Molecular and functional studies on a multifaceted kinase from *Helicobacter pylori*

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By

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DECLARATION

I, **Shivendra Tenguria**, hereby declare that the work presented in this thesis entitled **"Molecular and functional studies on a multifaceted kinase from *Helicobacter pylori*"** submitted by me under the guidance and supervision of **Dr. Niyaz Ahmed** is an original research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled **"Molecular and functional studies on a multifaceted kinase from *Helicobacter pylori*"** has been carried out by **Shivendra Tenguria**, under the guidance and supervision of **Dr. Niyaz Ahmed** at the Department of Biotechnology and Bioinformatics, School of Life Sciences, University of Hyderabad, for a full period prescribed under the PhD ordinance of this University.

We recommend this thesis for submission for the award of the degree of Doctor of Philosophy of this University.

Dr. Niyaz Ahmed

(Supervisor)

Head of the Department

Dean of the School

Dedicated to
my teachers and guide

Acknowledgement

Here comes the day for completion of 6 years long journey with efforts and support from my guide to lab mates and friends. This came out as a very splendid journey which moulded and casted me to jump with enthusiasm, once again into the ocean of hope, logic, exploration and research for welfare of human beings and nature, and to find way for the journey of life ahead.

For what I am today, with praise and immense honor from the core of my heart I pay regards to God.

To Him to hand me over to
Carving and caring hands of
Special person who supervises us (PBL)
The Lord of PBL

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That I'm to go to my Home…
By crossing each hurdle…
Sky or the light, mind or my heart
Body or the world, life or the death.

Thanks to you all
Smiling faces of Life Sciences
To all who helps each other
With clean and clear heart

ABBREVIATIONS

µg	Microgram
µl	Microliter
^{0}C	Degree centigrade
ATP	Adenosine triphosphate
BCA	Bicinchoninic Acid
BSA	Bovine serum albumin
CagA	Cytotoxin-associated gene A
CD	Cluster of Differentiation
cDNA	Complementary Deoxyribonucleic acid
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacidic Acid
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
HRP	Horseradish Peroxidase
ICD	Isocitrate Dehydrogenase
IgG	Immunoglobulin G
IL1β	Interleukin-1 beta
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
Kb	Kilo base pair

KDa	Kilodalton
LB	Luria Bertani Broth
LPS	Lipopolysaccharide
mM	Millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Center for Bio-informatics
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
nM	Nanomolar
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PVDF	Polyvinylidene difluoride
PZ	Plasticity Zone
RNA	Ribonucleic Acid
SDS	Sodium dodecyl Sulfate
Ser/Thr kinase	Serine/Threonine Kinase
TBST	Tris buffered saline with Tween-20
TCA	Trichloroacetic acid
TNFR	Tumor Necrosis Factor Receptor
TNFα	Tumor Necrosis Factor- alpha
Tris	2-amino-2-(hydroxymethyl)-1, 3-propanediol

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Chapter 1

Introduction

1.1 Introduction

Helicobacter pylori is a spiral shaped, Gram negative, micro-aerophillic bacterium that is found in almost two third of the world's population and causes infection of gastric mucosa (Marshall and Warren, 1984; Wroblewski *et al*., 2010). It is reported to be the most successful colonizer of the human stomach causing chronic gastritis, duodenal ulcers, intestinal metaplasia and gastric cancer as a consequence of long term colonization (Graham, 1997; Uemura *et al*., 2001). Due to the triggering role of *H. pylori* in gastric cancer, the bacterium has been classified as class I carcinogen by the WHO (1994).

According to GLOBOCAN report 2012 by International Agency for Research on Cancer (IARC), gastric cancer is the fifth most common cancer and the third leading cause of deaths due to cancer worldwide (Ferlay J, 2012). Research on *H. pylori* caught momentum after Barry Marshall and Robin Warren were awarded Nobel Prize in Physiology or Medicine in 2005 for their "discovery of the bacterium *Helicobacter pylori* and its role in gastritis and peptic ulcer disease."

1.2 Prevalence and geographical distribution

Helicobacter pylori is prevalent worldwide with varied geographical distribution. In developing countries, more than 80% population is considered colonized as compared to 20-50% in developed and industrial countries (Suerbaum and Michetti, 2002). According to GLOBOCAN 2012, overall prevalence of *H. pylori* infection shows large geographical variations. *H. pylori* Infection rates are highest in Africa, Asia and South America whereas lowest in North America, West Europe and Australia (Bauer, 2011). The prevalence of *H. pylori* infection was found to vary with geographical region, socioeconomic status, ethnic group, age and race (Goh *et al*., 2011; Malaty and Graham, 1994; Malcolm *et al*., 2004).

Low prevalence with constant infection rates in developed countries is a result of improved hygiene and sanitation and active elimination of carrier state by the use of antimicrobial therapies. The possibility of infection with *H. pylori* increases rapidly in the first 5 years of childhood and thereafter remains constantly high; on the other hand the infection rate is low in children from developed countries but increases slowly thereafter. This suggests that *H. pylori* is acquired early in the childhood and persists for the lifetime (Fiedorek *et al*., 1991) unless eradicated.

