8 Treg functional suppression assay

A volume 50 µl aliquots of the CFSE labelled Tresp cells were placed in 96 well plates. 50 µl of four dilutions of Tregs (1 x 10^6/ml, 0.5 x 10^6/ml, 0.25 x 10^6/ml and 0.125 x10^6/ml) were added to the Tresp cells in triplicate wells. 100 µl of Dynabeads mouse T-activator

CD3/CD28 beads (5 x 10^5 beads/ml; Cat 11452) was added to all wells. Unlabelled Tresp only labelled Tresp only and labelled Tresp with CD3/CD28 beads were included as controls. The plate was incubated at 37°C for 72 hours.

At the end of the 72 hours incubation period, the cells were collected into FACS tubes, fixed and re-suspended in 0.5 ml PBA.

Data on 50,000 events were acquired using Coulter FC500 flow cytometry (Beckman Coulter). Unlabelled cells, cells labelled with CFSE and stimulated, and cells labelled but not stimulated were included as controls. Data analysis was performed using Kaluza analysis 1.5a (Beckman Coulter).

3.3.9 Gating strategy

A gating strategy used to identify $CD4^+$ T -cells, $CD4^+CD25^{hi}Foxp3^+$ Treg and IL-10 producing T lymphocyte was prepared and was used for identifying the cells from the spleen samples as shown in Figure 3.2.

3.3.10 Statistical analysis

Graphs were prepared and statistical analysis was carried out using GraphPad Prism software. Data are shown with mean ±SEM. Statistical significance was determined using a one-tailed Student T-test for data with a non-Gaussian distribution and ordinary one-way ANOVA. Differences were considered significant at $p<0.05$*, $p<0.01$** and $p<0.001$***

3.4 Results

3.4.1 Effect of *H. pylori* VacA mutant SS1^{s1i1} and VacA wild-type SS1^{s2i2} strains on Gastric colonisation

Previously, VacA has been shown to promote gastric mucosa colonisation in infected mice (Raju et al., 2012). In this experiment, we use SS1 VacA mutant strain (SS1^{s1i1}) expressing s1i1 VacA type and SS1 VacA wildtype (SS1^{s2i2}) expressing s2i2 VacA type and reinvestigated the previous finding on the difference in the role of VacA a type in the colonisation of the bacteria. Our result was consistent with the earlier finding by Winter et al, who showed that SS1^{s2i2} strain was better able to colonise better than SS1^{s1i1} (Winter et al., 2014). There was a significant difference in the colonisation of mutant SS1^{s1i1} and wildtype SS1^{s2i2} with the average colonisation of the wildtype being increased by approximately one order magnitude compared to the mutant *H. pylori* at 3 months and 9 weeks post-infection (Figure 3.1).

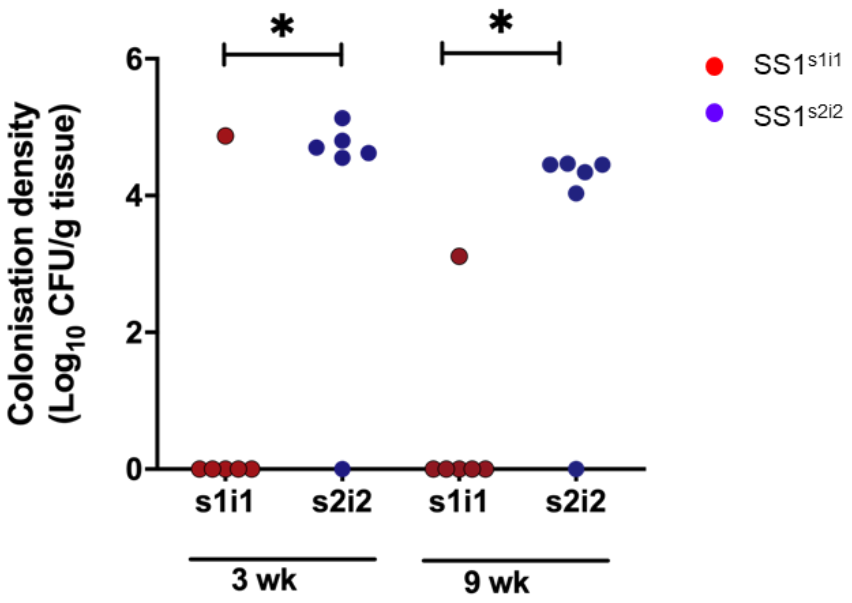

Figure 3.1 Colonisation density in the stomach of mice infected with two strains of **H. pylori** expressing different VacA type**.** The mice C57BL/6 were divided into two groups, one group were orally dosed with H. pylori SS1 wildtype ($SS1^{s2i2}$, in blue) and the other H. pylori SS1 mutant ($SS1^{s1i1}$, red) for 3 and 9 weeks. The colonisation density was measured at 3- and 9-weeks post-infection. The differences were considered significant at $p<0.05$.

3.4.2 Comparison of the IL-10-secreting T cell and Treg response to *H. pylori* infection in mice, and the impact of the vacA type

To investigate Treg and IL-10+ T cells levels in the spleens of 3 mice groups (broth placebo-dosed, SS1 wild-type strain-infected(s2i2 vacA) and SS1 (s1i1 vacA) mutant-infected group) at 3, 6 and 9 weeks post-infection, the cell suspension was stimulated with LPS, PMA and ionomycin for 6 hours, or was

left unstimulated. The cells were then stained with fluorochrome-conjugated antibodies and analysed by flow cytometry.

As shown in the representative gating strategy (figure 3.2), after gating from forward scatter and side scatter plots for the lymphocyte population, the percentage frequency of the $CD4^{+}$ $CD25^{hi}$, CD4+ $Foxp3^{hi}$, and $CD4^{+}$ $IL\text{-}10^{+}$ among the total lymphocyte population was calculated. Of the $CD4^{+}$ lymphocytes, the percentage frequency of the $CD4^{+}$ $CD25^{hi}$ $IL\text{-}10^{+}$ and $CD4^{+}$ $CD25^{hi}$ Foxp3hi were analysed for each mouse and comparisons were made between the three treatment.

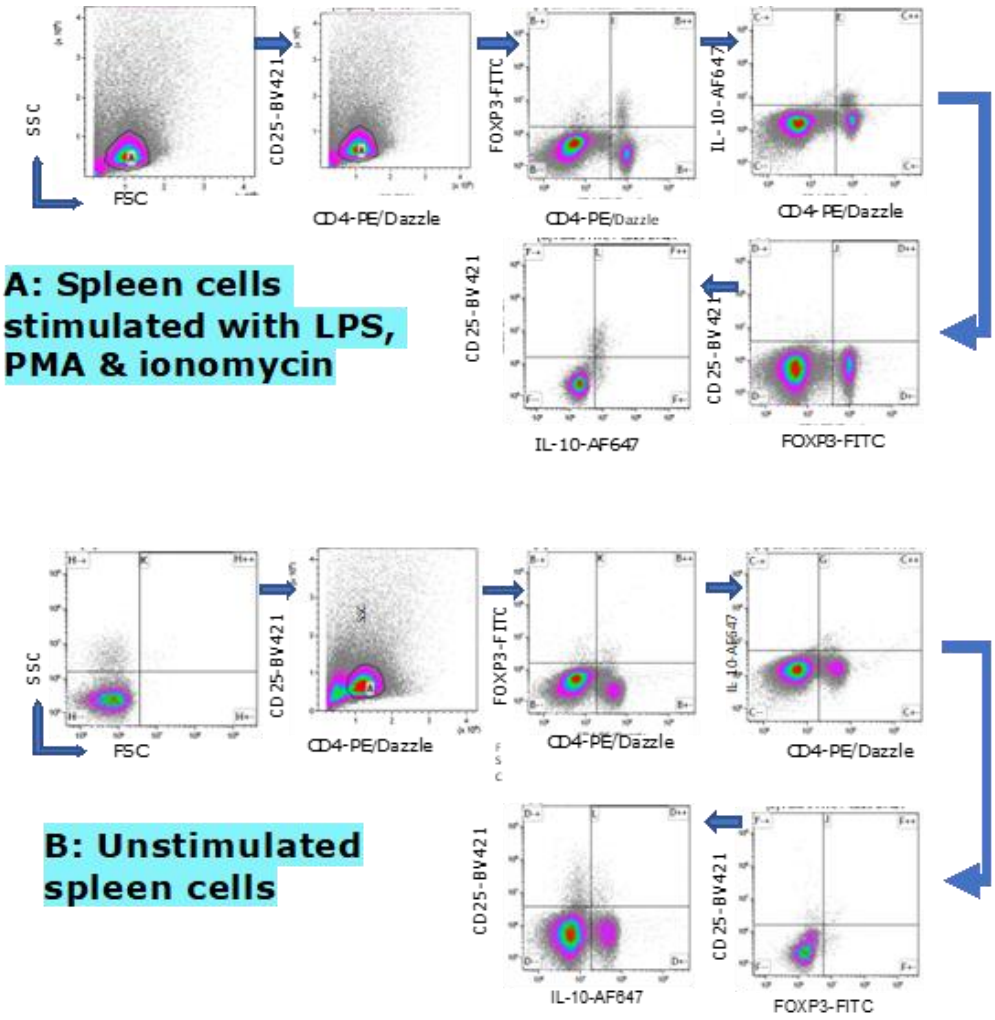

Figure 3.2 Representative flow cytometry plots depicting the gating strategy for quantification of regulatory T cells amongst stimulated (A) and unstimulated (B) spleen cells. Cells were stained with fluorochrome-conjugated antibodies as follows: anti-CD4-PE/Dazzle, anti-CD25-BV421, anti-Foxp3-FITC and anti-IL-10-AF647 and including respective isotype control reagents. (A) The lymphocyte population was gated based on forward scatter (FSC) and side scatters (SSC) plots. (B) $CD4^{+}CD25^{hi}$ events were then identified from the lymphocyte gate. (C) Quadrant settings for identification of the $CD4^{+}Foxp3^{+}$ population from gated lymphocytes (B), guided using isotype controls. (D) Quadrant settings for

identification of the $CD25^{hi}IL\text{-}10^{+}$ population from gated lymphocytes (B), guided using isotype controls.

3.4.2.1 Higher percentage frequency of $CD4^+$ $CD25^{hi}$ among unstimulated than stimulated spleen lymphocytes

Among the lymphocytes, a higher percentage frequency of $CD4^+$ $CD25^{hi}$ cells was observed in both *H*. *pylori*-infected groups, when the cells were left unstimulated compared to those that had been stimulated with mitogen. As shown in Figure 3.3 for 3 weeks, 6 weeks and 9 weeks post-infection there was a general decrease in the frequency of $CD4^+CD25^{hi}$ lymphocytes following stimulation. At 3 weeks post-infection there was a 2.6-fold difference ($p= 0.0003$) in cells from the wild-type infected group and a 2.1-fold difference ($p <0.0001$) in cells from those infected with mutant strain. At 6- and 9-weeks post-infection, there were also significant differences in cells from those infected with the wild-type strain ($p= 0.0005$, $p <0.0001$ respectively) and mutant ($p <0.0001$, $p <0.0001$ respectively). At 6 weeks the unstimulated wild-type 1.6-fold higher than the stimulated and 1.5-fold for the mutant. While at 9 weeks the fold difference was 2.6 with the wild-type and 2.6 with the mutant.

There was no statistically significant difference in the frequencies of $CD4^+CD25^{hi}$ lymphocyte between the groups of mice infected with the wild-type and mutant strains, at any of the time points post-infection (Figure 3.3). This was observed whether the cells had undergone mitogen stimulation before flow cytometric analysis or not.

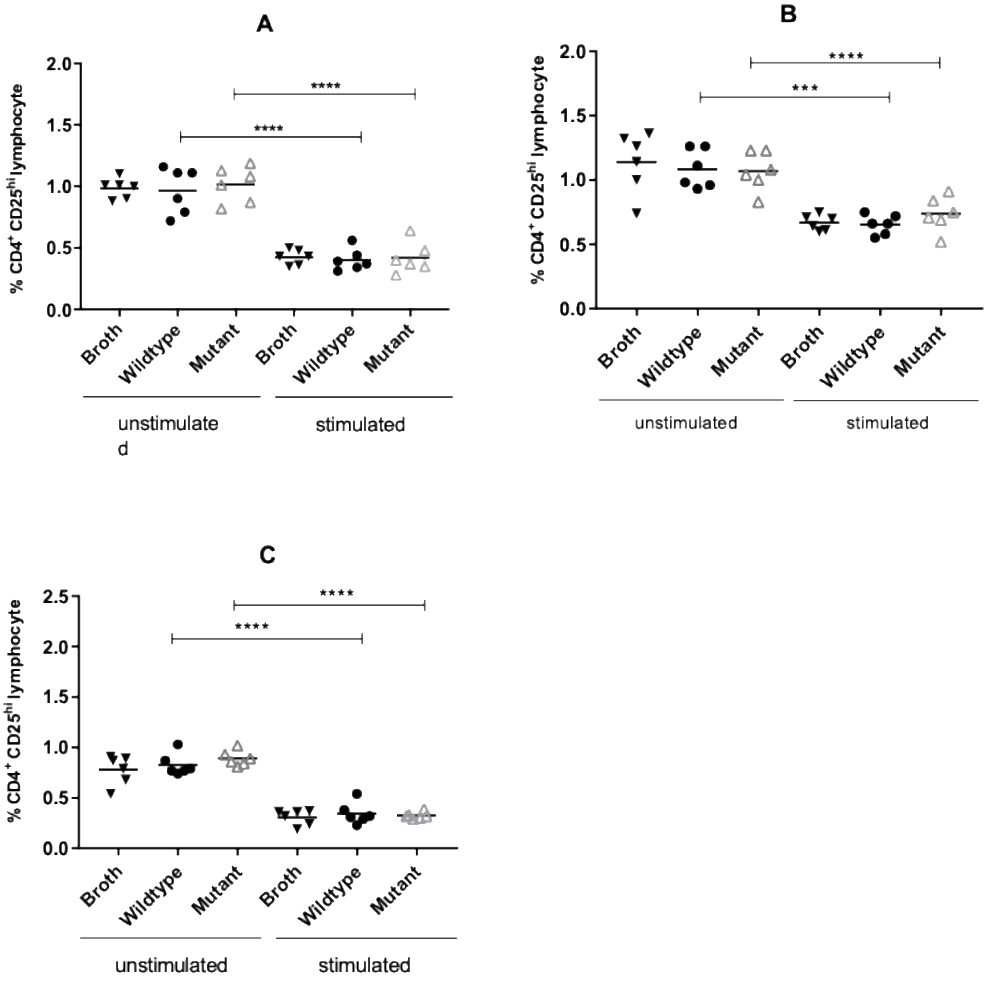

Figure 3.3. CD4+ CD25hi cell frequency in the spleen of groups of 3 mice treated with H. pylori or plain Brucella broth as placebo. Cells were stained with fluorochrome-conjugated antibodies as follows: anti-CD4-PE/Dazzle, anti-CD25-BV421, anti-Foxp3-FITC and anti-IL-10-AF647 and including the respective isotype control reagents. The percentage of $CD4^{+}CD25^{hi}$ cells in the total lymphocyte population was determined for each sample (A), (B) and (C) depict the frequency of $CD4^{+}$ $CD25^{hi}$ cells at 3 weeks, 6 weeks and 9 weeks post-

infection respectively. The horizontal bar represents median values Statistical significance was determined using a paired t-test. Differences were considered significant at p<0.05. **** P<0.0001, ***P=0.005.

3.4.2.2 Higher percentage frequency of $CD4^+$ $Foxp3^{hi}$ cells among unstimulated compared to mitogen-stimulated splenic lymphocytes.

Comparing the frequencies of $CD4^+$ $Foxp3^{hi}$ cells among the three treatment groups (Figure 3.4) showed that there was higher frequency amongst unstimulated rather than stimulated cells from the wild-type infected group by 3.5-fold (p=0.0009) for 3weeks, 5.3-fold (p=0.0002) for 6 weeks and 2.4-fold (p <0.0001) for 9 weeks post-infection). A similar observation was made when analysing cells from the group mutant strain infected 3 weeks 2.6-fold (p=0.0003), 6 weeks 4.1-fold p=0.0012 and 9 weeks 2.9-fold (0.0001).

When comparing data from the stimulated cells, there were no significant differences in the frequencies of $CD4^+$ $Foxp3^{hi}$ cells between the wildtype and mutant infected groups at any of the time's post-infection (Figure 3.4). Unstimulated cells showed the same result at 3- and 6-weeks post-infection, however, there was a significant 1.2-fold difference in $CD4^+$ $Foxp3^{hi}$ cells (p=0.0313) between the wild-type and mutant groups at 9 weeks.

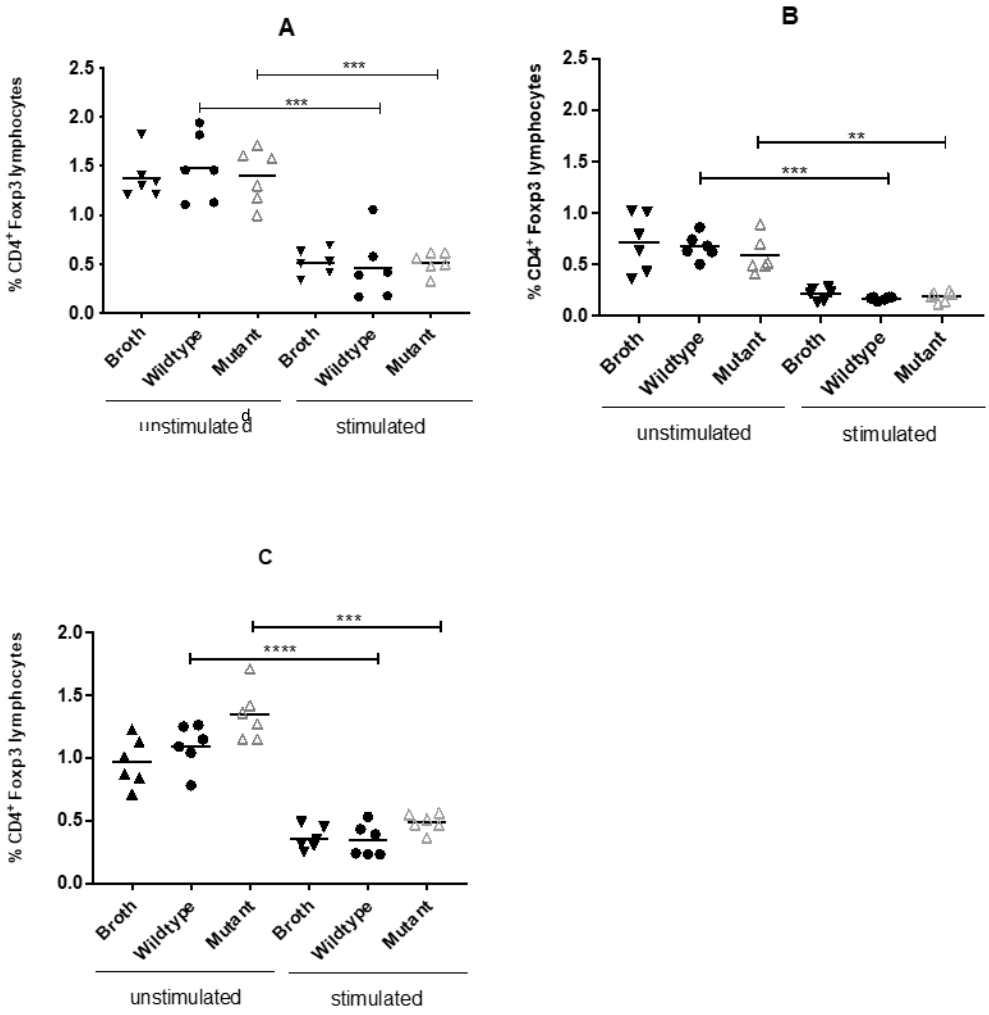

Figure 3.4. CD4+ Foxp3+ cell frequency in the spleen of groups of 3 mice treated with H. pylori or plain Brucella broth as a placebo. Cells were stained with fluorochrome-conjugated antibodies as follows: anti-CD4-PE/Dazzle, anti-CD25-BV421, anti-Foxp3-FITC and anti-IL-10-AF647 and including the respective isotype control reagents. The percentage of $CD4^{+}Foxp3^{hi}$ cells in the total lymphocyte population was determined for each sample (A), (B) and (C) depict the frequency of $CD4^{+}$ $Foxp3^{+}$ cells at 6 weeks and 9 weeks post-infection, respectively. The horizontal bar represents the median values.

Statistical significance was determined using a paired t-test. Differences were considered significant at $p<0.05$.

3.4.2.3 Mitogen stimulated splenocytes contained higher frequencies of IL-10^+ $CD4^+$ T cells.

As expected from previous work done by the group significantly higher frequency of IL-10 producing $CD4^+$ T lymphocytes was observed following the stimulation splenocytes. This was found at all three-time points (Figure 3.5), At 3 weeks 13-fold ($p=0.0001$) for the wild-type and 13-fold ($p=0.0001$) for the mutant, 6 weeks 10.6-fold ($p=0.0019$) for the wild-type and 11.3-fold ($p=0.0012$) for the mutant and at 9 weeks 11.3-fold ($p<0.0001$) for the wild-type and 12.2-fold ($p=0.0001$) for the mutant.

At the 3- and 6-week time points, there were no differences in the proportions of IL-10^+ $CD4^+$ T cells between the mice infected with the mutant and wild-type strains. At 9 weeks post-infection, however, mice infected with, the mutant (expressing the active s1i1 VacA) had a 1.6-fold higher percentage frequency of $CD4^+$IL-10^+ splenic lymphocytes ($p= 0.0004$).

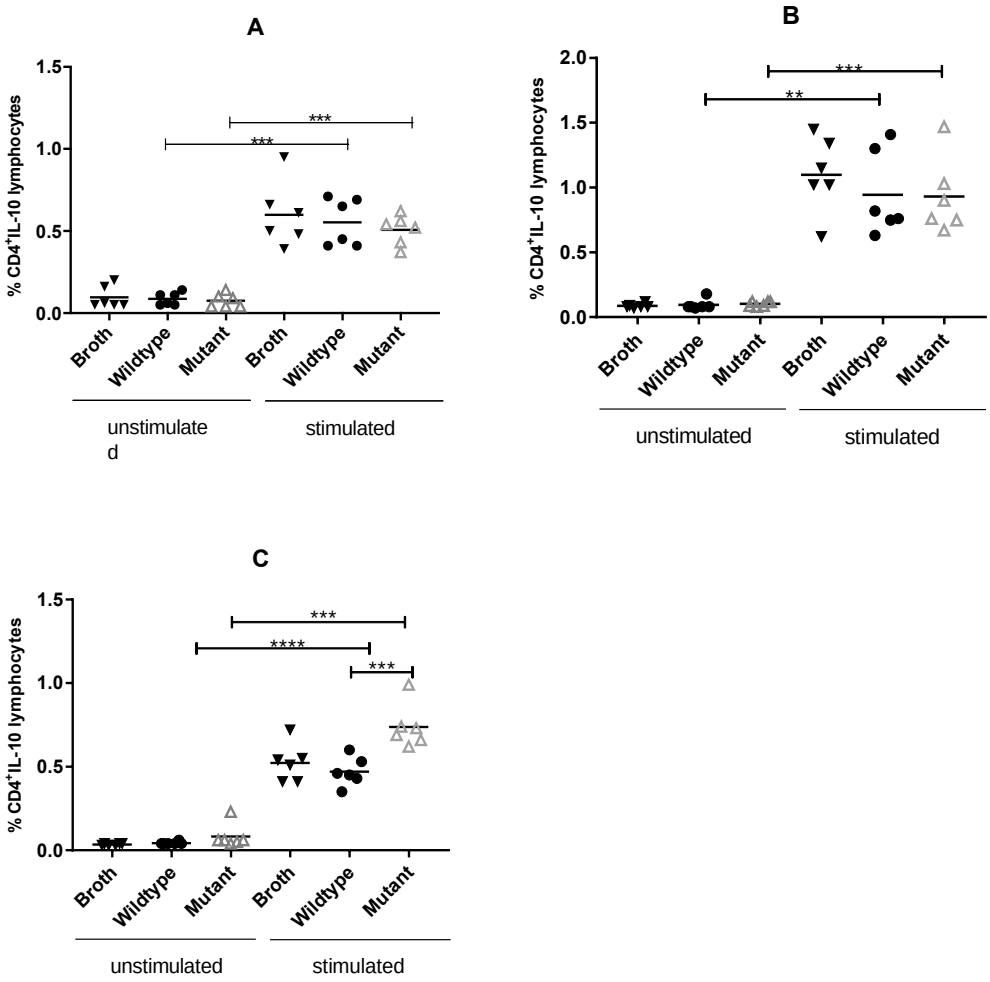

Figure 3.5. CD4+ IL-10+ cell frequency in the spleen of groups of 3 mice treated with **H. pylori or** plain Brucella broth as placebo. Cells were stained with fluorochrome-conjugated antibodies as follows: anti-CD4-PE/Dazzle, anti-CD25-BV421, anti-Foxp3-FITC and anti-IL-10-AF647 and including the respective isotype control reagents. The percentage of $CD4^{+}IL\text{-}10^{+}$ cells in the total lymphocyte population was determined for each sample (A), (B) and (C) depict the frequency of $CD4^{+}$ $IL\text{-}10^{+}$ cells at 3 weeks, 6 weeks and 9 weeks post-infection respectively. The horizontal bars represent median values. Statistical significance was determined using a paired t-test. Differences were considered significant at $p<0.05$.

3.4.2.4 Comparison of Foxp3-expressing $CD4^+CD25^+$ T cell frequencies

To discover potential differences in the Treg response to infection, the proportions of $CD25^{hi}$ and $Foxp3^+$ events amongst gated $CD4^+$ cells were analysed. The results were consistent with the previously described $CD4^+$ $CD25^{hi}$ data at all of the time points (Figure 3.6). Stimulation of the lymphocytes increased the proportion expressing Foxp3; but comparing the stimulated and unstimulated groups there was a decrease, for the wild-type, there was 13-fold ($p<0.0001$) decrease at 3 weeks, 10.8-fold ($p=0.0002$) at 6 weeks and 11.6-fold ($p=0.0195$) while amongst the mutant, at 3 weeks, 9.1-fold ($p<0.0001$) decrease, 10-fold ($p=0.001$) at 6 weeks and 10.4 ($p=0.0144$) at 9 weeks, but infection with wild-type or the mutant made no statistical difference.

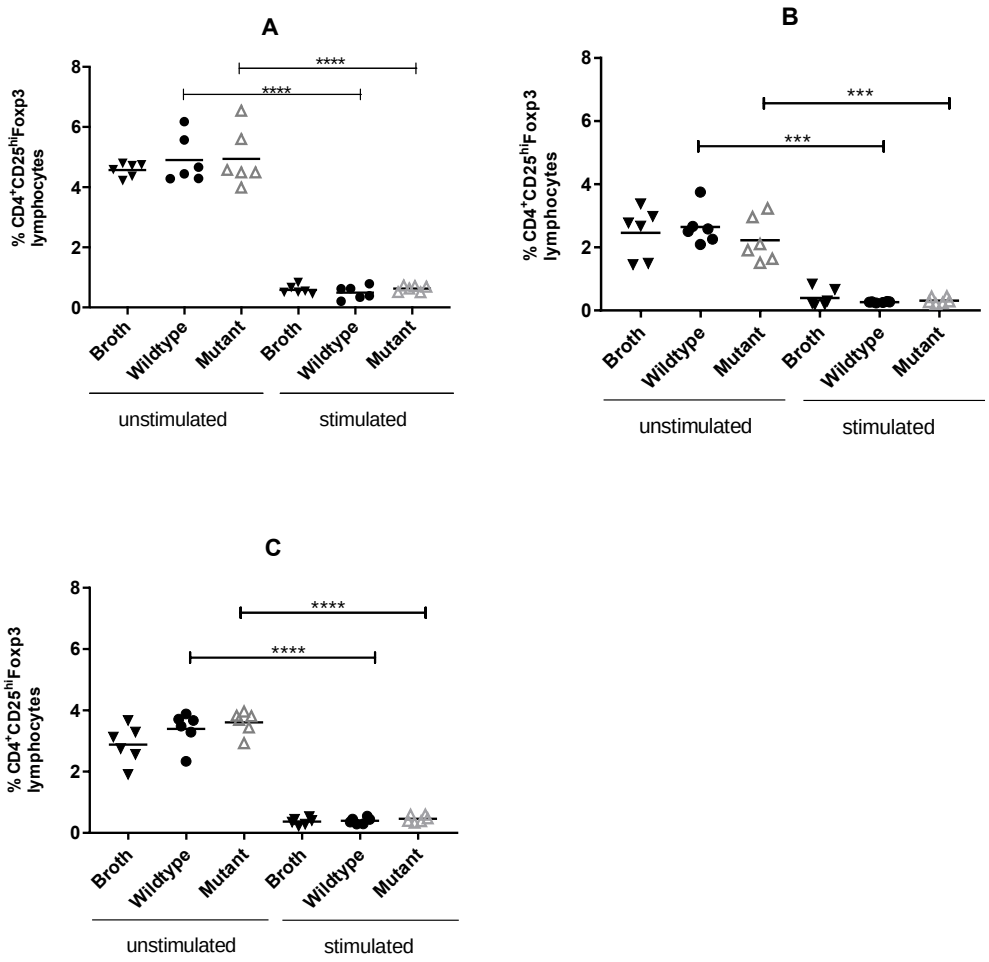

Figure 3.6. CD4+ CD25hi Foxp3+ cell frequency in the spleen of groups of 3 mice treated with **H. pylori** and plain Brucella broth as placebo. Cells were stained with fluorochrome-conjugated antibodies as follows: anti-CD4-PE/Dazzle, anti-CD25-BV421, anti-Foxp3-FITC and anti-IL-10-AF647 and including the respective isotype control reagents. The percentage of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ cells in the total lymphocyte population was determined for each sample (A), (B) and (C) depict the frequency of $CD4^+$ $CD25^{hi}$ $Foxp3^+$ cells at 3 weeks, 6 weeks and 9 weeks post-infection respectively. the horizontal bar represents median values. Statistical significance was determined using a paired t-test. Differences were considered significant at $p<0.05$.

3.4.2.5 Comparison of IL-10-producing $CD4^{+}CD25^{+}$ T cell frequencies

To ascertain the proportions of CD25hi and IL-10+ events amongst gated CD4+ cells were analysed. Analysis of the intracellular IL-10 in CD4+ T CD25+ T cell both for the stimulated and unstimulated situation, the result was consistent with previous findings in the group, a significant increase in the IL-10+ following stimulation (Figure 3.7).with 3.3-fold ($p=0.0014$) increase amongst the wild-type 3 weeks, 3.8-fold ($p=0.0001$) at 6 weeks and 2.3-fold ($p<0.0001$) at 9 weeks. Comparing the mutants, at 3 weeks there was 4-fold ($p=0.0029$) increase, 6 weeks 3.2-fold ($p=0.0009$) and 2.5-fold ($p<0.0001$) at 9 weeks.

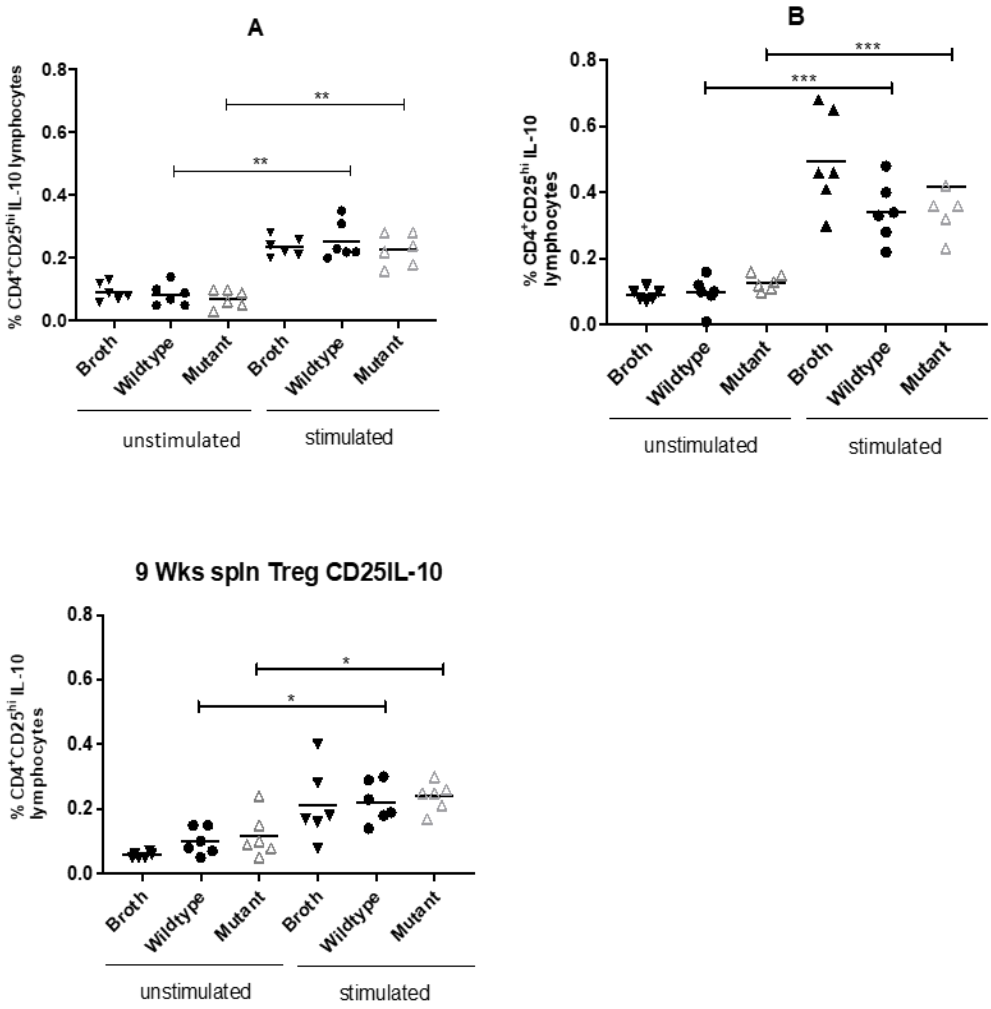

Figure 3.7. CD4+ CD25hi IL-10+ cell frequency in the spleen of groups of 3 mice treated with **H. pylori** and plain Brucella broth as placebo. Cells were stained with fluorochrome-conjugated antibodies as follows: anti-CD4-PE/Dazzle, anti-CD25-BV421, anti-Foxp3-FITC and anti-IL-10-AF647 and including the respective isotype control reagents. The percentage of $CD4^{+}CD25^{hi}IL\text{-}10^{+}$ cells in the total lymphocyte population was determined for each sample (A), (B) and (C) is the frequency of $CD4^{+}$ $CD25^{hi}$ $IL\text{-}10^{+}$cells at 3 weeks and 9 weeks post-infection respectively. The horizontal bars represent median values, and the statistical significance was determine using a paired t-test. Differences were considered significant at $p<0.05$.

3.4.3 Investigation of whether *H. pylori* infection influences the functional suppressive activity of regulatory T cells

To measure the suppressive function of $CD4^+$ $CD25^{hi}$ Treg cells isolated from the spleens of mice infected with the SS1 *H. pylori* strain or given plain broth as a placebo, they were mixed with $CD4^+$ $CD25^-$ T responder (Tresp) cells from untreated mice at different ratios (1:1, 1:2, 1:4 and 1:8) varying only the numbers of Tregs. The Tresp cells were labelled with the fluorescent dye CFSE and activated with anti-CD3/CD28 beads before adding the Treg and the cells were cultured for 96 hours.

To assess the suppressive capacity of the Treg cells, data on the percentage of Tresp cells that are suppressed response to the activation beads, and their fold expansion was analysed by flow cytometry.

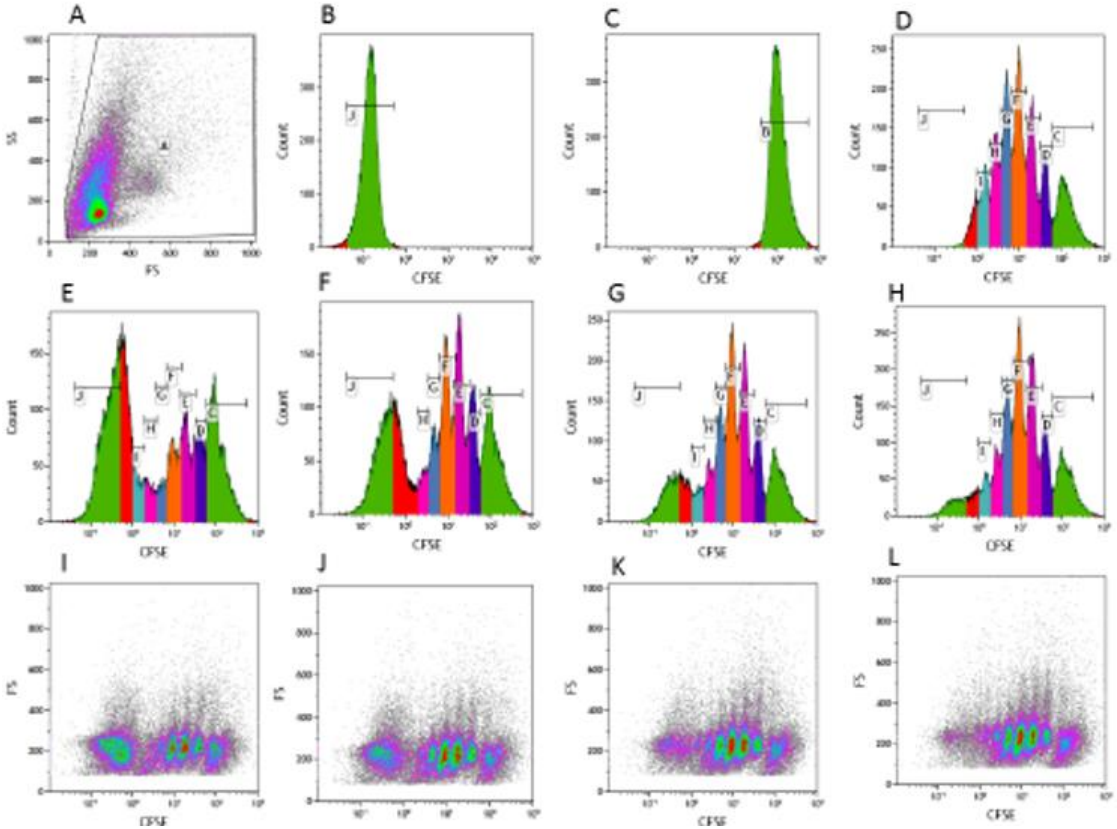
A
B
C
D
E
F
G
H
I
J
K
L
SS
FS
Count
CFSE

Figure 3.8. The Representative flow cytometry plot gating strategies for Treg suppression assay. The responder T (Trep) cells ($CD4^{+}CD25^{-}$) isolated from two untreated mice were labelled with CFSE, the labelled cells were cocultured with four dilutions of regulatory T(Treg) cells ($CD4^{+}$ $CD25^{hi}$) and stimulated anti-CD3/CD28 beads. (A) Lymphocytes were selected based on the forward scatter (FS) and side scatters (SS) as measures of size and granularity, respectively. (B) The histogram plot of the unlabelled Tresp negative control. (C) Histogram plot of labelled Tresp but unstimulated with anti-CD3/CD28. (D) Stimulated Tresp with no Treg. (E) Stimulated Tresp with the 1:1 Tresp: Treg ratio. (F) 1:2 Tresp: Treg ratio. (G) 1:4 Tresp: Treg ratio. (H) 1:8 Tresp: Treg ratio. (I) The corresponding dot plot of CFSE and FS for 1:1 Tresp: Treg ratio. (J) 1:2 Tresp: Treg ratio. (K) 1:4 Tresp: Treg ratio. And (L) 1:8 Tresp: Treg ratio

3.4.3.1 The percentage of cells suppressed by Treg

This is the measure of the percentage of the Tresp cell stimulated will divide at least once with minimal suppression of the Treg. It is used in determining the fraction of the original population that divided least once during the culture period. As shown in Figure 3.8, the graph showed increases in the percentage of cells dividing with decreases in numbers of Tregs present in the coculture. However, there was no statistically significant difference in function between the suppressive ability of the Tregs isolated from the spleens of *H. pylori* treated mice and those given the placebo (plain Brucella broth).

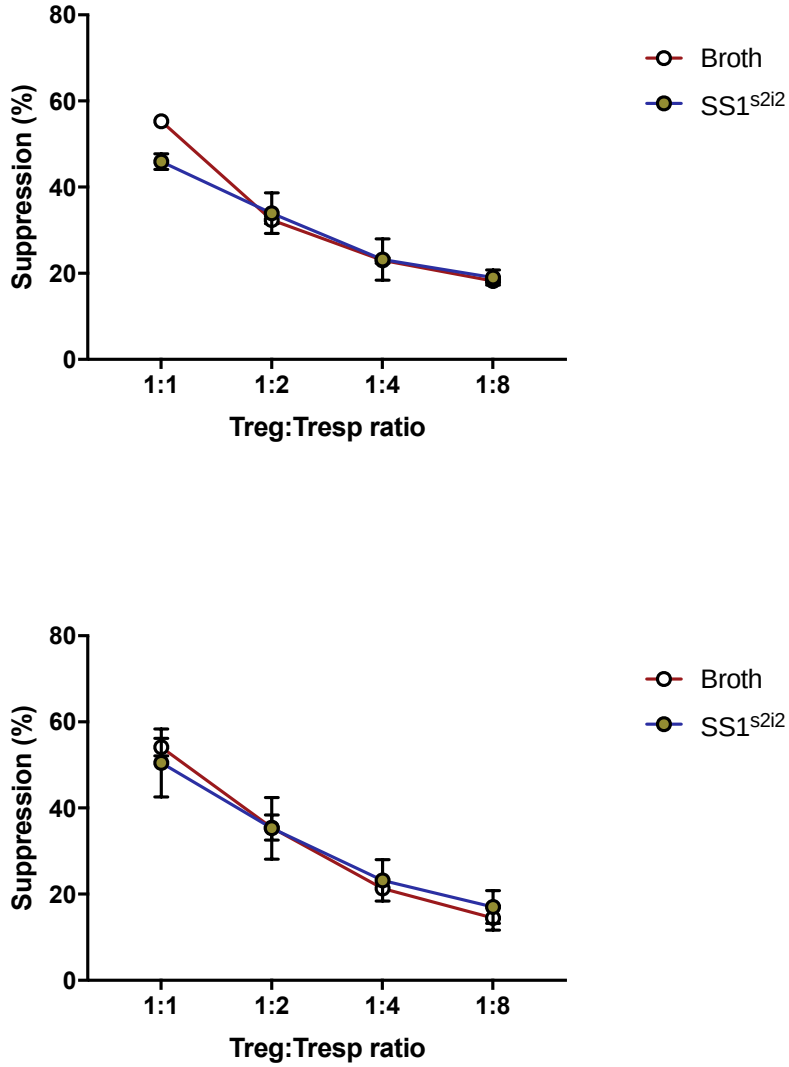

Figure 3.9. Group of six mice were infected with the SS1 strain of **H. pylori** in Brucella broth or administered plain broth as a placebo. 6 and 9 weeks later the mice were killed and the splenic $CD4^+$ $CD25^{hi}$ Treg cells isolated using magnetic bead isolation. Cells from two mice were combined to ensure sufficient recovery of cells. $CD4^+$ $CD25^-$ Tresp were also purified from the spleens of untreated mice and labelled with CFSE before stimulation using anti-CD3/28 beads. The suppressive activity of the Tregs cells from infected and placebo-treated mice was compared, co-culturing Treg and Tresp cells in a range of ratios for 96 hours. Effects on the Tresp cells expansion was assessed

using flow cytometry. Data show the mean and the standard deviation from the three replicates.

3.4.3.2 Fold expansion of Tresp cell

Fold expansion examines the ratio of final cell count to starting cell count during the culture. In this case, the fold expansion of the Tresp was calculated for both treatments and the result as shown in Figure 3.9 The overall fold expansion for each of the three-time points also showed increases in Tresp expansion when the numbers of Tregs in the coculture were reduced. As there was no statistically significant difference between Tregs from the spleens of infected or placebo-treated mice.

Furthermore, to assess the trend according to the three points, a plot the percentage cell divides and overall fold expansion for 1:4 Treg: Tresp for the 3 time points were made (Figure is shown in the appendix). There was no statistically significant difference between the time points.

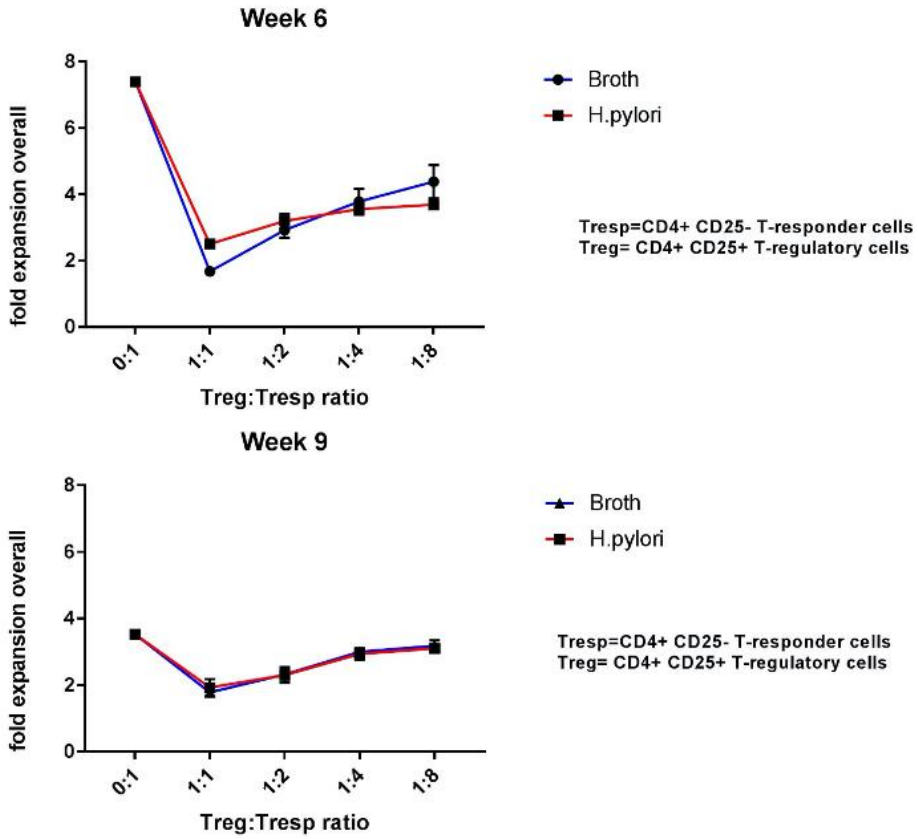

Figure 3.10. Group of six mice were infected with the SS1 strain of **H. pylori** in Brucella broth or administered plain broth as a placebo. 6 and 9 weeks later the mice were killed and the splenic $CD4^+$ $CD25^{hi}$ Treg cells isolated using magnetic bead isolation. Cells from two mice were combined to ensure sufficient recovery of cells. $CD4^+$ $CD25^-$ Tresp were also purified from the spleens of untreated mice and labelled with CFSE before stimulation using anti-CD3/28 beads. The suppressive activity of the Tregs cells from infected and placebo-treated mice was compared, co-culturing Treg and Tresp cells in a range of ratios for 96 hours. Effects on the Tresp cells expansion was assessed using flow cytometry. Data show the mean and the standard deviation from the three replicates.