Prevalence of *H. pylori* infection is associated with these gastric incidences but there are certain geographical regions with low gastric cancer incidence despite high prevalence of *H. pylori* infection, first such region was identified as African enigma by Holcombe (Holcombe, 1992). However, due to less life expectancy in Africa, African enigma is considered as false enigma. Moreover, other geographical regions known for *H. pylori* associated enigma include Malaysia, India, Columbia and Costa Rica (Ghoshal *et al*., 2010; Miwa *et al*., 2002).

This enigmatic association of *H. pylori* with the gastric diseases can be explained by variations in 1) oncogenic potential of different bacterial strains, 2) immunomodulation in response to *H. pylori* mainly through Th2 type, 3) dietary habits of populations from different geographical regions, and 4) genetic susceptibility of the host (Ahmed et al., 2008; Ahmed et al., 2009). For example, dietary component in Indian diet, curcumine, piperine and quercetine compounds from turmeric, black pepper and onion respectively exert anti-carcinogenic effect by involving various cellular mechanisms. Curcumine acts as anti-inflammatory by inhibiting NFκB complex and IL-8 induction in *H. pylori* infected gastric epithelial cells (Bengmark, 2006; Foryst-Ludwig *et al*., 2004). Onion consumption too has been shown to reduce gastric cancer risk in a human cohort study from Netherlands (Dorant *et al*., 1996). The enigmatic occurrence of the gastric diseases in

Asia is described as Asian enigma: Asian countries such as India, Thailand, Malaysia, Pakistan, Iran and Saudi Arabia show low incidence of gastric cancer despite high prevalence of *H. pylori*. This can be explained by the work done in the current decade which shows that the genotype of different bacterial strains is one of the causes behind Asian enigma. For example, in India, virulent *H. pylori* strains predominate in gastric disease patients as well as in control populations. The presence of Cag A antibodies in all populations suggested the involvement of factor(s) other than Cag A in the outcome of the gastric carcinoma (Ghoshal *et al*., 2008). In recent years, it has been reported that most of the *H. pylori* strains isolated from gastric disease patients with diverse cultural, geographic and linguistic background from India possess *cagA* and *vacA* s1m1 alleles while only 6.3 % isolates were *vacA* s2m1 positive (Devi *et al*., 2007). Dietary components, especially curcumin, vegetarian habits and low salts are also proposed to be associated with reduced risk of gastric cancer in north India as compared to south and east India where majority of population consume diet with an excess of rice, fish and more salt (Akhter et al., 2007; Singh and Ghoshal, 2006).

1.3 Transmission and routes of transmission

Infection and transmission of *H. pylori* is related to geographical area, socioeconomic conditions, antibiotic use, infection dose and hygienic family practices (Feldman *et al*., 1998). But the routes of transmission of the bacterium still remain unclear. On the basis of intrafamilial clustering, person to person and intrafamilial transmission seem to be the main routes of infection and transmission while waterborne, zoonotic or vector borne and iatrogenic transmission remain weak sources of transmission of the bacteria (Brown, 2000; Calvet *et al*., 2013).

Person to person transmission of *H. pylori* is shown to be occurring through family exposure (familial transmission), oral – oral, faecal – oral and gastro – oral routes (Brown, 2000).

It has been demonstrated that *H. pylori* can live in its infectious bacillary form for several days in milk and in coccoid form for several months in river and tap water (Vincent, 1995). In epidemiological studies conducted in Colombia, rural China and Peru, it has been shown that water source may be related to risk of *H. pylori* infection (Goodman *et al*., 1996; Klein *et al*., 1991; Zhang *et al*., 1996). These observations were supported by the presence of *H. pylori* in drinking and sewage water samples by PCR assays in Peru and Japan (Hazell *et al*., 1994; Sasaki *et al*., 1999; Westblom, 1997) especially in areas that have high rates of *H. pylori* infection. These sporadic evidences from different geographical areas suggest that water also acts as a source of *H. pylori* transmission but at low frequency.

The presence of *H. pylori* has also been reported in non-human primates and domestic cats (Fox, 1995; Handt *et al*., 1994; Handt *et al*., 1995), but they hardly act as major routes of transmission because of limited close contact between non-human primates and humans. The isolation of *H. pylori* from feline saliva and gastric sections and DNA from feline faeces and dental plaques has been demonstrated using domestic cats and naïve cats, experimentally, after infection (Fox *et al*., 1996).

Iatrogenic transmission is that when one person is infected by the use or introduction of improper sterilized endoscope, tube or specimen that are in contact with gastric mucosa from infected person (Akamatsu *et al*., 1996). Due to complex structure of the endoscope and difficulty in disinfecting it, endoscopy becomes a potential risk factor for acquisition of *H. pylori* which results in iatrogenic infection of about 1% endoscoped population (Langenberg *et al*., 1990). Following these iatrogenic infections, World Congress on Gastroenterology has recommended the disinfection of endoscope by soaking in 2% activated glutaraldehyde for 10 minutes after cleaning endoscope channels.

1.4 Colonization and adaptation of H. pylori to the human host

H. pylori colonize the stomach in childhood and persist thereafter throughout the life. It takes years to decades to develop symptomatic gastric disease in adults such as peptic ulcer and gastric cancer and hence it can be considered a perfect example of optimized adaptation of a bacterium to its human host by evading immune recognition (Blaser and Kirschner, 2007; Suerbaum and Michetti, 2002). *H. pylori* colonize and adapt to extreme acidic environment of the stomach due to its spiral shaped morphology with flagella that confer motility, environmental sensing and acid resistance. The bacteria neutralize acidic pH using urease enzyme which produces ammonia by hydrolysis of urea; ammonia thus produced buffers the intracellular and extracellular H^+ ions and neutralizes the acidic environment (Stingl *et al*., 2002). Entry of urease into the periplasm is regulated by a pH gated urea channel called UreI, which remains open at low pH for the influx of urea and closed at neutral pH (Stingl *et al*., 2002). *H. pylori* regulates this acid neutralization by producing up to 15% urea of total bacterial proteins (Stingl *et al*., 2002). *H. pylori* adhere to gastric epithelium with the help of several adherent molecules expressed on its outer membrane.

There are some bacterial factors like VacA, which increases bacterial fitness to the host by forming voltage dependent hexameric anion selective channels in the epithelium through which bicarbonate and other organic anions are released possibly for providing nutrients to the bacterium (Salama *et al*., 2001; Szabo *et al*., 1999). *H. pylori* has evolved specific mechanisms to circumvent stimulation of host immune responses in a controlled manner.

Apart from these mechanisms, immune homeostasis occurring due to a balance of proinflammatory and proapoptotic responses produced by the bacterium is considered one of the potential survival and adaptation strategy evolved by *H. pylori* (Ansari et al., 2014; Devi et al., 2014).

1.5 Coevolution of H. pylori with human host

Different *Helicobacter* species inhabit the gastrointestinal tract of their specific human and non-human hosts (Atherton and Blaser, 2009). Genetic diversity among these *Helicobacter* strains, as well as among humans decreases with geographical distances from East Africa (Linz *et al*., 2007) (Figure 1.1). These findings along with, genetic affinities among different *H. pylori* strains and lineages (Devi *et al*., 2007; Devi *et al*., 2006; Falush *et al*., 2003) suggest that *H. pylori* and humans had shared common ancestry for around 60,000 years before they started migrating out of Africa along with humans and have coevolved with their human host.

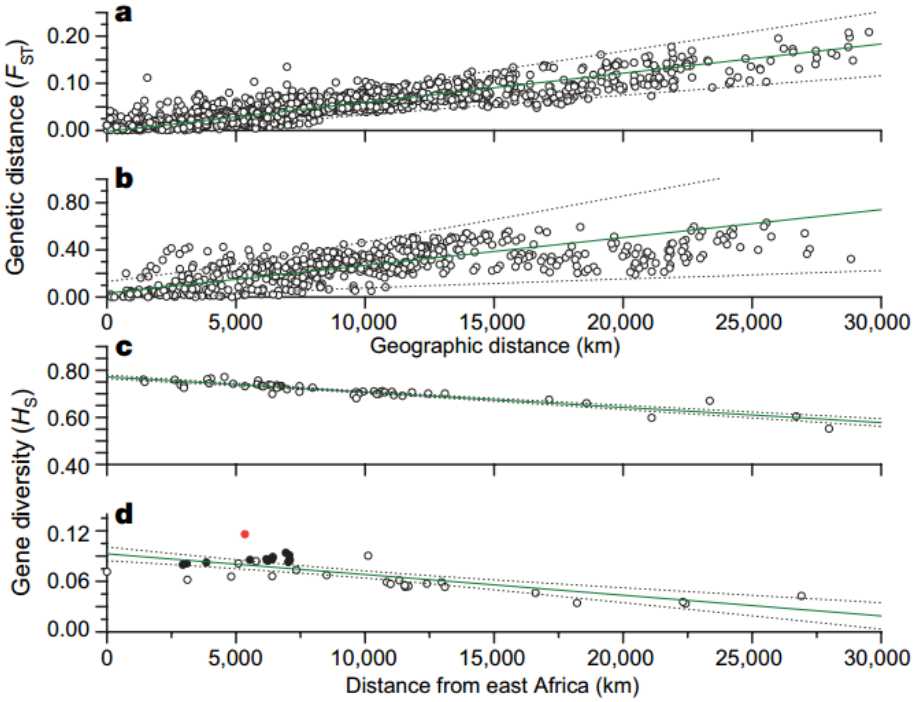

Figure 1.1: Parallel geographic patterns of genetic diversity in humans and *H. pylori*. a and b: Genetic distance in humans (a) and *H. pylori* (b) between pairs of geographic populations (F_{ST}) versus geographic distance between two populations. C and d: average gene diversity in humans (**c**) and *H. pylori* (d) within geographical populations (H_S) versus geographical distance from East Africa. In d, samples predominantly composed of hpEurope are indicated by filled circles, whereas red circle represents South Africa (Linz B *et al*., 2007).