3.5 Discussion

The aim of this study was primarily to access the immunoregulatory potential of the *H. pylori* protein, VacA. Several studies have shown that VacA is an effective immunomodulatory molecule (Oertli et al., 2013b, Kao et al., 2010), but there is very little information on the relative effects of the different types of toxin. This study used two strains of the Sydney Strain 1 (SS1) mouse-adapted model of *H. pylori*, which expressed the s2i2 and s1i1 forms of VacA. The SS1^{s2i2} strain is the wild-type, and this form is without vacuolating activity, whilst the SS1^{s1i1} mutant expressed active VacA (Day et al., 2001, Winter et al., 2014).

Although only a few *H. pylori* strains are capable of effectively colonizing mice to enable studies of this manner. SS1 strain was shown to consistently colonise several mouse strains including C57BL/6 mouse at high level with persistent infection that could last for months making it suitable for studies in immune system and effect of virulence factors (Lee et al., 1997). The SS1 strain used in this investigation is known to lack *cag*PAI functionality but expresses the nontoxigenic form of VacA (s2i2) (Arnold et al., 2011b). However, most human studies have demonstrated the association between active form of VacA (s1i1) and disease. Therefore, SS1^{s1i1} isogenic mutant expressing the toxigenic VacA^{s1i1} form was used in this study. The wild type SS1^{s2i2} *H. pylori* and the mutant SS1^{s1i1} *H. pylori* have been used extensively in investigation of VacA-specific experiments. The colonisation result in the experiment showed a lower colonisation density in the stomach the mice infected with SS1^{s1i1}

mutant compare to the $SS1^{s2i2}$ wild-type. This result is consistent with the findings by Winter et al, in which they showed that the strains producing s1i1 and s1i2 VacA has reduced colonisation densities while those of s2i2 readily colonised the mouse stomach (Winter et al., 2014). However, the result of the difference in colonisation is not expected to influence the overall outcome this studies since the same study by Winter *et al* as showed that there is no significant correlation between colonisation density and production of gastric pathology in mice infected with $SS1^{s1i1}$ producing toxigenic form of VacA or $SS1^{s2i2}$ nontoxigenic form of VacA.

The group and other literatures have previously shown that VacA is capable of suppressing the immune response *in vitro* and could be very important in the establishment of persistent colonization by *H. pylori* (Greenaway, 2014, (Salama et al., 2001b, Winter et al., 2014). Preliminary evidence from the group indicated that one of the mechanisms through which VacA achieves an immune regulatory function is the induction of Treg responses. It was shown that there was an increase in Foxp3 expression in PBMCs isolated from healthy patients with VacA-active strain compared to those with an inactive VacA strain (Greenaway 2014). This evidence serves as an indicator that VacA may be important in Foxp3 induction.

Furthermore, the group also had already shown that VacA also influenced the amount of IL-10 expressed by mouse splenocytes. The type of VacA expressed by *H. pylori* had an impact on the level of IL-10 expression by the splenocytes.

Therefore, in this present research, the hypotheses were that Splenocytes from the mice infected with *H. pylori* expressing active s1i1 VacA would respond better to mitogen stimulation than cells from mice infected with *H. pylori* expressing the s2i2, the inactive form of VacA. This would occur due to the presence of memory T cells which confers on it more responsiveness.

There would be higher proportions of $Foxp3^{+}$ CD4 cells among the lymphocytes from mice infected with the $SS1^{s1i1}$ strain expressing s1i1 VacA form compared to the $SS1^{s2i2}$ strain expressing s2i2 VacA form.

There would be higher proportions of IL-10^{+} CD4 cells among the lymphocytes from mice infected with the $SS1^{s1i1}$ strain compared to the $SS1^{s2i2}$ strain.

Interestingly, mitogen-stimulated cells from all the groups showed a decrease in the proportion of $Foxp3^{+}$ cells compared to their paired unstimulated cell preparations. However, there was no significant difference between the levels of FOXP3 expression when comparing the splenocytes from active VacA infected, non-active VacA infected or placebo-treated mice. This may be because VacA may not entirely be the only driving factor in FOXP3 expression and immunoregulation other *H. pylori* proteins such as GGT, PPT and KatA have been reported to have some immune regulatory functions (Oertli et al., 2013a, Oertli et al., 2012) (Greenaway, 2014, unpublished data).

Similarly, IL-10 expression by the splenocytes was analysed and showed that there was an increase in IL-10 expressing cells following stimulation. However, again the active VacA *H. pylori* strain made no difference. This agrees with the point made above, that infection with *H. pylori* could influence the induction of immunomodulatory cells but VacA alone may not be the sole factor. On the hand, a long-standing suggestion is that VacA is essentially pleiotropic may be in effect in this study (Isomoto et al., 2010).

Using CFSE suppression assay as described by Collision and Vignali (Collision and Vignali, 2011), the differences in the immunosuppression activities of Treg isolated from a group of mice infected with active *H. pylori* with active vacA gene and placebo-treated mice were investigated. The study here investigated whether the active VacA possessing *H. pylori* strain in mice induced a better Treg immunosuppressive activity compare to that of placebo-treated mice. The results from this experiment indicated obvious activities by Tregs on the Tresp cells, but there was no difference in the suppression activities between Tregs from the H. pylori and placebo groups. Research in the past by Boncristiano et al. and Sundrud et al. had shown that VacA activities have inhibited the clonal expansion of activated T cell. They showed that VacA is capable of inhibiting IL-2 in Jurkat cells in vitro. IL-2 is produced by T-cell in response to mitogenic stimulation and is required for T-cell proliferation(Boyman and Sprent, 2012, Boncristiano et al., 2003). Early research in the group also showed that PBMCs from VacA-negative patients

have decreased Foxp3 expression compare to that of VacA-positive patients, showing that VacA could be important Foxp3 induction (Greenaway, 2014 unpublished). Both results go on to suggest that VacA could have immunoregulatory activity in vivo and in vitro.

The result raises the concern on whether mechanism through which molecules of VacA inhibit T cell proliferation differ from the secreted VacA from *H. pylori*. There is also the case of the suitability of the laboratory animal in the simulation of the human infection. Mice are the natural host for *H. pylori* and may require a large dose of the bacteria for proper simulation. Infection in a human subject in most case occurs at an early age when the immune system is developing this is different for 6-8 weeks mice which have fully developed immune system. Another reason is that mice stomachs have a very dense microbial flora in a less-acidic mucosa compare to that of human.

The acidity in mice stomach is usually constant compare to human, which changes throughout the day. Finally, the difference between human and mice immune system can also influence the out of the experiment. For example, lack of IL-8 in mice. And better still mouse model and/or mouse-adapted strain of VacA-positive may not be a good model for this investigation (Arnold et al., 2005).

3.5.1 Conclusion

- There were no differences in the frequencies of Tregs in the spleen between the groups infected with *H. pylori* expressing s1/i1 or s2/i2 forms of VacA.

- There was a significantly increased frequency of $Foxp3^+$ cells in the stomach of infected mice compared to the uninfected group

- VacA type did not significantly affect the frequencies of Tregs in the spleen or gastric mucosa
- The infection did not influence the functional suppressive activity of Tregs from the spleen

- There was only a very small increase in Tregs in mice infected with the SS1 strain of H. pylori. This is possibly due to them being infected at 6 weeks of age (rather than as neonates), and the relatively short duration of the experiment.

Chapter 4

Serum *Helicobacter pylori* KatA, GGT. PPT, CagA and VacA antibodies as novel biomarkers

4.1 Introduction

Although the effect of *H. pylori*-specific antibody on the extent of bacterial colonisation is still debatable, studies have shown that *H. pylori* infection almost constantly induces robust mucosal and systemic antibody production (Czinn et al., 1993, Akhiani et al., 2004). In most epidemiological research studies, serological testing has remained the most important approach; as illustrated in most gastric cancer cases where the studies showed a significantly higher prevalence of *H. pylori* specific antibodies among patients compared to normal controls (Gao et al., 2009a). *Helicobacter pylori* infection is the major cause of a number of gastrointestinal tract diseases, such as chronic gastritis, peptic ulcer and gastric cancer (Suerbaum and Michetti, 2002). It is also known that over 50% of the world population is infected with *H. pylori.* Primarily, 85 % of those infected experience no symptoms or mild symptoms such as mild asymptomatic gastritis. In cases of more severe outcomes, 10-15% can develop peptic ulcer disease (PUD), 5% can develop atrophic gastritis and about 1-2% can develop gastric cancer(Amieva and El-Omar, 2008, Conteduca et al., 2013). Factors such as host factors and diet have been shown to be contributory to the risk of disease outcomes but researchers have also shown that bacterial virulence factors are a major independent determinant of disease and could serve as a predictive biomarker (Yamaoka,

2010). Virulence factors such as CagA, VacA, GGT, KatA and PPT among others are all recognized to play crucial roles in *H. pylori* pathogenicity (Atherton, 2006) and the serum antibodies to these *H. pylori* factors could serve as noninvasive biomarker for early detection of *H. pylori* diseases.

4.1.1 Anti-cytotoxin-associated gene A (CagA) antibodies

CagA is the most researched *H. pylori* virulence factor and as such possibly the most important virulence factor in the bacterium (Shiota et al., 2013). CagA positive *H. pylori* strains have been implicated in serious clinical sequellae, with damaging effects mediated directly by CagA (Kuipers et al., 1995, Huang et al., 2003). Parsonnet *et al* examined the serum of 179 infected and 63 uninfected individuals in a case-control investigation looking at the serum anti-CagA IgG response. Their results showed a more than a 5-fold increased risk of developing gastric cancer in individuals with CagA-positive *H. pylori* strains, compared to uninfected subjects. They also showed that subjects infected with CagA-negative *H. pylori* are not significantly more likely to develop gastric cancer than uninfected individuals (Parsonnet et al., 1997). Similarly, Gao *et al* investigated the association between chronic atrophic gastritis and serological status of antibodies to 15 *H. pylori* proteins using a novel multiplex serology method. Here, 534 cases of chronic atrophic gastritis and 1068 control subjects were examined for seropositivity for the 15 proteins. Among the virulence factors CagA, VacA, Helicobacter cysteine-rich protein C

(HcpC) and the chaperonin GroEL was shown to have a strong association with chronic atrophic gastritis (Gao et al., 2009b). Equally, another group investigated the seroprevalence of CagA, VacA, GroEL, Urease subunit (UreA), GGT and HcpC and its' relationship with gastric lesions, a precursor lesion of gastric malignancy, using the recomLine *H. pylori* test system. Their results showed that amongst the factors studied, only CagA and GroEL could be considered as an independent predictor for the development of gastric lesions (Pan et al., 2014).

4.1.2 Anti-vacuolating cytotoxin A (VacA) antibodies

VacA is another very well studied factor of *H. pylori*. The *vacA* gene is present in all strains but only the s1/i1/m1 allelic variant is regarded as encoding an active toxigenic form of VacA. The s1/i1 form of VacA is associated with severe disease outcomes such as peptic ulceration and gastric cancer (Atherton et al., 1995, Miehlke et al., 2000). Although known for its induction of vacuolation in gastric cells (de Bernard et al., 1997), VacA has also been implicated to cause disruption of mitochondrion functions, T-cell proliferation inhibition and induction of apoptosis (Gebert et al., 2003, Isomoto et al., 2010). Other reports also showed that VacA could be important in mediating the colonisation of *H. pylori* (Salama et al., 2001b, Salama et al., 2001a). There have been several mixed reports on the seroprevalence of VacA antibodies and its link with disease outcome. One report found 73.91% VacA seropositivity among gastric cancer patients (Suriani et al., 2008), and another reported an odds ratio of 3.19 (1.44-7.05) showing that VacA antibody could serve as an indicator for the

progression of chronic atrophic gastritis (Gao et al., 2009b). Pan *et al* reported a 38.9 % seroprevalence among patients with gastric lesions (Pan et al., 2014). The conflicting reports paved the way for our investigation, using a protein array method to ascertain the differences in the prevalence of VacA-specific antibody between *H.pylori*-positive and *H.pylori*-negative individuals.

4.1.3 Catalase (KatA)

Catalase is an enzyme present in all life forms that neutralizes extracellular hydrogen- peroxides. In *H. pylori* catalase, KatA, the enzyme protects the bacteria from oxidative damage in hostile immune environments which could have direct damaging effects on their genomic DNA (Harris et al., 2002, Wang et al., 2006, Wang et al., 2005). Therefore, KatA plays a vital role in *H. pylori* colonisation of the gastric mucosa (Hazell et al., 1991). KatA readily induces antibodies in *H. pylori*-infected individuals. Antibodies levels are similar in individuals from *H. pylori* -high-risk areas and in those residing in low-risk areas (Camargo et al., 2015), Camargo *et al* also reported associations of KatA with gastric diseases, showing it to be independent of other virulence factors such as CagA and VacA. Similarly, Zhang *et al* reported an association with gastric cancer and 14.59-fold increase in the risk of gastric cancer among those who were seropositive for KatA antibodies (Zhang et al., 2016). Their investigation was done employing an indirect ELISA test, using recombinant KatA which the

group acknowledged could lead to nonspecific signals from cross-reactivity. This problem could easily be eliminated by the protein array.

Although Kotiw *et al* reported that KatA-specific antibodies are readily induced in the serum of infected individuals, they still doubted the protective role of these antibodies (Kotiw et al., 2012). But earlier vaccine studies on rodents reported the opposite, demonstrating how anti-catalase antibodies could protect against a subsequent *H. pylori* infection (Chen et al., 2003).

4.1.4 γ-Glutamyltranspepetidase (GGT) antibodies

GGT is expressed by all strains of *H. pylori*. The enzyme contributes hugely in bacterial pathogenesis (Chevalier et al., 1999, Wustner et al., 2017). The activities of this enzyme alone, which include the utilisation of glutamine to generate glutamate and ammonia, conversion of glutathione to cysteinylglycine and by-products such as ammonia and reactive oxygen species, tend to have a massive effect on the surrounding host cells (Ricci et al., 2014). These effects contribute to final disease outcomes such as peptic ulcer and gastric cancer (Gong et al., 2010, Rimbara et al., 2013).

Pan *et al* demonstrated the virulence activities of GGT in promoting bacterial and cell apoptosis but failed to establish a relationship between GGT seropositivity and gastric cancer (43.3 %). In fact, they reported an inverse association with the presence of preneoplastic lesions. They speculated the anti-inflammatory effect is produced by the interference of GGT-specific antibodies with virulence activities (Pan et al., 2014).

4.1.5 Peptidyl prolyl cis trans isomerase (PPT) antibodies

PPT (also known as PPIase) is another enzyme possessed by both eukaryotic and prokaryotic cells, which catalysis the folding of proteins by cis/trans isomerisation of peptide bonds with prolyl residues. (Gothel and Marahiel, 1999). *H. pylori* PPT (product of gene HP0175) is one of the secreted proteins that the bacteria utilise in colonisation and pathogenesis (Basak et al., 2005). Studies of this protein in *E. coli* revealed that the protein perform more isomerase function but can equally independently perform chaperonin function (Ramm and Plückthun, 2001). The protein has been observed to bind TLR4 in the AGS human gastric adenocarcinoma cell line, triggering apoptosis and therefore it is also known as *H. pylori* cell-binding factor 2 (HpCBF2). This was demonstrated by antibody blockade of TLR4, which abrogated apoptosis on incubation with PPT. The level of cell death observed with PPT was significantly more than with other *H. pylori* virulence factors (Basak et al., 2005). TLR4 links innate immunity to adaptive immune responses, although the agonist widely known to associate with this PRR is bacterial (but not *H. pylori)* LPS. As PPT interacts with TLR4 in a similar way to LPS, it could follow a similar pattern of cell signalling pathway activation.

Atanassov *et al*, using PPT isolated from two strains of *H. pylori*; *H. pylori* HP141 and *H. pylori* ATCC 43579, showed the presence of PPT-specific antibodies by ELISA in *H. pylori*-infected individuals. They went further to show anti-PPT seropositivity value for gastroduodenal ulcers (GDU)patients of 33(60) and 30(55) seropositivity with *H. pylori* HP141 and *H. pylori* ATCC 43579

respectively and 8(27) and 7(23) with non-ulcer dyspepsia NUD. These values which are not sufficient to be a sole predictive biomarkerfor ulcer diseases but report that antibody frequency to PPT together VacA and β-ketoacyl-ACP S could make it possible to differentiate between the NUD and ulcer patients (Atanassov et al., 2002).

4.1.6 *H. pylori* 60190 and *H. pylori* Tx30a

Only a small percentage of *H. pylori*-infected individuals develop gastric cancer. This is known to be as a result of multiple factors which include, the host response to the infecting bacteria, the interaction between the host and bacteria and essentially the differing expression of the strains virulence factors (Blaser, 1997). One of the first virulence factors to be identified in the *H. pylori* was CagA, it is present in approximately 50% of bacterial isolates and is a constituent of the *cag* pathogenicity island (*cag*PAI) gene cluster (Covacci et al., 1993, Akopyants et al., 1998). The *cag*PAI encodes homologues of type IV bacterial secretion proteins (Censini et al., 1996). Several investigations have shown that individuals infected with CagA-positive strains are at a higher risk of developing disease outcomes, notably, gastritis, atrophic gastritis, peptic ulcer disease and gastric cancer (Kim et al., 2013a, Kuipers et al., 1995, Peek et al., 1995, Blaser et al., 1995) compared to those with CagA-negative strains which are seldom associated with disease.

Another vital virulence factor possessed by the strains is VacA, which is associated with disease (Atherton, 2006). Almost all strains possess the *vacA* gene, but only about half of the isolates express the active form of the protein. Allelic diversity in three main regions of the gene, which include the signal (s), the intermediate (i) and the middle (m) regions, results in different degrees of vacuolating activity (Atherton et al., 1995, van Doorn et al., 1998). For a long time, *vacA* s- and m- regions remained the two most important polymorphic regions associated with disease progression (Atherton et al., 1995, Blaser and Atherton, 2004, Sugimoto et al., 2009). Later, the i-region was recognised as the third determinant of *vacA* polymorphism, and shown to be a better predictor of disease severity (Rhead et al., 2007). Based on the amino acid sequence of each region it showed there could two more variants for each region: the s-region has two variants s1 and s2, the i-region has three variants i1, i2 and i3 while the m-region has two regions m1 and m2. (Atherton et al., 1995, van Doorn et al., 1998, Van Doorn et al., 1999). Studies have shown the effect of different genotypes emanating from the combination of the variants is the determinant of the pathogenesis of a strain rather than possession of the *vacA* gene. Similarly, different VacA genotypes also influence the vacuolating activity. As the case may be, *vacA* s1 encodes a vacuolating active protein while *vacA* s2 encodes a variant which does not have a vacuolating activity. It has also shown that the protein encoded by the latter has a different signal peptide cleavage site which produces an extension, of 12 -amino acid long and is probably the reason for vacuolation inhibition (McClain et al., 2001). Likewise, m1 variants are said to be contributors to the vacuolation in a wider range of

cell types than the m2 (Atherton et al., 1995). The i1 variant shows the highest vacuolating activity compared with i2, while i3 is thought to be rare (Rhead et al., 2007, Ferreira et al., 2012). Again the H. pylori 60190 and Tx30a used in this research work both posses *vacA* genes but are of s1/i1/m1 and s2/i2/m2 types respectively.

4.2 Hypothesis

The European Helicobacter Pylori Study Group (EHPSG) recommended serology assays and tests as one of the non-invasive means of H. pylori diagnosis (Malfertheiner et al., 1997). Several studies have shown that several *H. pylori* virulence factors elicit strong antibodies both in the blood and in the gastric mucosa(Mattsson et al., 1998, Lee et al., 1995). These antibodies are relatively associative rather than of being protective against *H. pylori* infections(Tiwari et al., 2007). Studies among certain gastroduodenal patients ulcers have shown that these serum antibodies against the factors preferentially recognize *H. pylori* antigens and this could be useful in differentiating one H. pylori disease from another (Atanassov et al., 2002).

Here, five H. pylori factors KatA, GGT, PPT, CagA and VacA were selected based on their relative prominence during *H. pylori* infection. We hypothesis that the level of antibody against *H. pylori* factors is an important indicator of the disease severity.

To test this hypothesis, we set the following objective

1. To establish the differences in levels of antibodies against *H. pylori* factors KatA, GGT, PPT, CagA and VacA among *H. pylori*-positive and negative subjects

2. To investigate whether a strong antibody response to each of the factors correlates with gastric pathology and disease outcome.

4.3 Materials and Methods

4.3.1 Biological samples collection

Peripheral blood samples were donated by 188 patients undergoing a routine upper gastrointestinal endoscopy at the Queen's Medical Centre, Nottingham. The most common reason for this was chronic dyspepsia. A combination of biopsy urease test, bacterial isolation and culture, histology and PCR results were used to determine the *H. pylori* infection status of each patient (Table 4.1). All samples were collected with approval from Nottingham Research Ethics Committee 2 (ref 08/H0408/195) and informed written patient consent. Blood samples were not collected from patients who had taken antibiotics and/or frequently on non-steroidal anti-inflammatory drugs (NSAIDs) and/or proton pump inhibitors (PPI) in the last 4 weeks prior to the sample collection. EDTA anticoagulant containing vacutainer tubes were used to collect the blood samples and the plasma was separated by centrifugation at 200 x g for 10 minutes. The plasma was aliquoted and stored at –80°C.

Table 4.1. The demographics of the patients

***H. pylori* status**	**Average age (years)**	**Female to Male Ratio**	**Disease status**
94 infected	52.2 (17-86)	51:43	35 duodonal ulcer 6 gastric ulcer 2 duodonal ulcer + gastric ulcer 51 No ulceration
94 uninfected	51.8(20-89)	57:37	No ulceration

4.3.2 Bacterial lysate preparation

H. pylori strains 60190 and Tx30a graciously provided by Dr Karen Robinson towards this experiment were prepared as follows: Briefly, *H. pylori* strains 60190 and Tx30a were cultured on blood agar plates in duplicate (Oxoid) and incubated in microaerophilic incubator, MACS VA500 microaerophilic workstation (DW Scientific) at 37 °C in a 10% O_2 and 5% CO_2 for 24 hours. The colonies were scraped off from the plates and resuspended

in ice-cold sterile phosphate-buffered saline (PBS) (Oxoid). The suspended bacterial cells were lysed on ice using the Soniprep 150 sonicator (SANYO, Watford, UK) at 4 x 10 seconds bursts at an amplitude of 10 microns with 30 minutes break time in between each sonication process. The concentration of the protein contents was measured using a BCA (Bicinchoninic Acid) Protein Assay and the concentrations were adjusted to a working concentration of 100 μg/ml.

4.3.3 Protein Sample preparation

The 3 protein samples (rKatA, rGGT and rPPT) prepared in Chapter 3, rVacA(s1m1i1 type), and lysates of two strains of *H. pylori* 60190 and Tx30a, were adjusted to the concentration of 100 μg/ml. 10 μl of each of the samples were aliquoted into a 96 well V-bottomed plate (Eppendorf) in quadruplicate. *Candida albicans* lysate was included as positive control as it is one of the commonest infection found in human (Hernday et al., 2010, Hernday et al., 2013) and landing light (2 μl/ml) was used as the edge marker on the slide.

4.3.4 Spotting of slide surface- contact printing

To print even protein spots on the surface of flat glass slides, a choice of the most suitable slide surface was made between amino-silane and epoxy-silane. The 8 headed solid pins were used, in a matrix of 2 columns and 4 rows, to print 16 pads, 2 columns by 8 rows on each of the 25 x 75 mm amino-silane or epoxy-silane activated glass slides which had been prepared in house. Briefly, the 96 well V-bottom PCR plates (Eppendorf) were placed in the plate

compartment of the BioRobotics Microgrid II microarray machine, the microspot solid pins were used to make double spotting to each array feature in a layout shown in Fig 4.1 at the humidity of 71-75 %. After spotting, the slides were removed and stored under vacuum until required.

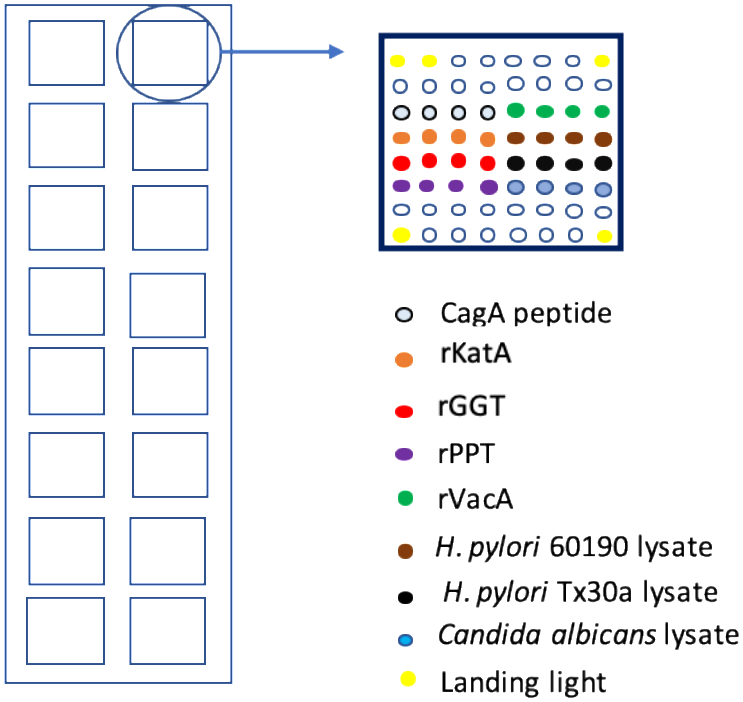

Figure 4.1. The design of the protein array. All of the 4 recombinant proteins (rKat, rGGT, rPPT and rVacA), a CagA peptide, 2 *H. pylori* whole cell lysates (strains 60190 and Tx30a) and 1 *Candida albicans* lysate (positive control) were spotted on each of the 16 fields on the slide while the empty spots were the negative controls. The landing light was used to mark the edges of the slide.

4.3.5 Array processing

The microwell gasket was assembled on the spotted glass slides, to allow testing of plasma samples from up to 16 patients. The surface of each slide was blocked with 240 µl of 3 % BSA, overnight at 4 °C. Following this, the microwells on the glass slides were washed sequentially for 3 x 1 min with PBS-Tween (0.05 % Tween 20 in phosphate-buffered saline) and 100 µl each of 1:100 dilutions of the plasma samples in LowCross-Buffer® (Candor) were added to the wells. Diluent only was added to one of the wells, a negative control. The slides were incubated on a shaker for 1 hour and then washed 3 x 1 min with PBS-Tween.

The slides were then probed with 100 µl of 1:10000 dilution of anti-human IgG-IR680 detection antibody (LI-COR Bioscience) diluted in LowCross-Buffer® (Candor) and incubated for 45 min on a shaker at room temperature. The detection antibodies were washed 5 x 1 min with PBS-Tween, rinsed with distilled water and then spun to dry using a microarray slide spinner (Labnet).

4.3.6 Fluorescence scanning

After staining the slides were scanned with a fluorescence imaging system LI-COR Odyssey SA (LI-COR Bioscience) at a resolution of 20 µm, focus of 3.1 nm and intensity of 11.5. The GenePix® ProMicroarray Image Software package was used to acquire the data. To determine the fluorescence signal for each antigen-antibody reaction, the average of the fluorescence signals for

the replicates were obtained. Furthermore, the signal-to-noise ratio was determined from the test samples and background control tests.

4.3.7 Data Analysis

The signals obtained from the reaction of plasma samples to immobilized antigens were compared using a Kruskal-Wallis test on GraphPad prism 8 software. A p value of less than 0.05 was considered statistically significant. Pearson's Statistical correlations were analysed using Minitab 7 software.

4.4 HBJM Results

4.4.1 Influence of slide surface on the protein array

CagA peptide, recombinant proteins VacA, KatA, GGT PPT and lysates of *H. pylori* strains 60190 and Tx30a were spotted onto two different surface chemistries and then the biochemical activities were evaluated. Aminosilane coated glass slides did not allow the sensitive detection of these proteins using serum samples from one *H. pylori*-posit

ive patient and *H. pylori*-negative patient (Figure 4.2 A). In contrast, the assays performed much better on epoxy silane coated slides, where there were brighter fluorescent signals (Figure 4.2 B). Hence, the epoxy silane coated surface was chosen for immobilising the antigens.

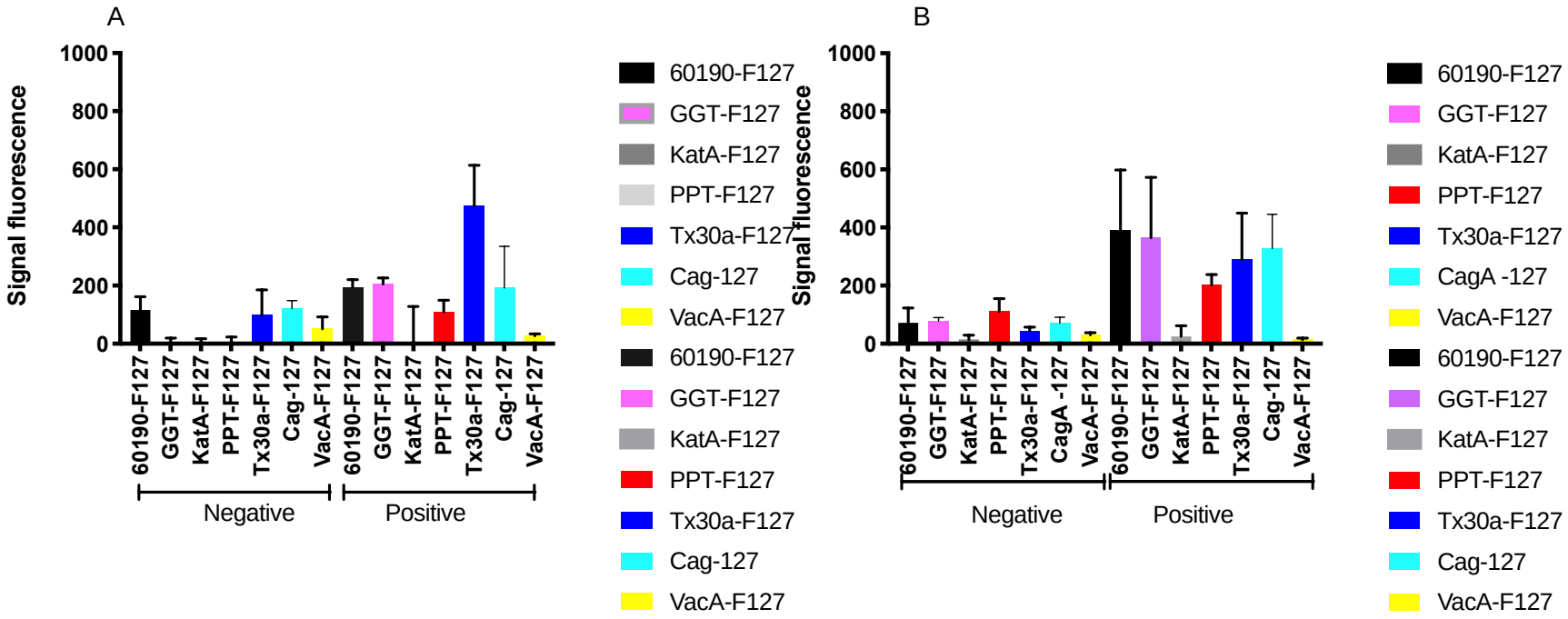

Figure 4.2. Evaluation of suitability of aminosilane and epoxysilane surface coatingsfor the slide for the protein microarrays. The proteins rVacA, rKatA, rGGT rPPT and the cell lysate (H. pylori 60190 and Tx30a) were spotted on an Aminosilane and epoxy-silane coated glass slide using BioRobotics Microgrid II microarray machine with solid microspot pins. The lysate and purified protein spots were blocked and assayed with a infected (confirmed H. pylori positive) and an uninfected (confirmed H. pylori negative) patients' serums and probed anti-human IgG-IR680 detection antibody (LI-COR Bioscience). The fluorescence signal readout was carried out using a fluorescence imaging system LI-COR Odyssey SA (LI-COR). The data were analysed on GraphPad Prism 8 software. (A). Result on the aminosilane coated slide. (B). Result on theepoxy-silane

4.4.2 Spotting of the proteins, CagA, VacA, KatA, GGT and PPT and H. pylori 60190 and Tx30a lysates

To analyse the serum antibodies against the recombinant proteins VacA, KatA, GGT and PPT, together with the two lysates of the *H*. *pylori* strains, 60190 and Tx30a, and CagA peptide, the concentrations of the molecules were adjusted to 100 μg/ml. A whole-cell lysate of the common yeast *Candida albicans* was included as a positive control antigen that the vast majority of individuals are exposed to. These were spotted on epoxysilane coated glass slide using a BioRobotics Microgrid II microarray machine with microspot solid pins. The spotted slides were incubated with serum samples from *H. pylori*-positive and *H. pylori*-negative patients, followed by anti-human IgG-IR680 conjugate and signals were visualised using a fluorescence imaging system. As shown in Figure 4.3 A after incubation with *H. pylori*-positive serum, fluorescence was detectable for almost all antigens spotted on the coated slide. In Figure 4.3 B, however, when slides were incubated with *H*. *pylori*-negative serum fluorescence was visible for only the positive control.

A B

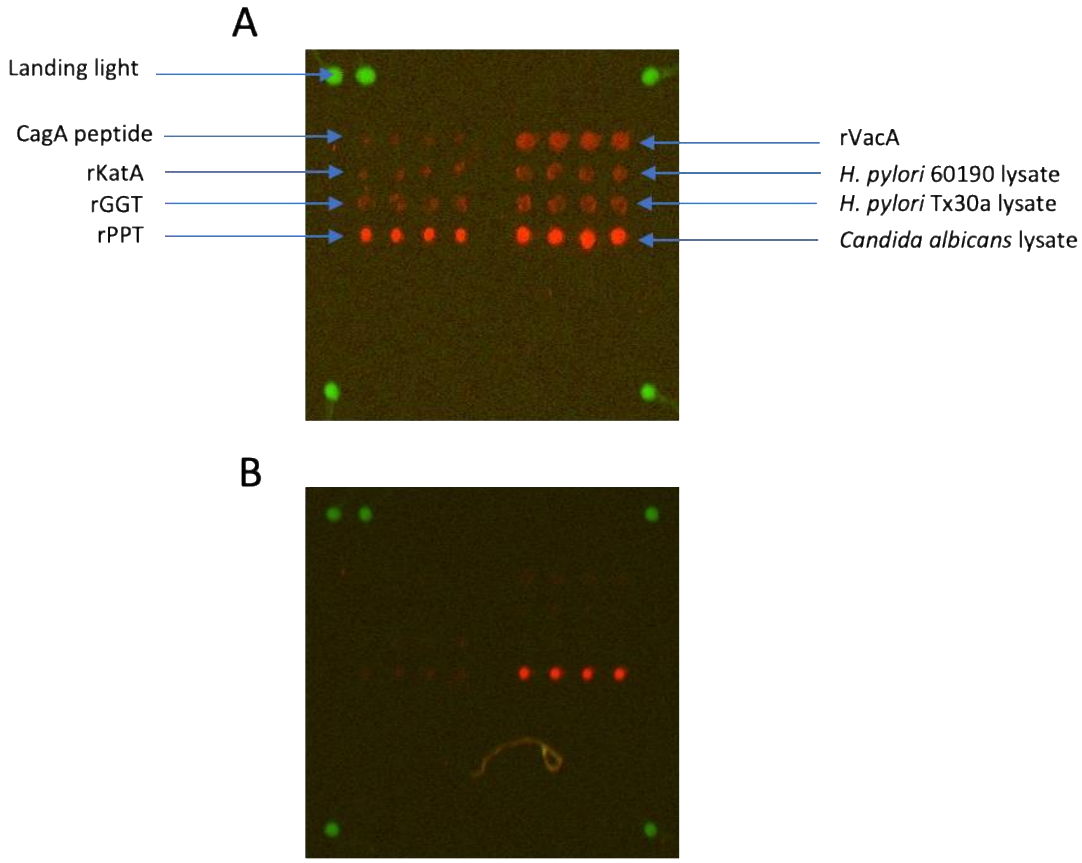

Figure 4.3. . Representative fluorescence image obtained from the protein array spotted with 100 □g/ml antigens**,** incubated with serum from patients, probed with anti-human IgG-IR680 detection antibody and then visualised using the LI-COR Odyssey SA (LI-COR) fluorescence imaging system**. A**. depicts fluorescence signals following incubation with serum from a typical H. pylori-positive patient. **B**. depicts the fluorescence signals after applying serum from an H. pylori-negative patient, where reactivity was detected only to the control Candida albicans spots.

4.4.3 Analysis of serum antibodies against *H. pylori* 60190 and Tx30a lysates in *H. pylori*-positive and *H. pylori*-negative patients

The first step was to ascertain the level reactivity of serum antibodies in *H. pylori*-positive and negative patients to two strains (60190 and Tx30a). The 60190 strain is CagA-positive and expresses the active s1/i1/m1 type of VacA, whilst the Tx30a strain is CagA-negative and expresses the least active s2/i2/m2 VacA type (Camorlinga-Ponce et al., 2004, Braga et al., 2014). Both strains are known to express GGT, KatA and PPT activities. To investigate the correlation between antibodies against *H. pylori* strain 60190 antigens and *H. pylori* strain Tx30a antigen, the lysates strains were spotted on epoxysilane coated glass slides and incubated with the 1:200 dilution of 94 *H. pylori*-positive and 94 *H. pylori*-negative patient's serum and further probed with the anti-human IgG-IR680 detection antibody. The fluorescence signals were measured using the fluorescence imaging system.

The data presented in Figure 4.4 show significant differences between sera from *H. pylori*-positive and -negative patients. This seems to suggest that antigens present in both lysates induce an antibody response that reacts with both types of bacteria. Analysis with a Kruskal-Wallis test showed 6.95-($p<0.0001$) and 3.93-fold ($p=0.0006$) higher concentrations of antibodies against *H. pylori* 60190 and *H. pylori* Tx30a lysates respectively, in sera from *H. pylori*-positive compared to *H. pylori*-negative patients.

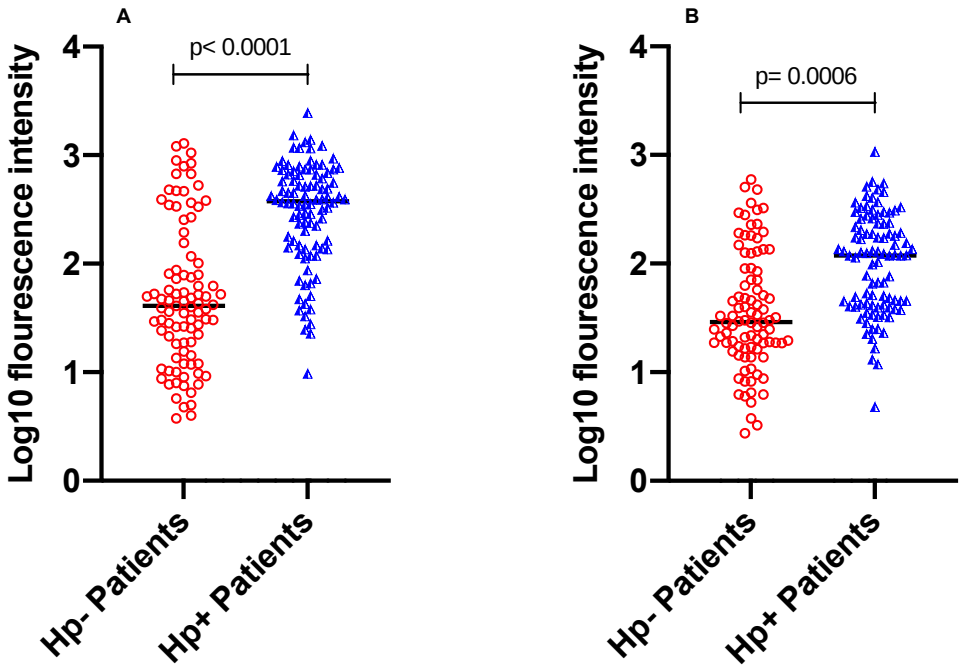

Figure 4.4. Seroprevalence of serum antibodies against **H. pylori** strains 60190 and Tx30a antigens **H. pylori**-infected and uninfected patients. The cell lysate H. pylori 60190 and Tx30a were spotted on an epoxy-silane coated glass slide using BioRobotics Microgrid II microarray machine with solid microspot pins. The lysate spots were assayed with a total of 188 (94 infected and 94 uninfected) patients' serums and probed anti-human IgG-IR680 detection antibody (LI-COR Bioscience). The fluorescence signal readout was carried out using a fluorescence imaging system LI-COR Odyssey SA (LI-COR). The horizontal bars represent median responses and statistical significance was calculated using the Kruskal-Wallis test on GraphPad Prism 8 software. (A). H. pylori 60190 lysate. (B). H. pylori Tx30a lysate.

Further investigation with Pearson's Statistical correlations analysis of the fluorescence signal of the *H. pylori*-positive for the lysates *H. pylori* 60190 and Tx30a strains. As shown in Figure 4.5 there was a correlation between the antibodies against the antigens present in lysates of both strains (P< 0.0001 r= 0.813). Which further suggests that, although Tx30a strains lacks CagA and express s2i2m2 VacA campare to 60190 strains which are cagA positive and express s1i1m1 VacA, there are similarities in the antigen responsible for the induction of the *H. pylori*-specific antibodies.

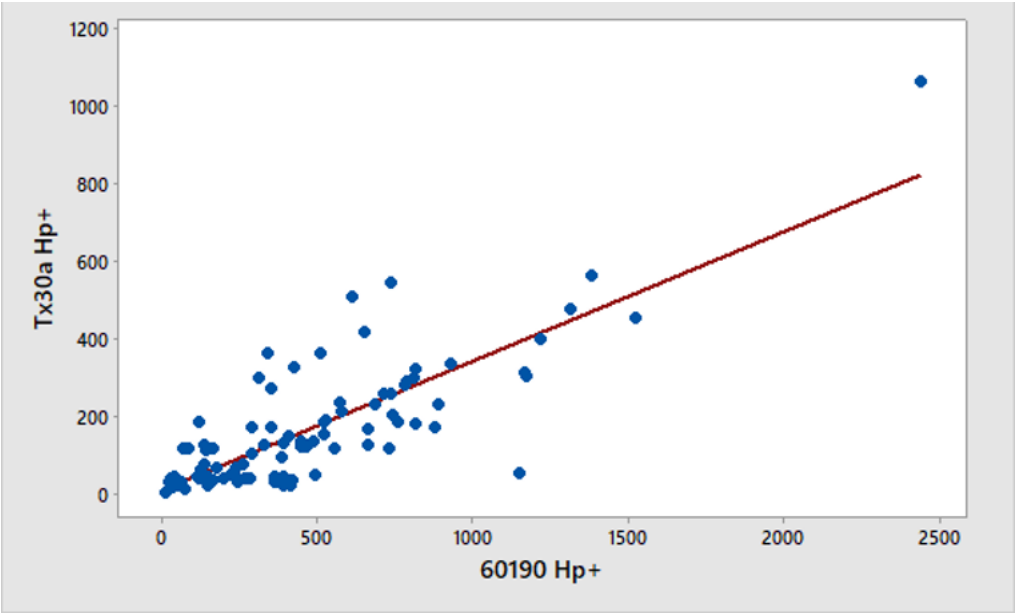

Figure 4.5. Positive Pearson's rank correlation between relative seropositivity of two strains of **H. pylori** 60190 and Tx30a antigens in **H. pylori**-infected

persons. The cell lysate H. pylori 60190 and Tx30a were spotted on an epoxy-silane coated glass slide using BioRobotics Microgrid II microarray machine with solid microspot pin. The lysate spots were assayed with a total of 94 infected patients' serums and probed anti-human IgG-IR680 detection antibody (LI-COR Bioscience). The fluorescence signal readout was carried out using a fluorescence imaging system LI-COR Odyssey SA (LI-COR). Pearson's Statistical correlations were analysed using Minitab 7 software. ($p < 0.0001$ $r = 0.813$).

4.4.4 Evaluation of serum antibodies against *H. pylori* molecules, CagA, VacA, GGT, KatA and PPT

In the previous section, it was shown that there was an increase in antibodies against *H. pylori* lysates from strains 60190 and Tx30a in the serum of *H. pylori*-positive patients. It was hypothesized that bacterial factors such as CagA, VacA, GGT, KatA and PPT could be major immunogens driving the antibody response to infection. Both of the strains express GGT, KatA and PPT, therefore their differences in CagA and VacA expression could be responsible for the differences in the levels of antibody signals detected.

This study aimed to determine the contribution of five important *H. pylori* factors, CagA, VacA, GGT, KatA and PPT, to the antibody response of *H. pylori*-positive patients and to investigate whether strong antibodies response from *H. pylori*-positive factors correlates with the pathology of the disease.

As shown on the Fgure 4.6 A, B, C, D and E, the *H. pylor*i infected individuals had significantly higher level of antibodies for CagA ($P<0.0001$), VacA (0.0289), KatA ($P<0.0001$), GGT ($P=0.0003$)and PPT ($P<0.0001$) than uninfected individuals.