Acquisition of *Cag*PAI, a determinant of virulence might have occurred recently, possibly due to close contact of humans with domesticated animals, crops and pests surrounding them in a geographical region specific manner. This notion also supports the existence of differential virulence potential of *H. pylori* strains from different geographical regions (Ahmed *et al*., 2009).

1.6 Clinical manifestation of H. pylori infection

The disruption of mucus layer in the *H. pylori* infected patients leads to development of ulcer disease in 10-20% cases and gastric cancer in 1-2% cases. The development of various gastric disorders depends on bacterial, host and environmental factors which also relate to the pattern and severity of gastritis (Kusters *et al*., 2006). Gastritis is characterized by the infiltration of neutrophils and mononuclear cells in the corpus and antrum region of the stomach. The gastritis is characterized mainly in two sub-categories: acute and chronic gastritis.

1.6.1 Acute gastritis

The acute phase gastritis results from deliberate or inadvertent acquisition of *H. pylori* resulting in inflammation of gastric mucosa or pan-gastritis and appearance of transient non-specific dyspeptic symptoms like nausea, vomiting and fullness. The acute gastritis often causes transient hypochlorhydria that may last for a few months. Sometimes, the acute gastritis can leave *H. pylori* in the gut to cause persistent colonization but how often the acute gastritis is followed by spontaneous clearance or persistent colonization is unclear. Also, the resultant outcome as acute gastritis depends on host, bacterial and environmental factors and it is possible that some individuals never develop persistent colonization by *H. pylori* (Kusters *et al*., 2006).

1.6.2 Chronic gastritis

Once *H. pylori* is persistently colonized in the stomach, it often develops chronic gastritis in

almost all individuals but 80-90% remain asymptomatic. Further clinical course of the infection is highly variable and depends on pattern of acid secretion, host and bacterial factors (Suerbaum and Michetti, 2002). In patients with high acid secretion, *H. pylori* colonizes in the antrum region of stomach, where a few acid secretory parietal cells are present leading to antral-predominant gastritis (Figure 1.2).

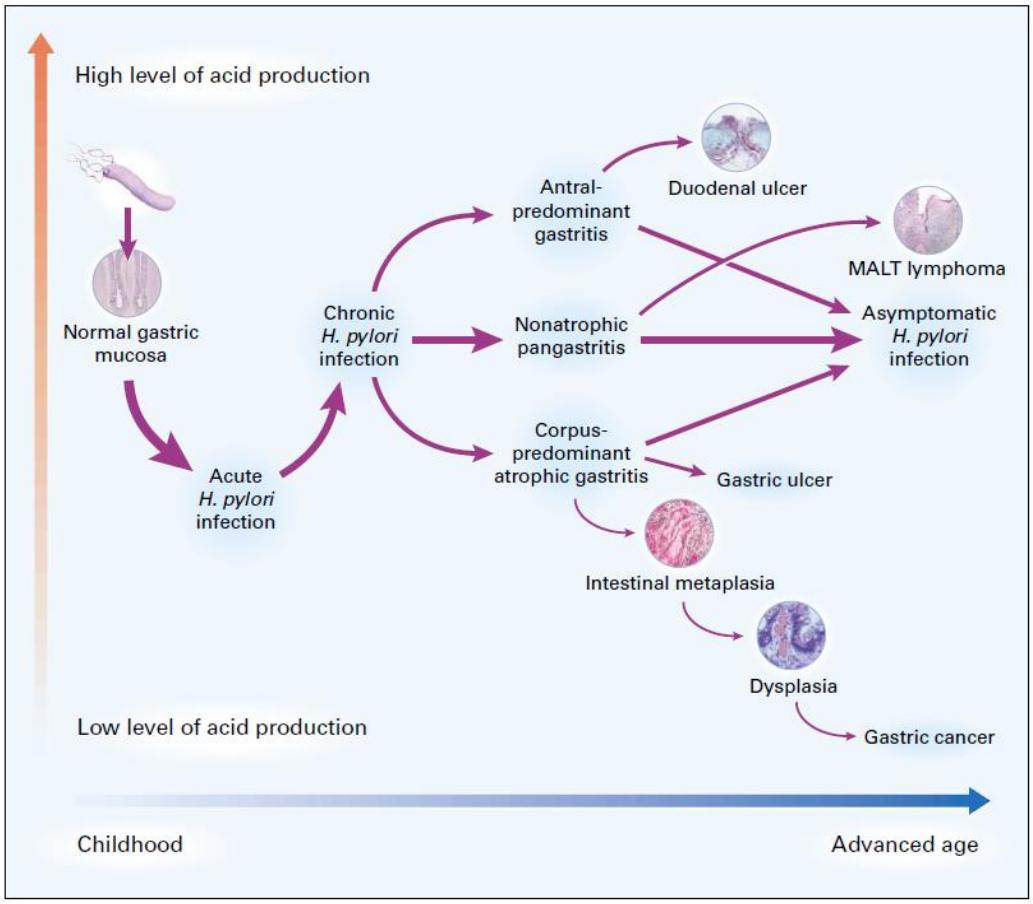

Figure 1.2: Progression of gastric disease with time and acid secretion (Suerbaum *et al*., 2002).