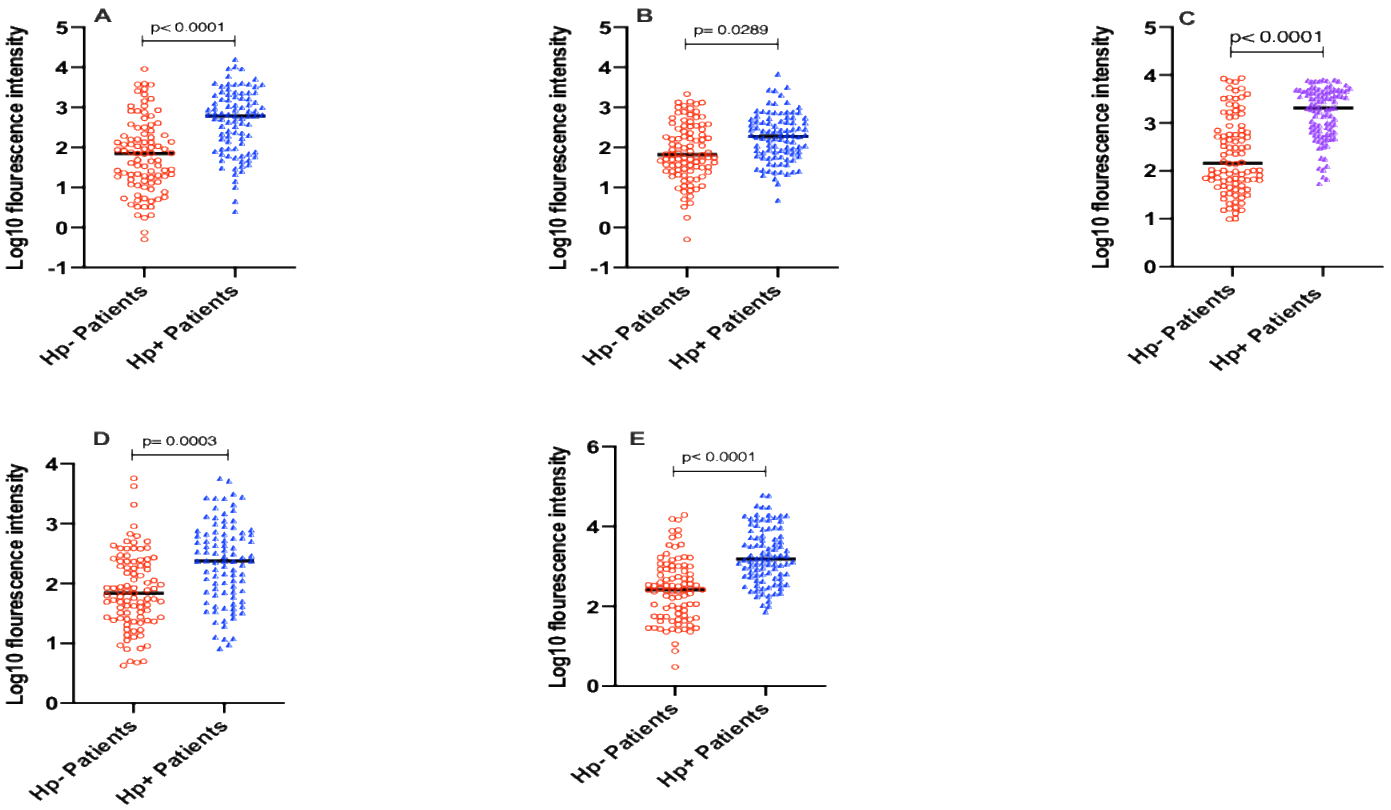

Figure 4.6. Serological responses to H. pylori CagA, VacA, KatA, GGT and PPT in H. pylori infected (Hp+) and uninfected (Hp-) patients. CagA peptide, and purified recombinant VacA, KatA, GGT and PPT proteins were spotted on epoxy-silane coated glass slides using BioRobotics Microgrid II microarray machine with solid microspot pin. The spots were probed with a total of 188 (94 infected and 94 uninfected) serum samples followed by anti-human IgG-IR680 detection antibody (LI-COR Bioscience). The fluorescence signal was detected using a

fluorescence imaging system LI-COR Odyssey SA (LI-COR). The horizontal bars represent median response levels and statistical significance was calculated using a Kruskal-Wallis test on GraphPad Prism 8 software. (A)CagA (B)VacA (C)KatA (D) GGT (E)PPT.

4.4.5 Correlations among Tx30a Hp+, 60190 Hp+, PPT Hp+, VacA Hp+, KatA Hp+, CagA Hp+, GGT Hp

To look at the association among and between the response to CagA, VacA, KatA, GGT and PPT, Pearson's statistical correlation was used to analyse the data from the *H. pylori*-positive patients. As shown in **Table 4.2** Only KatA/PPT and PPT/GGT data provided significant correlations, with r= 0.606 ($p<0.0001$) and r= 0.454 ($p<0.0001$) respectively. There were no correlations between CagA and any of the other antigens. Anti-VacA responses correlated weakly with those against PPT ($r=0.377$; $p<0.0001$) and KatA (r= 0.282; $p=0.006$).

Table 4.2 Pearson correlations in the data between the serological responses of H. pylori-positive patients to Tx30a Hp+, 60190 Hp+, PPT Hp+, VacA Hp+, KatA Hp+, CagA Hp+, GGT Hp

	Tx30a Hp+	60190 Hp+	PPT Hp+	VacA Hp+	KatA Hp+	CagA Hp+
60190 Hp+	0.739 0.000					
PPT Hp+	0.364 0.000	0.523 0.000				
VacA Hp+	0.143 0.170	0.379 0.000	0.377 0.000			
KatA Hp+	0.368 0.000	0.412 0.000	0.606 0.000	0.282 0.006		
CagA Hp+	0.076 0.469	0.136 0.192	-0.021 0.842	-0.035 0.736	0.066 0.528	
GGT Hp+	0.483 0.000	0.406 0.000	0.454 0.000	0.107 0.303	0.375 0.000	-0.001 0.993

4.4.6 Association between disease condition Cag and VacA type

Previously, the group had used both PCR and serology to characterised the *CagA* gene and VacA type in the the positive-*H. pylori* individuals. As shown in Table 4.3, out the 94 *H. pylori*-positive subjects involved in the studies, 35 has duodenal ulcers (DU), 6 were classified as gastric ulcer (GU), 2 has both duodenal ulcer and gastric ulcer (DU + GU) while 51 showed ulceration. The result is represented in the table below shows that $CagA^{+}$ /$VacA^{s1/i1}$ contain 50 (94), $CagA^{-}$ /$VacA^{s2/i2}$ was 21(94), $CagA^{+}$ /$VacA^{s2/i2}$ 15(94) and $CagA^{-}$/$VacA^{s1/i1}$ 8(94).

of the total *H. pylori*-positive subjects of which

Table 4.3 The association between duodenal ulcer and gastric ulcer and CagA and VacA status.

CagA and VacA type	**$CagA^{+}$ /$VacA^{s1/i1}$**	**$CagA^{-}$ /$VacA^{s2/i2}$**	**$CagA^{+}$ /$VacA^{s2/i2}$**	**$CagA^{-}$/$VacA^{s1/i1}$**	**Total**
No of Patient	50	21	15	8	94
Duodonal ulcer	20 (57.1%)	7(20%)	4(11.4%)	4(11.4%)	35
gastric ulcer	5 (83.3%)	0	1(16.7%)	0	6

Duodonal ulcer + gastric ulcer	0	2(100%)	0	0	2
% of total No of patient	53.3	22.3	15.9	8.5	

1

4.5 Discussion

From the results obtained this study, it is clear that immune response to *H. pylori* could easily be observed in distant blood peripheral other than the local areas of the gastric. This could be done by observing antibodies against one virulence factor as the quest for biomarkers against *H. pylori*-related diseases increases. The outcome of the investigation is in agreement other reports on the serum antibody prevalence of the both suspected *H. pylori* effector immune molecules and regulatory immune molecules (Gao et al., 2009b, Camargo et al., 2015, Mera et al., 2005, Atanassov et al., 2002). Therefore, this result could pave the way to the development of an accurate non-invasive diagnostic approach to *H. pylori*-related disease especially peptic ulcer. Besides, although some of the virulence factors are analogous of certain molecules in human the result in this chapter shows they are quite immunogenic and could serve as a vaccine candidate.

4.5.1 Influence of amino silane and epoxy silane coated slide glass slide surface on the protein array

Although glass is inert nonetheless it makes a great choice for any experiment involving optical sensors, since it is transparent and has little inherent fluorescence. Therefore, to make the surface suitable for a number biochemical investigation such as protein microarray, the surface is generally functionalised with a number chemicals, which should preserve the biological

activities of the protein, the protein natural conformation and the protein integrity while preserving the transparency of the glass (Jonkheijm et al., 2008). Protein is known to have exceedingly heterogeneous and complex structure hence, the need for a suitable surface for almost every investigation involving protein array. This surface should be able to fulfil a number of criteria to be deemed satisfactory, these criteria includes; maintaining the spotted protein activities, guaranteed stability for the spotted protein for a long time, ability to bind strongly with proteins, high signal to noise ratios and spotted protein retention (Jonkheijm et al., 2008, Robinson et al., 2002, Seurynck-Servoss et al., 2007a).

Aminosilane coated surface utilise physical adsorption as its immobilisation strategy with no covalent attachment, unlike epoxysilane coated surfaces. Physical adsorption involves a situation where the protein interacts with slide surface through hydrogen bonding, hydrophobic interaction or ionic interactions. This probably leaves the spotted proteins or blocking agent vulnerable to exchange with assay reagent (Ball et al., 1994). This could lead to poor sensitivity and further background noise. Seurynck-Servoss et al shows that although aminosilane require shorter immobilisation time of 1 hour than epoxysilane, aminosilane generates the lower signal intensity which could be due to lower density of the protein use, which is antibody (Seurynck-Servoss et al., 2007b), this is in agreement with our finding using different proteins, we obtained a far lower signal intensity on the aminosilane coated surface compare to the epoxysilane surface.

The physical adsorption interaction of aminosilane, on the other hand, could be advantageous in a situation where the activities of the spotted protein are affected by the covalent interaction, for instance in the of an antibody (Seurynck-Servoss et al., 2007a). For the case of the protein used in our experiment, it is unlikely that covalent bonding would affect the epitopes of the protein since unlike antibodies which have limited antigen-region and are easily denatured by a change in structure brought about by multiple covalent bonding. Therefore, since epoxysilane allows covalent stable binding through the primary amine and at the same time due not affect the protein activity it is believed to be the most suitable in terms of meeting up with at least two important criteria such as immobilization efficiency and protein function.

Chapter 5

Catalases, evolution from *Helicobacter pylori* to Humans

5.1 Introduction

Hydrogen peroxide (H_2O_2) is produced in living organisms as a by-product of aerobic metabolism and is the commonest found reactive oxygen species in all living species. Disproportionate H_2O_2 and its products are capable of causing damage to proteins, DNA, RNA, lipids and can trigger even apoptosis. Hence, the need for rapid elimination in all levels of living organisms became essential for survival (Cabiscol et al., 2000, Kirkman and Gaetani, 2007). On the other hand, in immune responses, H_2O_2 has evolved to play roles in signal transduction essential for the inflammatory response, immune cell activation, cell division and apoptosis (Veal et al., 2007).

One of the ways most living organisms use for elimination of this toxic molecules and similar molecule such as small organic peroxides is through enzymes namely hydroperoxides which act on the peroxides to release oxygen in a process known as dismutation (Zamocky et al., 2008).

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Hydroperoxidase has evolved independently of a number of protein families that are capable of performing the function of removing H_2O_2 and/or reducing it to an acceptable concentration useful for cellular signalling. These proteins are divided into three groups namely, typical catalases, catalase-peroxidase and Manganese containing catalases. The first two could be

classified as heme-containing while the latter is known as a non-heme containing catalase (Passardi et al., 2007).

Among these three, a typical catalase is one the most studied enzyme and it is the largest group which is found in a wide range of organisms from prokaryotes to eukaryotes (Zamocky and Koller, 1999). Among enzymes, catalase is known is to be one of the enzymes with the highest turnovers shown by the ability of 1 molecule of catalase to decompose more than 1 million molecules of hydrogen peroxide in a second (Goth and Nagy, 2012). In a phylogenetic analyses typical catalases are divided into three clades (Klotz and Loewen, 2003). Clade 1 is made up of bacteria, algae and plant catalases with a small subunit size of 55-69 kDa and using heme *b* as a prosthetic group. Clade 2 is contains bacterial and fungal enzymes with a larger subunit size of 75-84 kDa with heme *d* as the prosthetic group and a flavodoxin-like domain. Finally, clade 3 contains the most diverse group from archaebacteria, fungi, protists, plants and animals. This clade is also a very important group since it contains human catalases as well. Contrariwise it contains the small-subunit sizes of 43-75 kDa with heme *b* and NADPH as a cofactor.

Catalase is a highly conserved protein in both bacteria and mammal (Alyamani et al., 2007), it is found in the peroxisomes and cytosol of eukaryote and prokaryotes respectively. Its existence in peroxisomes is appropriate since peroxisomes are considered to be protective compartment with detoxifying enzymes such as catalase (Williams et al., 2012). Nevertheless, the presence of

catalase has been detected in some eukaryotic cytosol, as with fungi, *Saccharomyces cerevisiae (*Taub et al*.,* 1999*).*

H. pylori catalase (KatA) has been recognised as one of the most important virulence factors that aid the survival of the bacteria. Harris et al showed that wildtype SS1 strain of *H. pylori* consistently colonised the gut of C57/BL6 mice at a significantly higher level compared to the mutant deficient of KatA and KatA-associated protein (KapA). They showed it is not essential for initial colonisation but it was demonstrated to be essential for the maintenance of longer colonisation (Harris et al., 2003). Interestingly, recent studies. by Richter et al, described the moonlighting of KatA, in which they demonstrated KatA protection of *H. pylori* from complement-mediated killing through a vitronectin-dependent manner (Richter et al., 2016).

5.2 Hypothesis

The major role of catalase is the removal of hydrogen peroxide from living organisms thereby protecting it from the consequences of a high concentration of H_2O_2 (Zamocky and Koller, 1999). Unfortunately, some people are without this essential protection due to lack of this enzyme, in a condition called acatalasemia. This is a rare congenital abnormality typified by the lack of the catalase enzyme. This was first described by Takahara in 1952 which showed the development of progressive oral gangrene among people who are catalase deficient on infection with bacteria capable of inducing hydrogen peroxide (Takahara, 1952). In the blood, deficiency of the catalase enzyme could lead to aggregation of erythrocytes due to high hydrogen peroxide generation and subsequent development of disease due to blood flow obstruction (Masuoka et al., 2006). Apart from this development of oral gangrene, Goth et al in their work demonstrated the link between acatalasemia and diabetes mellitus while working with some Hungarian acatalasemia patients. They inferred that the inability of catalase to protect the pancreatic β-cell from the destruction of hydrogen peroxide (Goth and Nagy, 2012, Goth, 2001). Others reported the generation of reactive oxygen species (ROS) by hyperglycaemia which further causes cell damages (Hunt et al., 1988, Jaganjac et al., 2013).

H. pylori KatA is a 505 amino acid tetrameric enzyme with heme prosthetic, KatA makes approximately 1% of the total protein expressed by *H. pylori (*Alyamani et al*.,* 2007*).* Preliminary protein bioinformatics analysis of *H. pylori* KatA shows it shares 78-85% amino acid sequence identity with that from other *Helicobacter* species, about 65% identity with other bacterial catalases and it is believed it could share high amino acid sequence. It differs from other bacterial catalase proteins by having an additional 17 amino acid sequence at the C-terminus, containing a poly-lysine motif. The combination of shared homology with human catalase and the additional poly-lysine motif, is believed to impart different functional characteristics on *H. pylori* KatA

We hypothesize that *H. pylori* catalase (KatA) could offer protection similar to that of human catalase. To test this hypothesis, we used the following objective

Objective

- To use bioinformatics to evaluate the conservation of catalase among different *Helicobacter* species and between *H. pylori* and other organisms.
- To evaluate the cross-reactivity of anti-human catalase antibodies with catalases from different organisms
- To evaluate the presence of antibodies against selected catalases in the blood *H. pylori*-infected patients

5.3 Materials and Methods

5.3.1 Conservation of *Helicobacter pylori* Catalase among other *Helicobacter* species

The conservation of the catalase (KatA) sequence among 9 *Helicobacter* species was evaluated by a sequence-based method. This includes multiple sequence alignment using the Clustal Omega program on the UniProtKB through the ExPASy SIB Bioinformatics Resource Portal. Two *H. pylori* strains: *Helicobacter pylori* (strain ATCC 700392 / 26695) and *Helicobacter pylori* (strain J99 / ATCC 700824) were first aligned to ascertain the degree of similarity. Then using *Helicobacter pylori* (strain ATCC 700392 / 26695) as a reference strain (which is also the source of the catalase I have used throughout my PhD experiment); the catalases of *Helicobacter felis* (strain ATCC 49179 / NCTC 12436 / CS1), *Helicobacter cinaedi* CCUG 18818 = ATCC BAA-847, *Helicobacter hepaticus* (strain ATCC 51449 / 3B1), *Helicobacter suis* HS5, *Helicobacter muridarum (*ATCC 49282)*, Helicobacter cholecystus* ATCC 700242 and *Helicobacter canadensis* MIT 98-5491 were analysed.

The percentage of similarities and identical positions were first evaluated from the alignments alongside the active sites and metal-binding sequence.

5.3.1.1 Conservation of *Helicobacter pylori* Catalase among other *Helicobacter* species and other organisms

Following the protein bioinformatic analysis of *Helicobacter* spp., I went further to look at the analysis of above-named *Helicobacter* spp. in addition to the human catalase, *Corynebacterium glutamicum* catalase and *Aspergillus niger*. Again, the percentage similarities and identical position were evaluated in comparison *H. pylori* catalase. Furthermore, the catalases' active sites and metal-binding sequence were determined. Then from the aligned sequences the domain, helix, Beta strand and chain were assessed.

5.3.1.2 Structural Bioinformatics

Likewise, to enhance the understanding of the structure *H. pylori* KatA, human catalase, *Corynebacterium glutamicum* and *Aspergillus niger* catalase, and for better comparison, 3D structural bioinformatics was carried using the Phyre2 with the FASTA sequence from the NCBI protein database; *H. pylori* KatA (accession number: TLR88905.1), human catalase (accession number: NP_001743.1), *Corynebacterium glutamicum* (accession number: AIK83984.1) and *Aspergillus niger* (accession number: AAA68206.1)catalase. The 3D structures were captured and evaluated for differences, then the secondary structure and disorder prediction analysis was evaluated.

5.3.1.3 Experimental validation of conservation catalase among organisms

5.3.1.3.1 Catalase selection criteria

To assess the conservation we use the cross-reactivity of anti-catalase antibodies among different catalases from different organisms, commercially available catalase from human erythrocytes (Sigma-Aldrich) (EC 1.11.1.6) concentration ≤10 mg/mL, catalase from *Aspergillus niger* (Sigma-Aldrich) (EC 1.11.1.6), concentration ≥4,000 units/mg protein, catalase from *Corynebacterium glutamicum* solution, deep brown, concentration ≥500000 U/mL were purchased and *H. pylori* catalase produced in our laboratory representing human, fungi, gram-positive and gram-negative, respectively.

5.3.1.3.2 *H. pylori*-positive and *H. pylori*-negative Serum sample preparation

From the blood samples collected in **section 4.1** *H. pylori*-positive and *H. pylori*-negative serum samples and were diluted by 1:200 in PBS-Tween (0.05% Tween™ 20, 0.01 M phosphate buffer with pH 7.4 at 25°C).

5.3.1.4 Characterisation of the purified protein

5.3.1.4.1 BCA protein assay, Catalase activity, SDS-PAGE and PageBlue™ Protein Staining

The concentration of the respective catalases was measured using Pierce™ BCA protein assay kit (Thermo Scientific) following the manufacturer's

instructions. Then using 3% hydrogen peroxide the catalase activities were assessed following the procedure previously used in **section 2.3.1.7.3** Subsequently, SDS-PAGE was to evaluate the purity of the commercially available catalases, following the technique previously described **section 2.3.1.7.1**

5.3.1.4.2 Further purification of the *Aspergillus niger*

Because all the catalase appeared to be clean on the gel photo except the *Aspergillus niger* catalase. Consequently, the *Aspergillus niger* suspension was further purified by size exclusion chromatography (SEC) as previously described in **Section 2.3.1.6.** The eluted protein was assessed for purity using SDS-PAGE as described in **section 2.3.1.7.1.** The concentration of purified proteins was determined using Pierce™ BCA protein assay kit (Thermo Scientific) following the manufacturer's instructions.

5.3.1.5 Western blot analysis of catalases Anti-human catalase antibody

To test whether antibodies against human catalase also react well with catalases from *H. pylori* and other microbes. SDS-PAGE previously described in **section 2.3.1.7.1** was to resolve to the proteins, Human catalase, *Aspergillus niger* catalase, *Corynebacterium glutamicum* catalase and *H. pylori* recombinant catalase. The resolved proteins were transferred to the nitrocellulose paper following the procedure described in **section 2.3.1.7.2.**

According to the western blot procedure described in **section 2.3.1.7.2,** the proteins on the nitrocellulose paper were probed with anti-human catalase

antibody (rabbit polyclonal antibodies)(Sigma-Aldrich). Then secondary antibody was anti-rabbit IgG-horseradish peroxide conjugate (Sigma) visualise by chemiluminescence readout and the bands were compared with the prestained protein marker to ensure it is at the expected molecular weight.

5.3.1.6 Western blot evaluation of *H. pylori*-positive and negative patients on human, *Aspergillus niger, Corynebacterium glutamicum* and *Helicobacter pylori* catalase

The human, *Aspergillus niger*, *Corynebacterium glutamicum* and *Helicobacter pylori* catalase were resolved on the SDS-PAGE gel following the procedure described in **section 2.3.1.7.1** using a double pockets SDS-PAGE combs (Figure 5.1.) a uniform lane of the resolved protein.

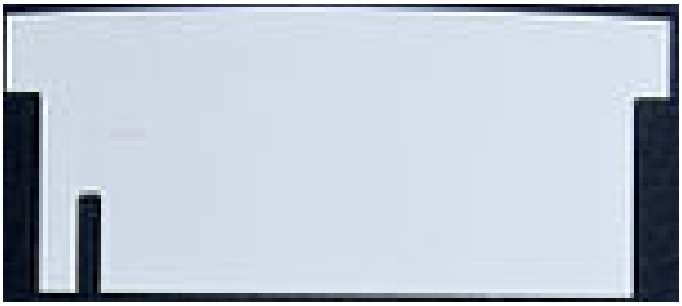

Figure 5.1. Figure 5.1 A double pocket comb used in creating a single well.

The proteins on the gel were transferred to a nitrocellulose paper following the procedure described above. The nitrocellulose paper

upon which the proteins were transferred to was cut into strips of 0.5 cm each. Two strips selected from each of the respective catalases were marked and placed one each on two different containers labelled Positive and Negative and was blocked for 1 hour on a shaker at room temperature with blocking solution described in **section 2.4.2.2.** A total 20 patient samples were used in this study, 10 confirm *H. pylori*-positive and 10 *H. pylori*-negative sample, following the blocking 5 ml of the of 1:200 dilution of the serum sample prepared in **section……** was added and incubated overnight at 4 °C on the shaker. At the end of the incubation, the blot was washed in PBS-Tween 3 x with 5 minutes shaking incubation in between. The blot was placed in a secondary antibody, anti-human IgG-horseradish peroxide conjugate (Sigma) and incubated on a shaker for 1 hour. Successively, the strips were washed 5 x in PBS-Tween with an interval of 5 minutes and 1 x in PBS.