Chronic gastritis is divided majorly into four types: *H. pylori* induced gastritis, autoimmune gastritis due to autoimmune disorders like Crohn's disease, multifocal atrophic gastritis and chemical induced gastritis due to NSAIDs (aspirin) use or alcohol abuse (Furuta and Delchier, 2009). *H. pylori* induced gastritis is the most common type of chronic gastritis characterized in

most cases by chronic and active inflammation of corpus and antrum regions of the stomach which further enhances hypochlorhydria parallel to the acute phase gastritis. In patients with low gastric acid secretion, high number of *H. pylori* colonizes in the corpus region causing corpus predominant pan-gastritis or atrophic gastritis. This can initiate a sequence of events starting from intestinal metaplasia, dysplasia and eventually leading to gastric cancer in 1-2% of *H. pylori* infected individuals. The reduced acid secretion can result either by loss of parietal cells due to atrophic gastritis or by the use of acid suppressive drugs such as Proton Pump Inhibitors (PPIs) (Kuipers et al., 1995; Kusters et al., 2006).

1.6.3 Peptic ulcers

Peptic ulcers are the mucosal defects in the stomach that penetrate muscularis mucosa and are at least 0.5 cm in diameter. These are of two types: gastric ulcers and duodenal ulcers. Gastric ulcers appear mostly on less curved mucosal region of the stomach at the transition zone of antrum and corpus while duodenal ulcers occur in the duodenal bulb (Van Zanten *et al*., 1999). Developments of ulcers depend on various host and bacterial factors. These ulcers develop mostly on the sites of more severe inflammation. Patients with low acid secretion develop gastric ulcers in the transition zone of antrum and corpus region while regions in the high acidic environment in the distal part of the stomach experience duodenal ulcers (Van Zanten *et al*., 1999). Gastrointestinal bleeding, perforations inside the stomach and stricture formation are the main complications arising in the ulcer disease. Endoscopic therapy is the primary treatment for bleeding ulcers which is aimed at the establishing cause and cure for bleeding (Kusters *et al*., 2006). As mentioned above, the incidence of peptic ulcers as well as *H. pylori* infection rate have declined over the past three decades but the cases of ulcer bleeding and perforations, however remain more stable, possibly due to increased and widespread over the counter use of NSAIDs or

aspirin which also produces similar effects. Thus, in recent years the reduction in *H. pylori* associated bleeding ulcers has been compensated by increase in NSAIDs associated bleeding cases (Hung *et al*., 2005; van Leerdam *et al*., 2003). It has been shown that *H. pylori* and NSAIDs are the two major agents to induce gastro duodenal ulcers. Both these agents produce cumulative ulcer-inducing effect and neither of the two alone is sufficient to prevent these ulcer complications (Huang *et al*., 2002).

1.6.4 Intestinal metaplasia

Intestinal metaplasia is transformation of stomach epithelium to a type that resembles to intestine as in case of Barrett's esophagus. It can be stimulated by atrophic gastritis in conditions of reduced acid secretions, to destroy mucosal architecture and gastric glands and replacing them with intestinal type-epithelium (Kusters *et al*., 2006). Progression to intestinal metaplasia depends on pattern and distribution of acid secretion and chronic active inflammation. It first appears on more severe inflamed sites with reduced acid secretion leading to dysplasia and ultimately gastric cancer (Kusters *et al*., 2006). Intestinal metaplasia is associated with increased risk of developing gastric cancer. Though intestinal metaplasia is the initial stage for gastric cancer development, there are emerging evidences that it may be reversed on long term follow up with *H. pylori* eradication and along with the use of anti-oxidant agents (Walker, 2003).

1.6.5 Gastric cancer

The association of *H. pylori* infection with gastric cancer has been reported by several early studies (1993; Forman *et al*., 1990). The colonization of *H. pylori* increases the risk of gastric cancer 10 folds and, on the basis of several studies, *H. pylori* has been classified as Class I carcinogen by the WHO (1994).

H. pylori induced chronic inflammation has been reported in gastric carcinogenesis, as IL-1β, a prominent cytokine secreted in chronic inflammation acts as a potent inhibitor of gastric acid secretion and has been shown to trigger DNA damage of gastric epithelial cells by increased production of free radicals (reactive oxygen and nitrogen species) (Baik *et al*., 1996; Katsurahara *et al*., 2009), also causing hyper proliferation of gastric epithelial cells (Brenes *et al*., 1993). Additionally, the reduced acid secretion permits growth of other bacteria that enhance the production of highly carcinogenic N-nitroso compounds (Wang *et al*., 2014; Yeomans *et al*., 1995).

Mechanisms other than inflammation could also promote *H. pylori* induced carcinogenesis. *H. pylori* has been noted to reduce DNA repair *in vivo* and *in vitro* as well (Ladeira *et al*., 2004; Obst *et al*., 2000; Toller *et al*., 2011). Recently, much attention has been given to the relationship between *H. pylori*, stem cells and cancer. It has been proposed that *H. pylori* alters the maturation process of epithelial stem cells by damaging the parietal cells (Correa and Houghton, 2007; Houghton *et al*., 2004).