The chemiluminescent readout was achieved following the previous procedure described in **section 2.3.1.7.2**

5.4 Result

5.4.1.1 Bioinformatic analysis of conservation among *Helicobacter* spp

Nine Helicobacter species were analysed using Clustal-Omega, with well-known human pathogens: *Helicobacter pylori* (strain ATCC 700392 / 26695). Additionally, *Helicobacter felis*; a bacterial species commonly isolated from mammals such as cat, dog and rabbits, although capable of causing human diseases (Lee et al., 1988), *Helicobacter cinaedi;* another well-known Helicobacter isolated from hamsters and monkeys which is also known to cause disease in immunocompromised individuals (Kawamura et al., 2014), *Helicobacter hepaticus;* this is a well-known mouse Helicobacter, shown to induce lower bowel inflammation(Fox et al., 2011), *Helicobacter suis; H. suis* is commonly isolated from pig and non-human primate, it is well studied *Helicobacter* sp capable of causing diseases similar to *H. pylori*, but unlike *H. pylori, H. suis* is notable for infecting parietal cells, causing degeneration of the cells (Joo et al., 2007), *Helicobacter muridarum,* known for its fastidious nature and first isolated from the murine mucosa (Lee et al., 1992), *Helicobacter cholecystus;* first described as the filamentous *Helicobacter* sp isolated from the gallbladder of a hamster, it is associated with the occurrence of cholangiofibrosis and centrilobular. pancreatitis(Franklin et al., 1996) and *Helicobacter canadensis;* this specie is primarily isolated from poultry and its products (Atabay et al., 1998), although isolation from barnacle, Canada geese

and from rodent faeces have been recorded (Waldenstrom et al., 2003), while it is not known to cause disease in humans infection cannot be ruled out (Inglis et al., 2006). The percentage identity between *H. pylori* and other *Helicobacter* spp used in this study ranged from 97% to 50% having an identical position of amino acid sequences ranges from 492 amino acid as found in *H. pylori* J99 to 255 amino acids found in *Helicobacter cholecystus* as shown in **Table 5.1**

The analysis of the showed that all the catalases share the same active site and metal-binding sequence, but interestingly only *H. pylori* have a beta-strand region, helix region and sequence are not consecutive (sequence conflict). *H. pylori* also do not possess domain sequence contrasting to the other *Helicobacter* species in the analysis which have domains throughout most their amino acid sequence.

Table 5.2 Sequence alignment summary of the 9 *Helicobacter* species showing the percentage identity and identical position with *Helicobacter pylori* 26695 strain.

Organism	Entry Number	Entry name	Amino acid Sequence	Identical position *WITH* *H. pylori* (strain ATCC 700392 / 26695)	% Identity *WITH* *H. pylori* (strain ATCC 700392 / 26695)
Helicobacter pylori **(strain ATCC 700392 / 26695)**	P77872	CATA_HELPY	505	-	-
Helicobacter pylori **(strain J99 / ATCC 700824)**	Q9ZKX5	CATA_HELPJ	505	492	97.426
Helicobacter felis **(strain ATCC 49179 / NCTC 12436 / CS1),**	E7AAY6	E7AAY6_HELFC	485	331	65.415
Helicobacter cinaedi **(CCUG 18818 = ATCC BAA-847)**	I7H4C3	I7H4C3_9HELI	481	311	61.584
Helicobacter hepaticus **(strain ATCC 51449 / 3B1),**	Q7VK49	Q7VK49_HELHP	478	298	59.01
Helicobacter suis **(HS5),**	E7G3S7	E7G3S7_9HELI	494	323	63.708
Helicobacter muridarum (**ATCC 49282),**	A0A099TYR4	A0A099TYR4_9HELI	482	312	61.782
Helicobacter cholecystus **ATCC 700242**	A0A3D8IWC8	A0A3D8IWC8_9HELI	468	255	50.296
Helicobacter canadensis **MIT 98-5491**	C5ZW34	C5ZW34_9HELI	474	271	53.452

5.4.1.2 Bioinformatic analysis of conservation among *Helicobacter* spp catalase, other microbes and human catalase

Next, the catalases of from other organisms were added to the analysis; a Gram-positive bacterium catalase from *Corynebacterium glutamicum (C. glutamicum),* a fungi catalase *Aspergillus niger (A. niger)* and human catalase. Briefly, *C. glutamicum* is a non-pathogenic non-spore-forming Gram-negative bacterium which is widely used in the industries for the production of industrial amino acids (Vertes et al., 2012) and it was named glutamicum because of its discovery as a producer of glutamate in the 1950s and was previously named *Micrococcus glutamicus* (Yang and Yang, 2017). *Aspergillus niger* is also another industrial important microbe, it is a filamentous fungus efficient in utilising low-cost substrates in the production of a large quantity of proteins, metabolite and organic acid (Meyer et al., 2015) and was popularly known for citric acid production(Ciriminna et al., 2017), although its infection is not common to compare to others species of Aspergillus such *A. fumigatus* and *A. flavus* there have been reported infections in humans (Person et al., 2010).

Again, the sequence alignment analysis of the 3 catalases from human, *C. glutamicum* and *A. niger* with other *H. pylori* and other *Helicobacter* spp showed that the active site and metal-binding sequence are well conserved across all the species (Appendix). Interestingly, there are so many similarities shared only between *H. pylori* catalase human catalase that is not shared with catalase of other *Helicobacter* spp and organisms. These include the possession

of a beta-strand, helix, turn and sequence conflict in its sequence and absence of a domain which is found in every other catalase

Interestingly, to support the similarity between *H. pylori* KatA and Human catalase, as shown in Table 5.3 there is about 48% amino acid sequence identity this is higher than fellow bacteria *Corynebacterium glutamicum* (42%) and the fungi *Aspergillus niger* (26%). This similarity could be as a result of coevolution of Human and *H. pylori* over a long period of time.

Table 5.3 Sequence alignment summary of the 9 *Helicobacter* species showing the percentage identity and identical position with *Helicobacter pylori* 26695 strain.

Organism	Accession Number	Entry name	Amino acid Sequence	Identical position *WITH H. pylori* (strain ATCC 700392 / 26695)	% Identity *WITH H. pylori* (strain ATCC 700392 / 26695)
***Helicobacter pylori* (strain ATCC 700392 / 26695)**	P77872	CATA_HELPY	505	-	-
Human (Homo sapiens) Catalase	P55303	CATA_HUMAN	527	259	48.411
***Corynebacterium glutamicum*. catalase**	A0A160PMU0	A0A160PMU0_CORGT	516	227	42.351
Aspergillus niger catalase	P55303	CATR_ASPNG	730	195	26.423

5.4.1.3 3D structure of the H. pylori KatA, human catalase, Corynebacterium glutamicum and Aspergillus niger

The 3D structure of the *H. pylori* KatA, human catalase, *C. glutamicum* and *A. niger* were obtained from the Phyre2 using the individual FASTA sequence from the NCBI database. The result as shown below (Figure 5.2). The four structures showed similarities in the basic structure but it is worthy to note when the secondary structures were analysed. The secondary structure and disorder prediction analysis of each of catalases revealed an interestingly close similarity between human catalase and *H. pylori* catalase having an equal percentage of both alpha-helix and beta-strand (31% and 16% respectively) Table 5.4 and Appendix (Figure E, F, G and H).

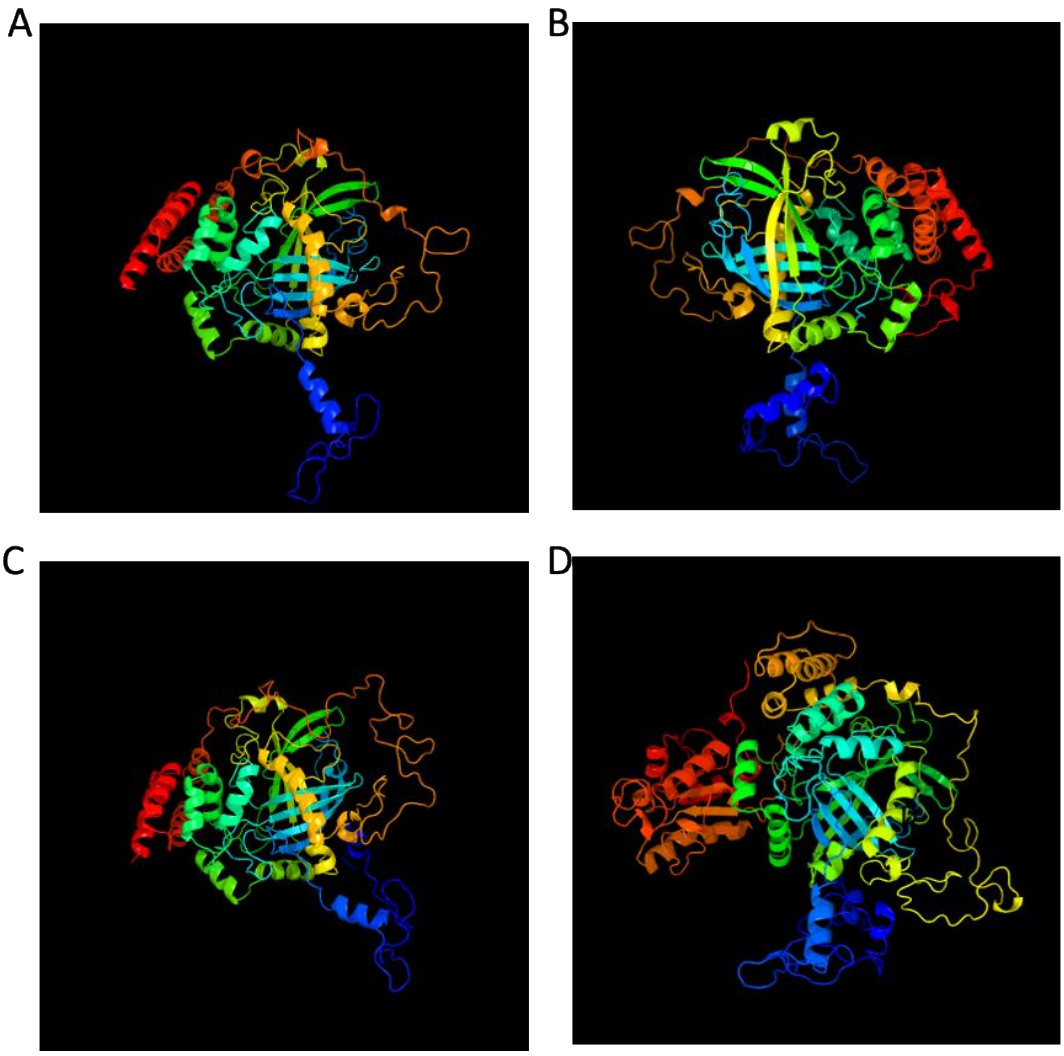

Figure 5.2. Phyre2 3D structural analysis of catalases (A)*H. pylori* KatA, (B)human catalase, (C) *Corynebacterium glutamicum* and (D)*Aspergillus niger* on the Phyre2. Image were coloured rainbow N⟶ C. Percentage of each of residues have been modelled at >90% confidence.

Table 5.4 The secondary structure and disorder prediction analysis catalase from different organisms.

Organism	**Disordered**	**Alpha helix**	**Beta strand**
H. pylori	25%	31%	16%
Human	24%	31%	16%
C. glutamicum	22%	34%	14%
A. niger	28%	35%	14%

5.4.1.4 Quantitative and qualitative evaluation of the commercially available catalases

The concentration of the commercially available catalases; Human catalase, *Aspergillus niger* catalase, *Corynebacterium glutamicum* catalase and recombinant *H. pylori* KatA prepared in **section 2.4.1** were measured using Pierce™ BCA protein assay. Then the catalase activity was assessed by 3 % hydrogen peroxide presumptive test, the result showed positive activities for all the catalases and was confirmed using commercially available Amplex® Red Catalase. Then the proteins were resolved on the SDS-PAGE to evaluate the purity (figure 5.3). Only *Aspergillus niger* catalase was not up to 95% pure as shown in figure 5.3 lane 4.

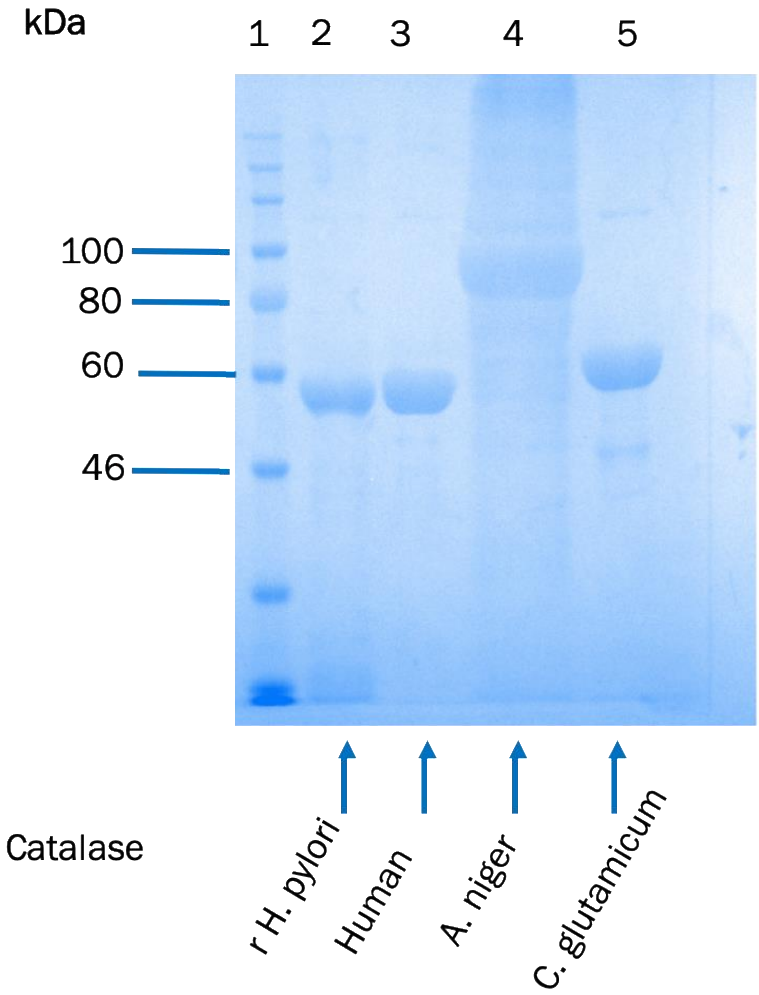

Figure 5.3. A typical SDS-PAGE showing the resolved catalases from **H. pylori***, Human,* **Corynebacterium glutamicum** and **Aspergillus niger**. Commercially available catalases from human erythrocyte, Aspergillus niger, Corynebacterium glutamicum and H. pylori were resolved on the SDS-PAGE and proteins were visualised using PageBlue™ Protein Staining Solution. Lane1, prestained protein standard; lane 2, H. pylori catalase (KatA); lane 3, Human catalase; lane 4, A. niger catalase; lane 5, C. glutamicum catalase.

Following the SDS-PAGE revelation of the impurities in the *Aspergillus niger* a decision was made for further purification of the protein. Size exclusion chromatography (SEC) was for its further purified since it works on the simple principle of differences in the sizes of the molecules involve or sometimes the molecular weight. Then protein was purified using SEC, following the procedure in section 2.4.1.6 The concentration was measured using Pierce™ BCA protein assay kit. The elution product was resolved on the SDS-PAGE gel together with other catalases and stained using PageBlue™ Protein Staining Solution as shown in figure 5.4.

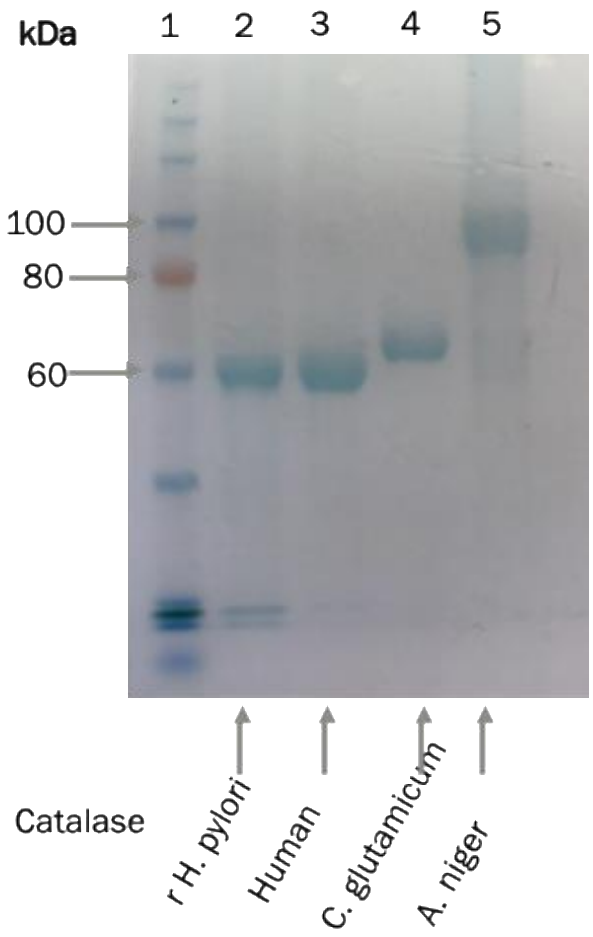

Figure 5.4. A typical SDS-PAGE showing the resolved catalases from *H. pylori, Human, Corynebacterium glutamicum and Aspergillus niger.*

Commercially available catalases from human erythrocyte, *Aspergillus niger, Corynebacterium glutamicum* and *H. pylori* were resolved on the SDS-PAGE and proteins were visualised using PageBlue™ Protein Staining SolutionLane 1, prestained protein standard; lane 2, H. pylori catalase (KatA); lane 3, Human catalase; lane 4, *C. glutamicum* catalase; lane 5, *A. niger*. Catalase

5.4.1.5 The Western Blot analysis of the catalases from *H. pylori,* human erythrocyte, *A. niger* and *C. glutamicum*

In order to evaluate The Western Blot analysis of the cross-reactivity among the antibodies for of catalases from *H. pylori,* human erythrocyte, *A. niger* and *C. glutamicum*

5.4.1.5.1 Reaction with anti-human catalase antibody with other microbe catalase

In order to evaluate whether antibodies against human catalase (rabbit polyclonal antibodies) also react well with catalases from *H. pylori* and other microbes, *Aspergillus niger, Corynebacterium glutamicum* and *H. pylori*. Therefore, western blot analysis for the four catalases was carried out using anti-human catalase IgG as the primary antibody. The bands were compared with the prestained protein marker to ensure it is at the expected molecular weight. The result showed there was no cross-reactivity between antibodies against human catalase and catalase from *Aspergillus niger, Corynebacterium glutamicum* and *H. pylori* figure 5.5.

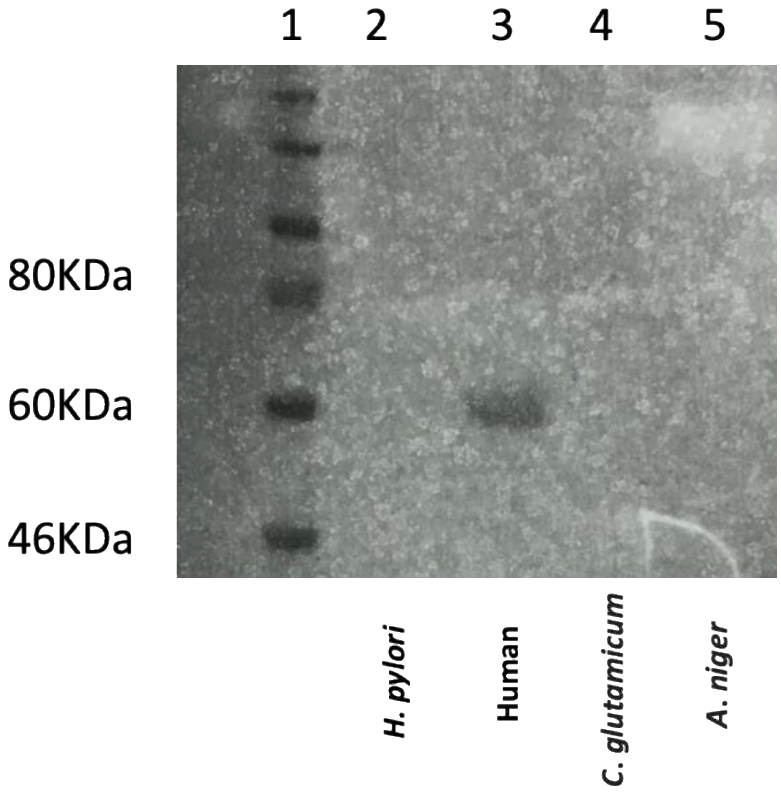

Figure 5.5. A typical western blot chemiluminescence film showing the catalases from **H. pylori***, Human,* **Corynebacterium glutamicum** *and* **Aspergillus niger** probed with anti-human catalase IgG to evaluate cross-reactivity among the catalases. Commercially available catalases from human erythrocyte, Aspergillus niger, Corynebacterium glutamicum and H. pylori were resolved on SDS-PAGE, then transferred to a nitrocellulose paper and probed with anti-human catalase IgG. Lane 1, prestained protein standard; lane 2, *H. pylori* catalase (KatA); lane 3, Human catalase; lane 4, C. glutamicum catalase; lane 5, A. niger.

5.4.1.5.2 Reaction with serum antibodies from *H. pylori*-positive and *H. pylori*-negative patients

Following the confirmation that polyclonal anti-human catalase antibody does not cross-react with catalase from *Aspergillus niger, Corynebacterium glutamicum* and *H. pylori*. Especially with the higher-level sequence similarity between KatA and human catalase amino acids, it is expected they could share a few epitopes which could result to cross-reactivity between anti-KatA catalase present in the serum of *H. pylori*-positive patient.

Western blot was used to analysis the cross-reactivity of the anti-catalase antibody from *H. pylori*-positive and *H. pylori*-negative patients. The results shown in Fig 5.6 indicated strong bands on the *H. pylori*-catalase (KatA) with the *H. pylori*-positive serum compare to the *H. pylori*-negative serum. Surprisingly, there are bands on the *H. pylori*-negative serum.

Interestingly, there were either weak bands or no band on the human catalase with *H. pylori*-positive serum and no band on the negative serum. Similarly, *C. glutamicum* weakly bands or no band on both positive and negative serum. *A. niger* showed strong both the positive and negative serum.

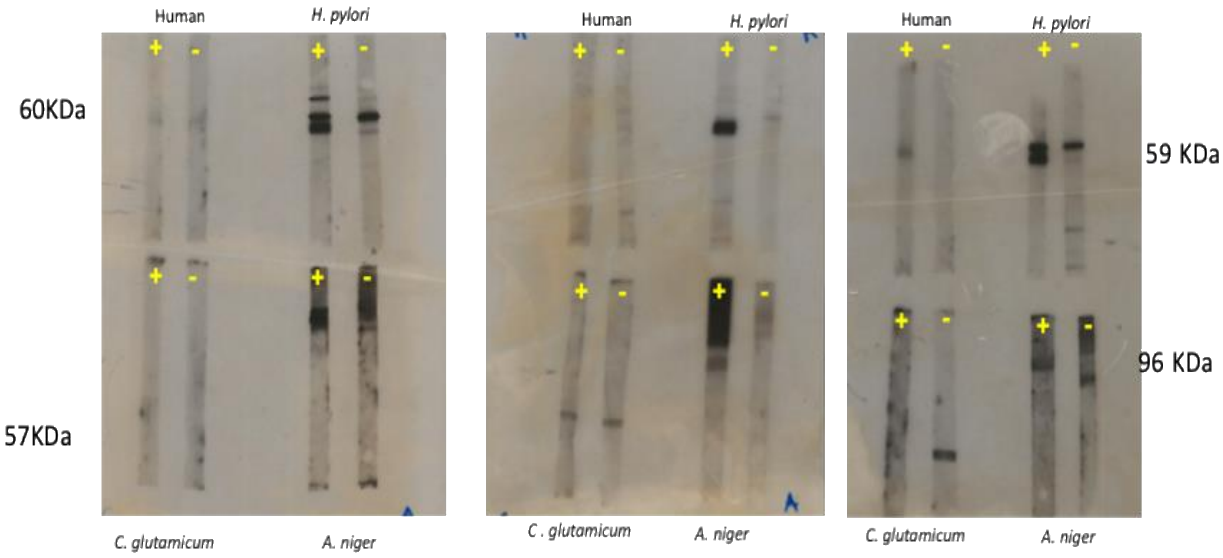

Figure 5.6. A typical western blot chemiluminescence film showing the catalases from *H. pylori*, Human, *Corynebacterium glutamicum* and *Aspergillus niger* probed with *H. pylori*-positive (+) and H. pylori-negative (-) patients' serum to evaluate cross-reactivity among the catalases. The catalases are commercially available catalases from human erythrocyte, *A. niger*, *C.* glutamicum and recombinant H. pylori catalase. The catalases were resolved on SDS-PAGE, then transferred to a nitrocellulose paper and probed with 1:100 dilution of H. pylori-positive patient serum.