1.6.6 Gastric MALT lymphoma

Gastric Mucosa Associated Lymphoid Tissue (MALT) lymphoma is a rare disorder of the gastric epithelium which occurs only in less than 1% of *H. pylori* positive patients (Parsonnet and Isaacson, 2004). *H. pylori* infected patients have increased risk of developing the disease and also, all MALT lymphoma patients have been shown positive for *H. pylori* that confirms the association of the disease with *H. pylori* colonization (Eidt *et al*., 1994).

In 60-80 % of the cases, complete remission of MALT lymphoma has been reported by eradicating the bacterium, and complete remission from early phase of stage one (IE MALT) has

been reported in several studies (de Mascarel *et al*., 2005; Fischbach *et al*., 2004; Nakamura *et al*., 2005; Wundisch *et al*., 2005). In these studies, the long term follow up for remission of MALT lymphoma is mandatary because in 10-35 % cases, recurrence of the disease has been reported (Raderer *et al*., 2005).

1.6.7 Gastro-esophageal Reflux Disease (GERD)

GERD is characterized by flow of gastric acid and stomach content from the stomach to the esophagus which may be in response to mucosal damage. *H. pylori* presence is inversely associated with the GERD disorder which might be due to the fact that *H. pylori* induced chronic inflammation suppresses the acid production in the stomach which in turn doesn't irritate the mucosa of the stomach. Thus *H. pylori* prevents the development of GERD. This has been proved by the increased incidences of GERD and its complications in GERD patients who underwent *H. pylori* eradication (Labenz *et al*., 1997). However, eradication of *H. pylori* in gastritis patients who already had GERD, didn't exacerbate GERD. This points, that *H. pylori* can be eradicated, irrespective of the GERD status from gastric disease patients (Kuipers *et al*., 2004).

1.7 Diagnosis of H. pylori

There are several tests to diagnose *H. pylori* in human stomach. All of these tests can be divided into two categories; first, invasive tests that involve the isolation of gastric tissue specimen and second, non-invasive tests involving peripheral samples (Kusters *et al*., 2006) (as mentioned in Table 1.1). The choice of test depends on clinical set up and subject's choice. Generally, for diagnosis purpose, only one test is sufficient, but for research purposes, a combination of two tests is recommended. One rapid and cheap test to diagnose *H. pylori* is rapid urease test, also known as CLO (Campylobacter-Like Organism) test. In this test, sample or gastric biopsy is kept

in a medium containing urea and pH indicator like phenol red. In samples positive for *H. pylori*, bacterial urease enzyme hydrolyze urea into ammonia and carbon dioxide, thus producing ammonia and thereby increasing the pH that converts yellow sample to red (positive) (Midolo and Marshall, 2000). Urea breath test is another noninvasive and rapid test to diagnose *H. pylori* but is expensive due to high cost of ^{13}C production. Urea breath test utilizes urease enzyme to break ^{13}C labelled urea into ammonia and carbon dioxide. The ^{13}C labelled CO_2 diffuses into

Table 1.1: Methods used to diagnose *H. pylori* infection (Kusters *et al*., 2006)

Diagnostic method	**Sensitivity**	**Remarks**
Invasive methods		
Rapid urease test	>90%	Cost effective and rapid, require additional test to confirm infection of *H. pylori*
Histology	>95%	Gold standard for routine use in hospitals, requires expert pathologist and also provide data on inflammation and gastric atrophy
Culture biopsy	>95%	Alternate gold standard but requires expert microbiologist
Noninvasive methods		
Urea breath test	>95%	Alternative gold standard, generally used for testing *H. pylori* eradication, limited use due to high cost
Fecal antigen test	>90%	Simple and can be used for children
Serology	80-90%	Mainly used for epidemiological studies, cannot be used for *H. pylori* infected patients because of immunological memory

blood and excreted by lungs as breath which can be detected and measured by isotope ratio mass spectrometer (IRMS) (Savarino *et al*., 1999).

Molecular microbiologists diagnose *H. pylori* using molecular techniques such as PCR and approaches based on nucleic acid sequences assumed to be based on the fact that each microbial species has unique genetic makeup. PCR can be used to detect a single gene or its part or a non-coding sequence. These PCR based tests are extremely sensitive and don't need live bacteria (Datta et al., 2003a). This technique has been used with gastric biopsy, saliva, stool and gastric

juice samples (Dube *et al*., 2009). *H. pylori* has been detected using conventional PCR, reverse transcriptase PCR (RT-PCR), real time PCR (qPCR), nested PCR and multiplex PCR (Bolek *et al*., 2007; Wong *et al*., 2001). Multiplex PCR is fast and accurate and is widely used (Smith *et al*., 2002) while qPCR offers speed, robustness, sensitivity and specificity but needs extensive optimization (Espy *et al*., 2006). Serological test is suitable for epidemiological research with large sample size. Stool test is used for children to detect the presence of *H. pylori* antigens (Kusters *et al*., 2006).

1.8 Treatment of H. pylori infection

Antibiotic therapy for *H. pylori* eradication embraced several changes in course of time since the bacteria was discovered. At present, triple or quadruple therapy is used for *H. pylori* eradication with efficiency of 85-90%. In first-line triple therapy, clarithromycin and metronidazole are two most frequently used antibiotics along with either a proton pump inhibitor (PPI) or a bismuth compound for 7-10 days with recommended dose twice a day (Suzuki *et al*., 2010). In cases wherein *H. pylori* developed resistance to clarithromycin, it can be replaced with either tetracycline or amoxicillin. A combination of rifabutin and amoxicillin is used if *H. pylori* developed resistance to both clarithromycin and metronidazole, (Guo *et al*., 2004; Megraud, 2000). Triple therapy has been reported to fail in almost 20% cases which is followed by the second line quadruple therapy using two antibiotics, tetracycline and metronidazole along with a bismuth compound and a PPI (Suzuki *et al*., 2010). Bismuth is used as anti-bacterial for *H. pylori* which works by inhibition of protein and cell wall synthesis and formation of plasma membrane, and impairment of *H. pylori* adherence to gastric epithelium (Lambert and Midolo, 1997). Most frequently used PPIs in triple and quadruple therapy include omeprazole, lansoprazole, ranitidine bismuth citrate, pantoprazole, levofloxacin or rabeprazole. In the cases of failure of second line

therapy, the patients are evaluated by case to case approach for antibiotic sensitivity and treated according to the recommendations of European guidelines for management of *H. pylori* infection (Gisbert and Pajares, 2002; Malfertheiner *et al*., 2012). At present, fluoroquinolones based rescue therapy is also being used to further prevent the resistance of *H. pylori* to quinolone (Chuah SK, 2014).

None of the antibiotic therapy is 100% effective because the bacteria live in high acidic conditions and inside mucosal layer. The antibiotic resistance has been acquired due to indiscriminate use of these antibiotics for the treatment of infectious diseases (Megraud, 2004) and natural competence of *H. pylori* which enables the bacteria to acquire markers for antibiotic resistance (Kim *et al*., 2001). Thus success of antibiotic therapy depends on patients' compliance and susceptibility of antibiotics. Any adverse effect of antibiotics may lead to non-compliance and failure of the treatment.

1.9 Genetic diversity of H. pylori

The most characteristic feature of *H. pylori* genome is its heterogeneity or genomic diversity. Any two positive individuals harbor different strains of the bacteria. However, the difference may be small (Kansau *et al*., 1996). The extent of genomic heterogeneity in *H. pylori* can be understood by a case study of *H. pylori* infected African duodenal ulcer patient. *H. pylori* 908 strain was isolated from the antrum region of the stomach in 1994, followed by recrudescence of two new serial isolates *H. pylori* 2017 and *H. pylori* 2018 from antrum and corpus region respectively in 2003 from the same patient, 10 year apart (Avasthi *et al*., 2011). This suggests that a single strain of *H. pylori* can evolve into new and different strains to adapt to changing host.

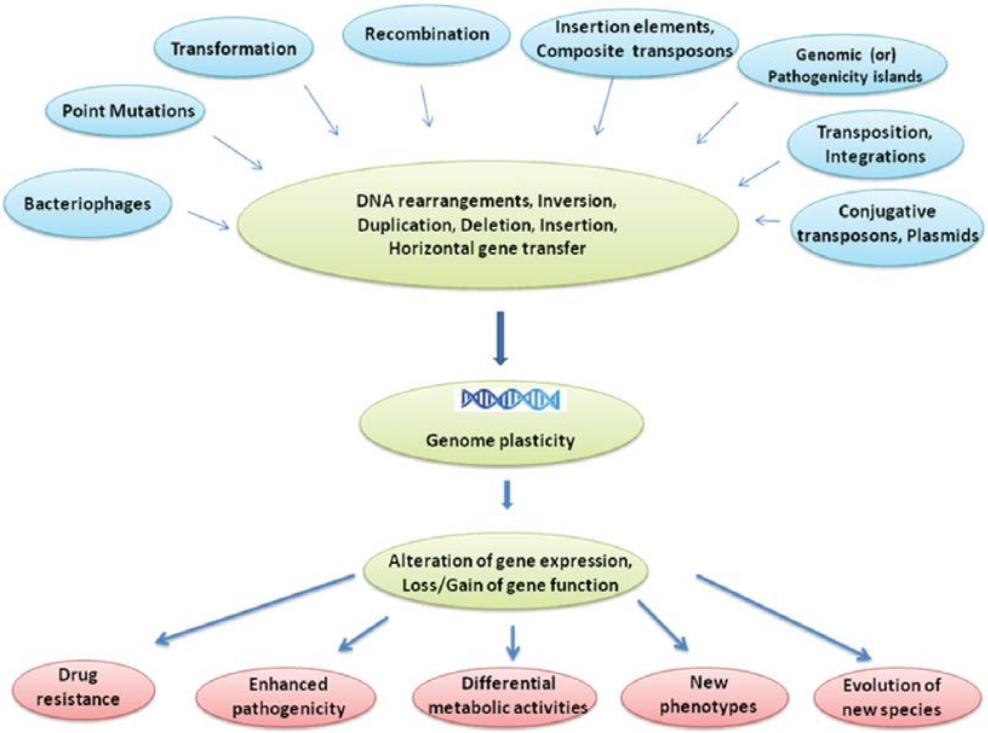

Figure 1.3: Mechanisms that confer genetic diversity to *H. pylori* (Ahmed N *et al*., 2012).