5.5 Discussion

The major aim of the studies was to investigate the similarity between catalase from *H. pylori* (KatA) and other organisms especially that of human to ascertain their relatedness. More importantly to investigate whether the antibody against *H. pylori*-KatA anti-body present in the serum of *H. pylori*-positive patient could cross-react with human catalase. The result of these studies presented evidence for further understanding *H. pylori* coevolution with human and this could be a benefit in the understanding of the effect of *H. pylori* KatA in immune regulation.

5.5.1 Bioinformatic analysis

The bioinformatic analysis of the catalases of *Helicobacter* species from different mammalian hosts showed a percentage similarity in the amino acid sequence of about 50 to 65%. This can actually be said to be relatively low when compared to the other proteins shared among the species such as urease subunit beta (UreB) and gamma-glutamyltranspeptidase (GGT), whose similarities range between 75-88% and 65-71% respectively. but the major difference is the absence of the beta-strand, helix and sequence conflict in other Helicobacter species catalases. Likewise, it is only `*H. pylori* catalase that does not possess domain sequence regions. The high percentage similarity

could be due to common origin of the *Helicobacter* species which is also shown by their tendency to caused similar inflammatory response which resembles the type caused by *H. pylori* (Solnick and Schauer, 2001). On the other hand, the difference marked differences seen between *H. pylori* catalase and other species could be explained by the host specificity and several years of coevolution with its human host (Kodaman et al., 2014).

Interestingly, *H. pylori* catalase s high percentage identity between its amino acid sequence and that of human catalase (48%), which is surprisingly high when compared to the percentage identity with other microorganisms. Actually, the result in this study showed that percentage identity between *H. pylori* catalase and catalases of *C. glutamicum* and *A. niger* is 42% and 26% respectively, which are both lower than that of human. This is further supported by the observation that among all the catalase sequences used in the analysis, it was only *H. pylori* and Human catalase that posses a beta-strand, helix, turn and sequence conflict in its sequence interestingly both lack the regions of the domain which are found in every other catalase. This observation could be appreciated in the light of long term adaptation of *H. pylori* to its human host (Atherton and Blaser, 2009); several factors of the bacteria have been modified to enable it to survive and coexist with its host. A typical illustration was in the recent report by Richter et al, which showed that *H. pylori* KatA is a receptor to an extracellular matrix and plasma protein, the vitronectin, which the bacteria uses to mask itself against the complement-mediated killing (Richter et al., 2016). This phenomenon is a clear diversion from the orthodox

function of catalase which is the antioxidant activity in every living organism. Similarly, it could be the close similarity between human and *H. pylori* catalase means that antibody against one could react to the other.

The tertiary and secondary structure of protein especially the alpha-helix and beta-strand have a huge influence on the epitope. This could be due to high chemical bond energy found in alpha helices and beta strands of a protein in addition to factors such as hydrophobicity, polarity, turns and surface accessibility (Poland et al., 2001). Consequently, the tertiary 3D and secondary structure of each of the catalase from the 4 organisms were analysed using Phyre2, this showed *A. niger* is made up of 28% disorder, 35% alpha-helix and 14% beta-strand, *C. glutamicum* 22% disordered, 34% alpha-helix and 14% beta-strand, *H. pylori* KatA 25% disordered, 31% alpha-helix and 16% beta-strand while human catalase is made up of 24% disordered, 31% alpha-helix and 16% beta-strand.

Earlier analysis considering only amino acid sequence, primary structure, showed 48% similarity between human and *H. pylori* catalase. Primary structure of proteins does not put into consideration of molecular interaction which gives rise to the secondary structures, hence cannot efficiently establish epitopes on the protein. Consequently, the similarity in the percentage of alpha helices, beta-strand and disordered sequence could be a good prediction on the resemblance between the two proteins. This also could explain the interaction between the pathogen (*H.pylori*)and host (*Homo sapien*), especially why the antibodies produced by the pathogen are not

effective. Again coevolution between the two organisms (Kodaman et al., 2014) is likely to account immunomodulation and likelihood of *H. pylori* to persist in the incidence of infection.

5.5.2 Investigation cross-reactivities of the anti-KatA antibody with catalase from different organisms

Previously in chapter 4, microarray was used to investigate the anti-KatA antibody present in the serum of *H. pylori*-positive and *H. pylori*-negative patients. The result from this study showed that *H. pylori*-positive patients have 4-fold higher anti-KatA antibody in their serum compare to that of the *H. pylori*-negative patients. Therefore, since KatA elicits such a high level of antibody and with the scepticism among several researchers on the protective capability of the KatA-specific antibodies to protect against *Helicobacter pylori* infection (Kotiw et al., 2012). The hypothesis that *H. pylori* KatA specific antibodies could behave functions that are normal antibody functions. With a high level of similarity between the human and *H. pylori* catalase, one possible function of anti-KatA antibodies could cross-reaction with human catalase to block its functions.

The result from the western blot investigation of the cross-reactivity of the anti-KatA specific antibody using serum from both *H. pylori*-positive and *H. pylori*-negative patient presented an inconclusive result. There is obvious inconsistency in the result of *C. glutamicum* and *A. niger,* while most the *C.*

glutamicum showed the band at both positive serum and negative serum, the *A. niger* results showed no band with some of the positive serum and weak band in some of the negative serum. The positive western blot bands on the catalases of *C. glutamicum* and *A. niger* catalase is unexpected since both could be described as non-pathogenic (Person et al., 2010, Burkovski, 2013). Further experimental evidence through additional studies could provide some answer on the role of catalase on immunomodulation in *H. pylori*-infected patient.

Chapter 6

Final Discussion

6.1 Important and Novel

The fundamental objective of this study was to ascertain the other factors *H. pylori* utilises in inducing immune suppression. `To do this, a hypothesis which states that *H. pylori* molecules have both direct and indirect immune regulatory effect on the cells of the immune was developed. The results obtained from the study in chapter 2 shows that KatA significantly suppresses IL-2 a proinflammatory cytokine, first recognised for its function in the T-cell expansion (Smith, 1988). At the same time, the study also supports the already existing finding of the immune-suppressive ability of GGT while showing that PPT is ineffective in immune suppression. Although the mechanism behind KatA immune suppression was not investigated due to time constraints, this study is the first to demonstrate another function for this ubiquitous protein. Furthermore, data from chapter 4 showed that five *H. pylori* factors CagA, VacA, KatA, GGT and PPT were the potential biomarker for the screen peptic ulcer and potentially Gastric cancer, using serum antibodies against these factors. In Chapter 5, the bioinformatic analysis of catalase proteins of H. *pylori*, other *Helicobacter* species, *C. glutamicum*, fungi-*A. niger* and human reveal a consistent shift in similarities of *H. pylori* KatA and away from microorganisms and unanticipated shift in similarity towards human catalase. These similarities between *H. pylori* KatA and Human catalase might be in important in conferring additional functional characteristics during an infection such as immunoregulation through moonlighting on the surface of *H. pylori* to avoid

immune cells activation (Richter et al., 2016), direct suppression of immune and probable compensation in the case of acatalasemia (Goth and Nagy, 2012).

6.1.1 Effect of H. pylori VacA types on the immunoregulation

H. pylori infection stimulates robust immune response although this response is known to often fail in the eradication of the bacteria leading to persistent colonialization of the gastric mucosa and/or gastric damage (Perez-Perez et al., 1988, Tinnert et al., 1997, Blanchard et al., 1999, Jakob et al., 2001, Suerbaum and Michetti, 2002). One of the most studied methods utilised by *H. pylori* in surviving the immune assault is the immune suppression (Algood and Cover 2006). Müller et al in their review described series of processes *H. pylori* utilised in the manipulation of both innate and adaptive immune cell signalling pathway to establish persistent infection (Müller et al., 2011a).

The study in **Chapter 2** provided evidence which suggested that KatA could directly inhibit proinflammation cytokines. That is KatA may have immunoregulatory properties capable of manipulating of immune cells during *H. pylori* infection. Most of the early studies have focused on VacA and GGT are the main factor providing the immunoregulatory properties in *H. pylori* infection (Salama et al., 2001a, Zheng and Jones, 2003, Gebert et al., 2003, Sundrud et al., 2004, Gerhard et al., 2005, Torres et al., 2007, Oertli et al., 2013a, Wustner et al., 2017). The best of my knowledge this was the first report on immunoregulatory properties of *H. pylori* KatA outside it activities of hydrogen peroxides elimination. Although the result of the experiment showed

the significant reduction of IL-2 production at concentration 50 μg/ml, it is worthy of note that KatA is secreted in high level making up to 1% of the total bacterial secreted protein (Hazell et al., 1991). Gebert et al reported that in addition to inhibition of T cell proliferation, VacA abrogated the nuclear translocation of nuclear factor of activated T cells (NFAT) leading to down-regulation of IL-2 transcription (Gebert et al., 2003). Other reports on GGT only agreed on its ability to inhibit cell proliferation without impairing the IL-2 production (Schmees et al., 2007, Sundrud et al., 2004), this is not in agreement with the result of the experiment in Chapter 2 which showed that high concentration 50 μg/ml, GGT significantly inhibit IL-2 secretion in Jurkat cells.

Although the biological mechanism through which these two protein factors of H. pylori were not investigated, there is a need for further studies for additional understanding the pathways of immunosuppression of the molecule this could help in the development of new therapeutic alternatives for the treatment of associated diseases.

6.1.2 The anti-inflammatory property of H. pylori proteins

A number of human diseases such as pathogens infection, allergy, cancer etc have been associated with changes in the Treg number and function (Banham, 2006). Treg has been shown to be vital in the immune regulation during *H. pylori* and subsequent pathogenesis (Kandulski et al., 2010, Overacre and Vignali, 2016, Vignali et al., 2008). An earlier study by Liu et al in C57BL/6 mice revealed an increase in CD^+ $Foxp3^+$ Treg frequency both in the lamina propria

and the spleen of the infected cohort compared to the placebo receiving group. Similarly, their work also showed a higher Treg number when stimulated with *H. pylori* antigen in vitro (Liu et al., 2015). Their study focused largely on the difference in the Treg and does not put into consideration the difference and effect of VacA type being expressed by the infecting *H. pylori*, the experiment in **Chapter 3** which utilised similar model but measured the difference in VacA type.

The investigation by Lui et al reported an increase in Treg number in the spleen by just 2.98 ±16 % which was not a significant change between the infected group and the placebo receiving group, this is consistent with the result of the investigation in Chapter 3, which showed no significant difference in Treg in *H. pylori* exposed mice compares to the non-exposed group after 9 weeks but consistently show an increase in the number of $CD4^+$ $Foxp3^+$. Furthermore, there was also no difference in Treg number from the spleen of mice infected with *H. pylori* expressing distinctive types of VacA.

Unlike the nTreg which differentiate in the thymus before it migrates to the peripheral, iTreg are mostly induced at the peripheral from the pool of peripheral $CD4^+$ CD^-Focp3^- precursor (Mayne and Williams, 2013, Schmitt and Williams, 2013). The principal function of Treg cells are its immunosuppressive activities through IL-10 and other associated cytokines such as TGF-β (Ray et al., 2010).(Ray et al., 2010) To ensure that the effect of the VacA types was not the case of number rather the functionality, the level of immunosuppressive IL-10 cytokine production was investigated. The result of this investigation

showed no difference in the level of IL-10 secretion from the Treg derived from the mice groups when stimulated with PMA-ionomycin/LPS mitogen. This result was in contrast to results from other investigations which indicated that on stimulation of Treg derived from mice exposed with *H. pylori* there would increase the IL-10 production. (Rad et al., 2006, Wang et al., 2013a, Liu et al., 2015).

Compare to the mitogen, PMA-ionomycin/LPS used the experiment, other studies have shown that a more potent activator of *H. pylori*-experienced Treg to the secrete of IL-10 is the *H. pylori* antigen rather than the PMA-ionomycin. Liu et al showed that spleen lymphocytes isolated from *H. pylori*-infected mice stimulated for 15 hours with *H. pylori* antigen secreted a statistically significant amount of IL-10 and TGF-β compare to the PMA-ionomycin stimulated spleen lymphocyte from *H. pylori*-infected and unstimulated uninfected mice (Liu et al., 2015, Raghavan et al., 2003). The results point to the fact that the IL-10 production in stimulated Treg is a function of the presence of memory Treg rather than other possible sources such as induced Treg.

Conclusively, functional analysis of the $CD4^+$ $CD25^+$ Treg isolated from *H. pylori* exposed and non-exposed mice showed an obvious suppression of T cell proliferation but does not show any difference immunosuppressive activities in both groups.

6.2 Critique of the experimental design

Recombinant proteins production has been one of the mainstays in biomedical and other scientific research in general. One of the ways of purification of recombinant protein has been the addition of extra amino acid residues to the N- or C-terminus of the protein by modification of the DNA sequence. The extra sequence of amino, known as tags, confer particular properties to the protein. One of such properties seen with the tags is exemplified by polyhistidine (His-tag) addition to the N-terminal which bestow which enable purification of recombinant protein from crude cell lysate using immobilized metal affinity chromatography (Munro and Pelham, 1984). Booth et al showed that there are differences in the stability and functions of N-terminal His-tag protein and native protein (protein with its histags cleaved with TEV protease) (Booth et al., 2018). Consequently, the experimental design of Chapter 2, may be affected since the assays carried out in the investigation are functional assays intended to mimic the role of the protein in its native form in vivo.

Furthermore, the one the hypothesis in this work put into consideration that the possible reason for the unique behaviour of *H. pylori* KatA could be because it differs from other bacterial catalase proteins by having an additional 17 amino acid sequence at the C-terminus, containing a poly-lysine motif and this may impart different functional characteristics. Therefore, if it is believed that this poly-lysine sequence could affect the functionality and behaviour of the protein, the same could be ba said of the polyhistidine at the C-terminus.

The immune suppression assays have been essential in the evaluation of activities Treg. The *in vitro* studies involving suppression assays have been deemed technically difficult due to whole line problems ranging from induction to cell purification (McMurchy and Levings, 2012). The use of PMA/ionomycin/LPS in Chapter 2 is a polyclonal mix of mitogens which could easily mimic *in vivo* natural environment. Ionomycin is a Calcium (Ca^{2+}) ionophore produced by *Streptomyces conglobatus* fermentation, with high affinity for calcium (Liu et al., 1978). In rest T cells ionomycin induces the activation of protein kinase C(PKC) via phosphorylation of CD4 and CD8 proteins. The function of PMA is to advance the activation by induction of T cell proliferation through NFAT nuclear translocation (Chatila et al., 1989, Wang et al., 2013a). The LPS is the most common component present in all Gram-negative bacterial wall, it is a PAMP that is well known for it the ability to bind to TLR4 leading to the activation of major immune responses (Ngkelo et al., 2012). Although this polyclonal mix has been shown to efficient in induction of immune response, its aptness to experiment in Chapter 3 could be deemed slightly inapt since the experiment is designed to evaluate specific activities of *H. pylori* factors. Thus as stated earlier, *H. pylori* antigen could have been better.

Finally, the western blot experiments in Chapter 5 appear to be the only qualitative rather than quantitative. Consequently, the results of this investigation do not permit an adequate conclusion. To overcome this, the use quantifiable western blot technique such as electronic imaging systems that

could enable analysable digital images. Such an image could then allow normalization of the bands and further analyses to acquire quantitative data.

6.3 Future work

On the direct immunosuppressive ability of *H. pylori* KatA, GGT and PPT the future work would be mostly on the framework for the understanding of how KatA mediate immunosuppression of the T-cell. Focus on KatA is important since immunosuppression ability of GGT has already been established (Schmees et al., 2007) and PPT has no T-cell suppression from our experiments or any literature report. To investigate further with KatA, two areas are important.

Firstly, Understand the key KatA binding receptor or cellular structure in human T cell and other immune cells. This would entail the investigation of the location of the receptor, that is whether extracellular and intracellular. Additionally, establishing the means through which KatA could reach its receptor. This is important in the light of T-cells being nonphagocytic cell and would require a special mechanism to accumulate KatA intracellularly to the level needed to exert immunosuppression.

Another import area to look at is the mechanism through which KatA establishes immune suppression. Although VacA and GGT are known to suppress T-cell via cycle arrest (Gebert et al., 2003, Gerhard et al., 2005, Schmees et al., 2007), this suggests that KatA could follow similar mechanism

but still, it could novel if another different means id discovered for T-cell suppression.

Largely, the structure and function of mice and the human immune system are relatively comparable, in that both species remarkably a prototypical of each in immunology experiment (Haley, 2003). However, there are still some obvious differences in the activation and development of their immune system which could hinder the extrapolating of experimental results from mouse studies to human. A prominent example is the variation in the lymphocyte and neutrophile balance in adult human and mice which has 30-50% lymphocyte, 50-70% neutrophils and 75-90% lymphocyte, 10-25 neutrophils, respectively (Doeing et al., 2003). Due to some these variations, it is worth investigating the correlation between VacA types and Treg numbers in PBMCs of infected human patients. This could bring to light the answer to the important question of which type of VacA is driving the skewing of the immune toward regulatory immune system.

Furthermore, with VacA already been known to promote the Treg differentiation in cases of *H. pylori* infection (Oertli et al., 2013a), and *in vitro* investigation of the effect of recombinant VacA variants on human dendritic cell maturation and the differentiation of naïve T cells, would elucidate possible outcome *in vivo*.

Equally, the suppressive activity of human Tregs generated via DCs exposed to *vacA* mutants could be measured to assess tolerogenic activities of the protein on DCs.

Reference:

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Appendix

Table 6.1: katA, ggt and ppt primer sequence used in the study

Primer type	Primer sequence
GGT Primer	
Forward	5’ - TGAAAGGAAAACCCATGGGACGGAG -3’
Reverse	5’ - CCAAGGAAAGAATTTGGTACCTTTG -3’
KatA Primer	
Forward	5’ GATCCATATGGTTAATAAAGATGTG -3’
Reverse	5’CACACAAAAAAGAAAAAGCATCACCATCACCATCACTAATAAGGTACCTG-3’
PPT Primer	
Forward	5’ - TAAGGATCCGCTAGCATGAAAAAAAATATCTTAAAT -3’
Reverse	5’ - TAGAATTCTTACTTGTTGATAACAATTTTA -3’.

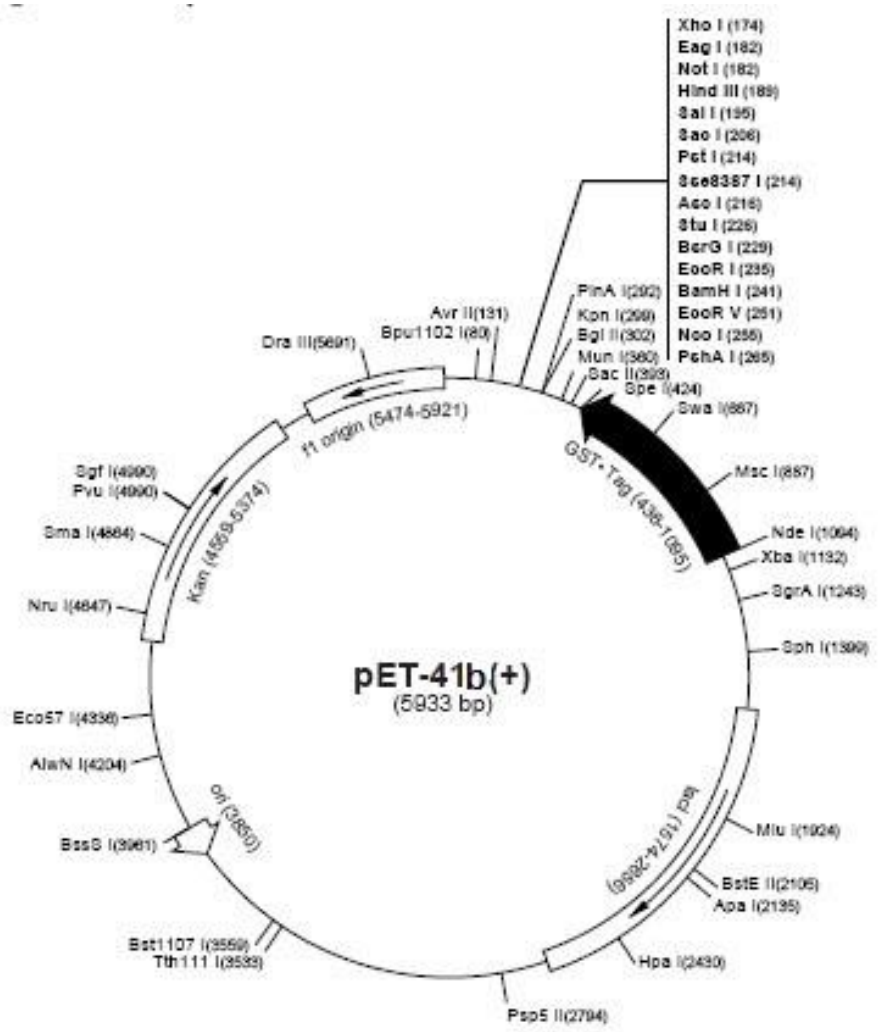

Figure A. Map of the pET-41b plasmid expression vector (Novagen)

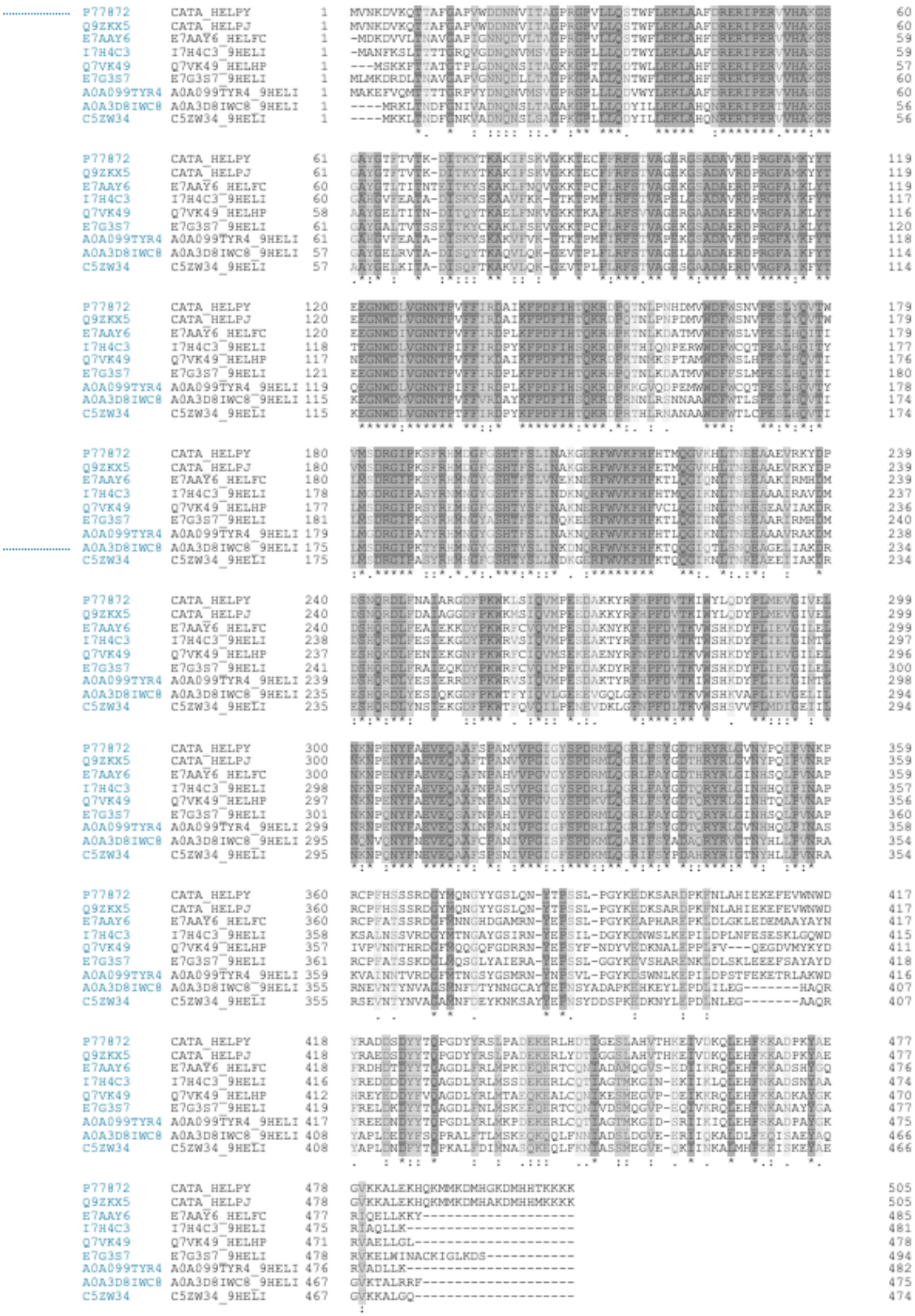
P77872 CATA_HELPY
Q9ZKX5 CATA_HELPJ
E7AAY6 E7AAY6_HELFC
I7H4C3 I7H4C3_9HELI
Q7VK49 Q7VK49_HELHP
E7G3S7 E7G3S7_9HELI
A0A099TYR4 A0A099TYR4_9HELI
A0A3D8IWC8 A0A3D8IWC8_9HELI
C5ZW34 C5ZW34_9HELI

Figure B

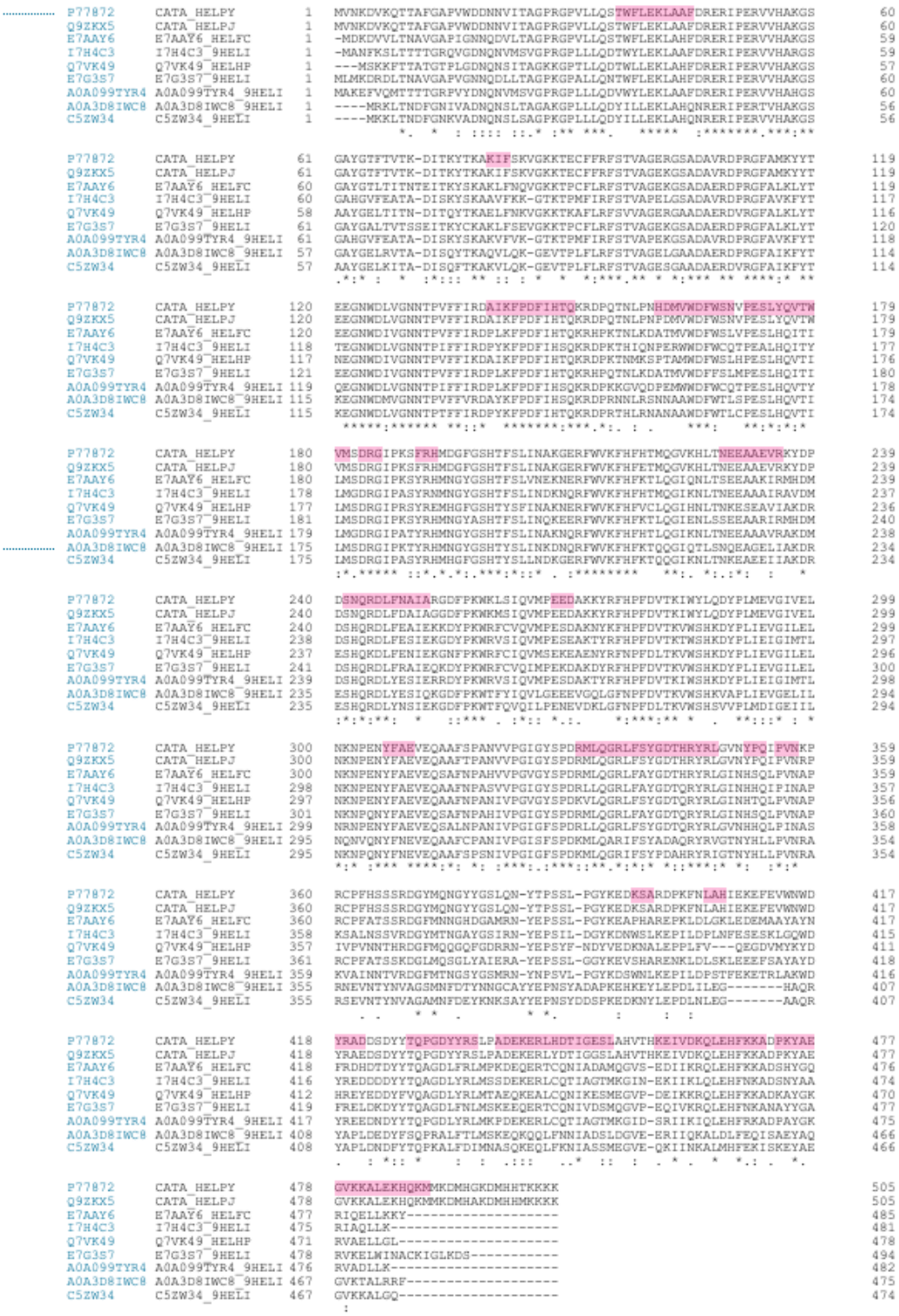
P77872 CATA_HELPY
Q9ZKX5 CATA_HELPJ
E7AAY6 E7AAY6_HELFC
I7H4C3 I7H4C3_9HELI
Q7VK49 Q7VK49_HELHP
E7G3S7 E7G3S7_9HELI
A0A099TYR4 A0A099TYR4_9HELI
A0A3D8IWC8 A0A3D8IWC8_9HELI
C5ZW34 C5ZW34_9HELI

Figure C

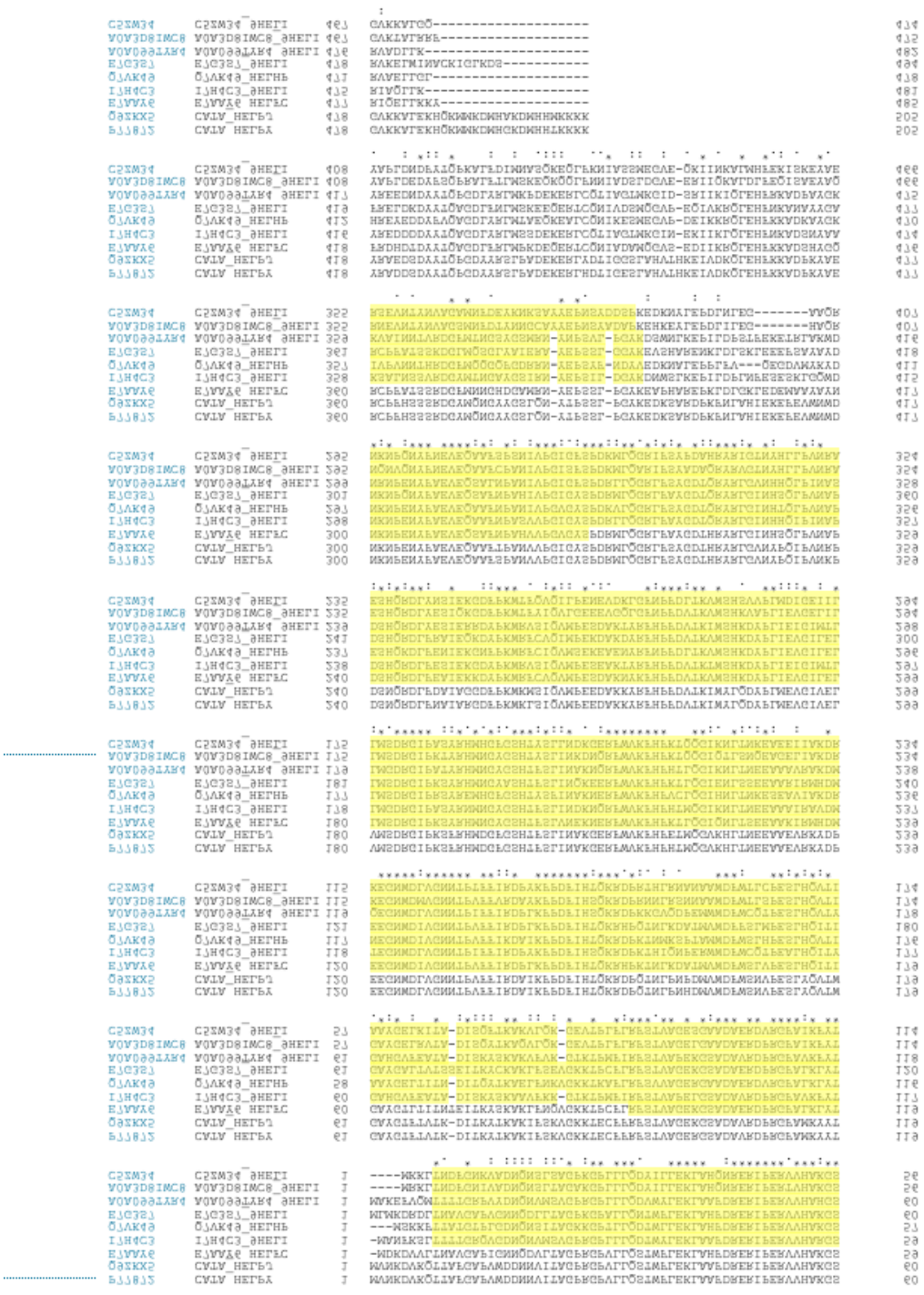

Figure D

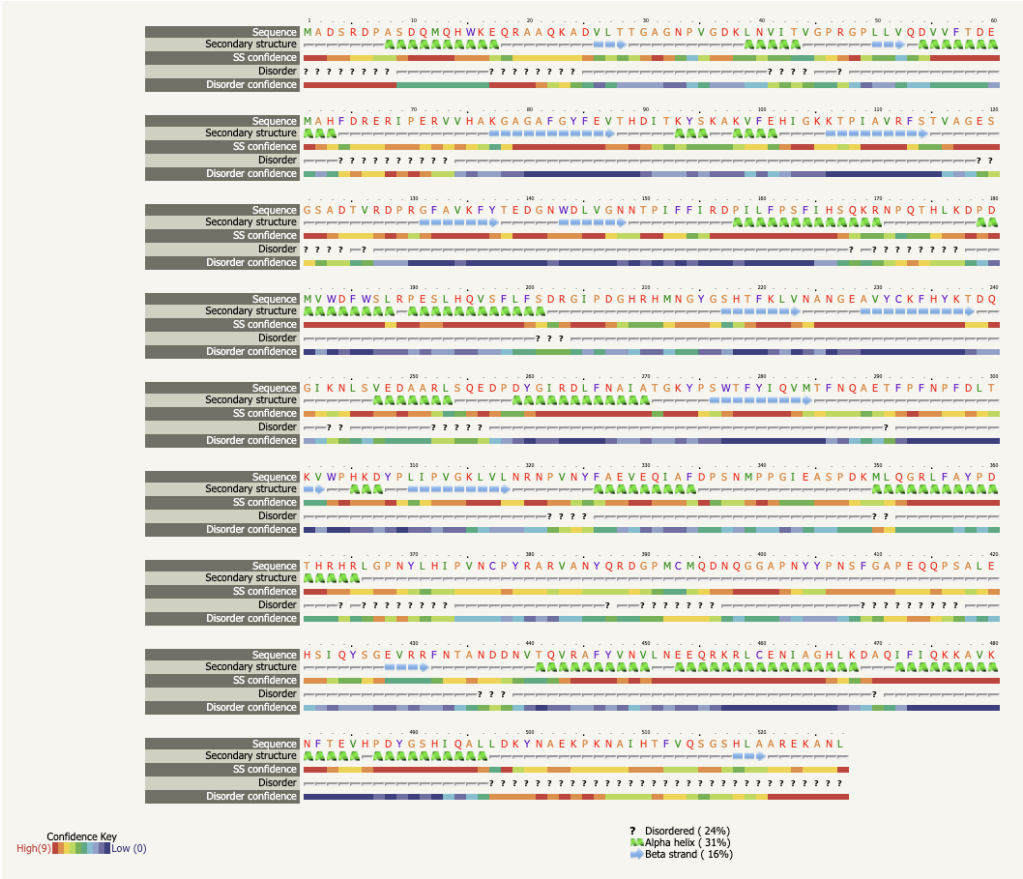

Figure E Secondary structure and disorder prediction of Human (*Homo sapian*) erothrocyte catalase

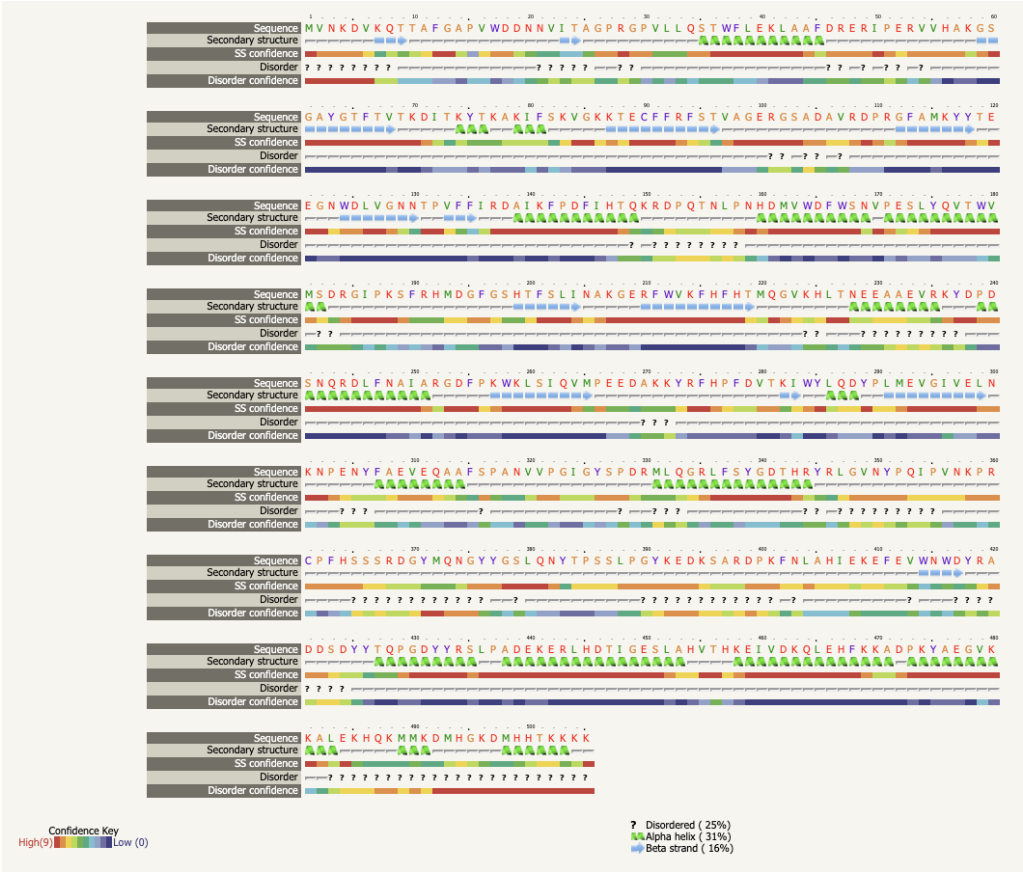

Figure F. Secondary structure and disorder prediction of *H. pylori* catalase (KatA)

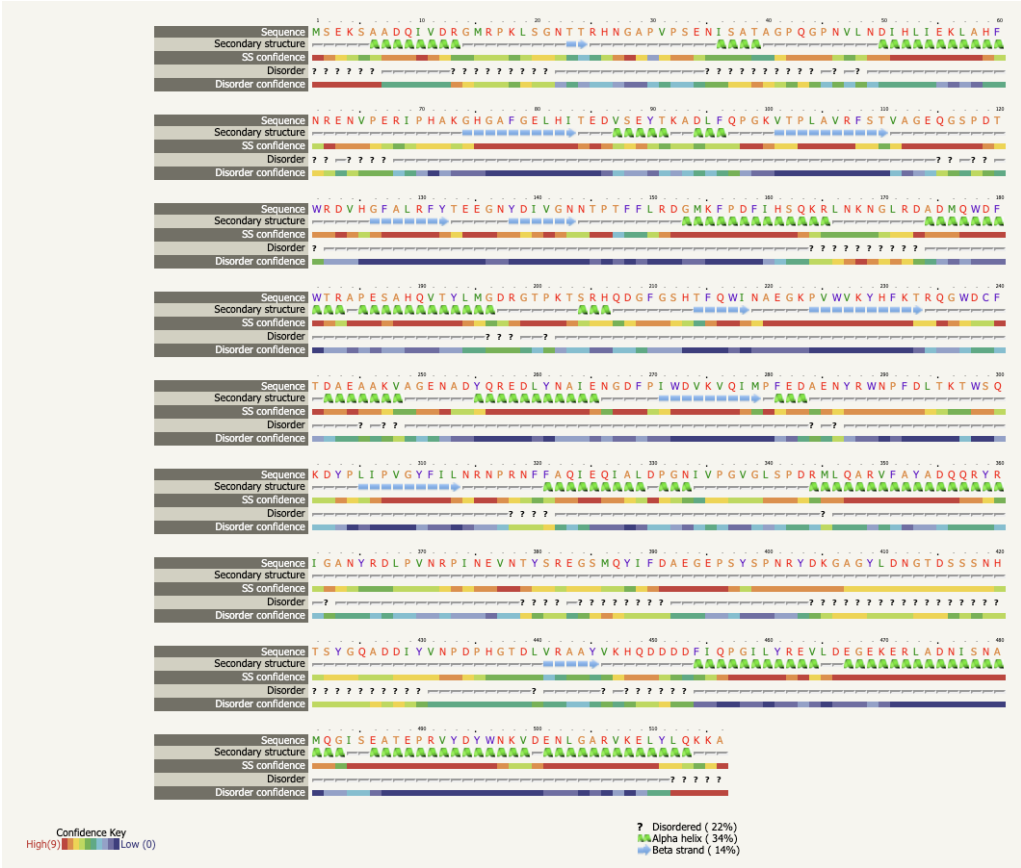

Figure G Secondary structure and disorder prediction of *C. glutamicum*

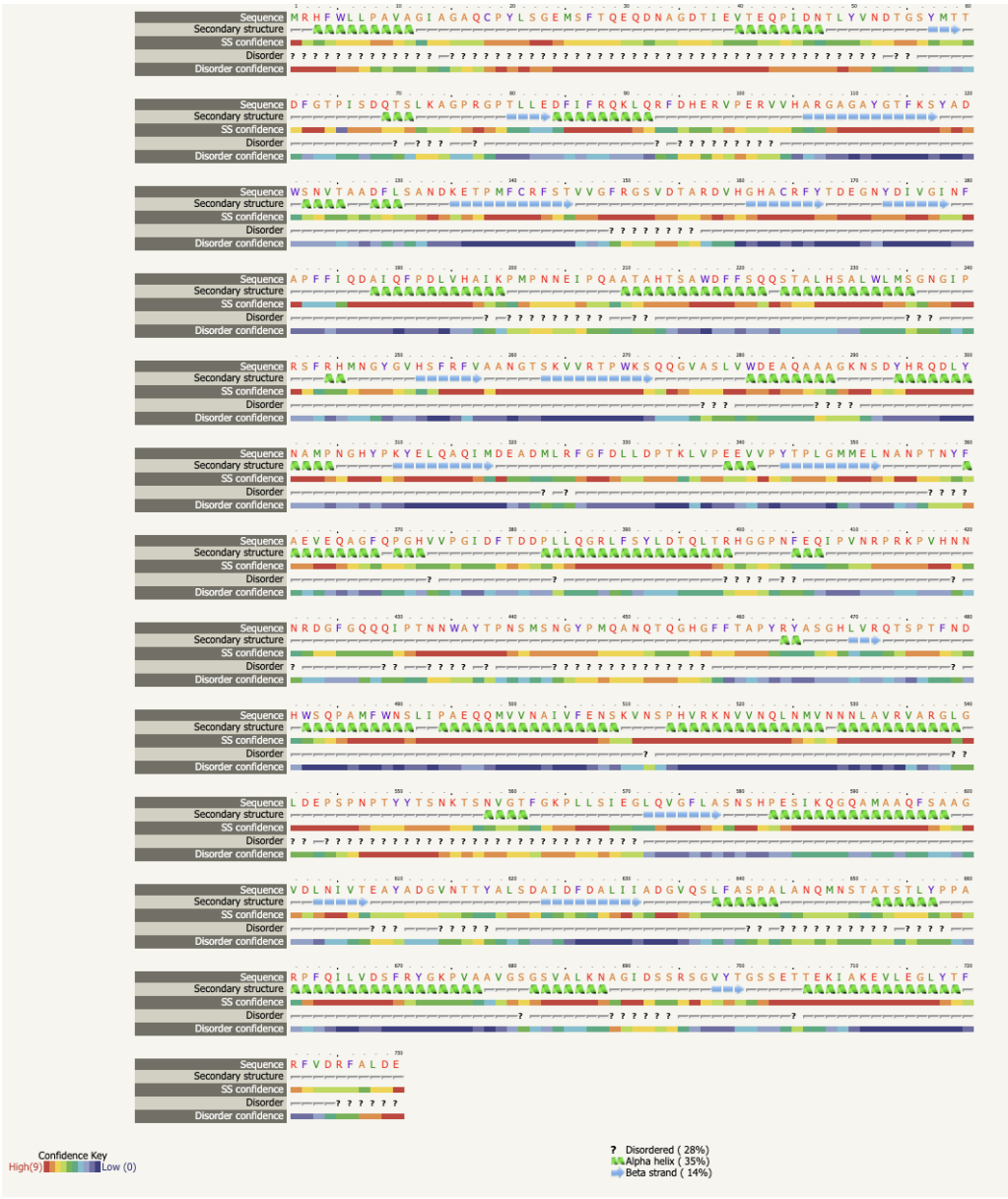

Figure H Secondary structure and disorder prediction of *A. niger*