The genetic heterogeneity of *H. pylori* has been reported to be acquired through various genetic mechanisms (Figure 1.3) (Ahmed N, 2012). These different mechanisms include mutation, transformation, recombination, transposition and horizontal gene transfer and result into DNA rearrangements such as inversion, duplication, deletion and insertions leading to alternation in gene expression and loss or gain of gene function which in turn could be responsible for evolution of differential metabolic activities, drug resistance, novel phenotype and ultimately evolution of new strains of *H. pylori* (Ahmed N, 2012).

This high genetic heterogeneity of *H. pylori* hints at the capacity of the bacteria towards adaptive evolution in rapidly changing gastric environment (Akopyants *et al*., 1995; Baltrus *et al*., 2008) and is achieved predominantly by frequent recombination and high rate of mutation (Akopyants *et al*., 1995; Falush *et al*., 2001). The recombination frequency in *H. pylori* has been observed

more than that of any other bacterium known to date (Falush *et al*., 2001; Suerbaum and Achtman, 1999). The reason behind the high frequency of recombination is natural competence of *H. pylori* for transformation which allows the bacteria to uptake foreign DNA and incorporate into their genome by recombination (Suerbaum and Achtman, 1999). This competence of *H. pylori* is conferred by *com*B cluster, a genomic island for competence which is independent of any other genomic islands. This island is indispensable for *H. pylori* competence by allowing uptake of plasmid or chromosomal DNA through natural transformation and/or horizontal gene transfer. This *com*B gene cluster consists of genes *com*B7 to *com*B10 (Hofreuter *et al*., 1998) and every gene from this cluster is essential for competence and natural transformation (Hofreuter *et al*., 2001). Later, one more gene HP0017, a homologue of virB4 was found to play role in competence (Hofreuter *et al*., 2003). It is important to note that *com*B cluster was reported in all *H. pylori* strains, and this conserved nature of *com*B cluster in *H. pylori* explains high genetic diversity and genomic fluidity *via* frequently occurring phenomena such as natural transformation and transposition (Falush *et al*., 2001; Gressmann *et al*., 2005) to adapt and colonize the changing host- niche environment.

Horizontal gene transfer mediated by recombinational insertion results in formation of genomic islands. In *H. pylori* three types of genomic islands have been reported which encode components of T4SS; *com*B gene cluster, which has been already described above, *cag*PAI and plasticity zone (Ahmed N, 2012). Genes of these genomic islands play major role in origin of genetic diversity in *H. pylori* which ultimately culminates in generation of novel phenotypes with differential bacterial fitness in changing host response and environmental conditions (Ahmed N, 2012).

*Cag*PAI is a 40 kb genomic island that encodes components of T4SS which facilitate the injection of CagA and other bacterial molecules into the host gastric epithelium. This island is thought to be acquired by ancestral *H. pylori* populations that arose on different continents before agriculture began in the civilized world (Ahmed *et al*., 2009). Subsequent changes in environment, food habits and host response might have led to continent specific *CagA* and *Cag*PAI genotypes which were better adapted to a particular geographical region as evidenced by existence of Western and East Asian type *CagA* (Hatakeyama, 2004). This hints that *cag*PAI acts as a platform to accommodate genetic diversity in *H. pylori* genome. Moreover, more than one third of the *cag*PAI genes, mainly those encoding cell surface proteins have undergone diversifying selection. Furthermore, considerable genetic diversity has also been reported in housekeeping genes of *H. pylori* (Olbermann *et al*., 2010).

The third type of genomic island, the plasticity zone, which is acquired by horizontal gene transfer also plays major role in genetic diversity of *H. pylori* genome. Horizontal gene transfer is characterized by the presence of DNA regions with 35% G+C contents against 39% described on an average for a *H. pylori* genome. One of these regions is plasticity zone, which was identified by comparing genomes of two strains, *H. pylori* 26695 and *H. pylori* J99 (Alm *et al*., 1999; Tomb *et al*., 1997). The plasticity zone contains approximately 50% of strain specific genes. Kersulyte *et al* reported that plasticity zones act as novel transposable elements (TnPZs) and 65% of *H. pylori* stains had the genes derived from these plasticity zones (Kersulyte *et al*., 2009). Moreover, most of the ORFs of the plasticity zone encode putative proteins with unknown function and some ORFs show protein level homologies to recombinases, integrases and topoisomerases (Occhialini *et al*., 2000). These observations on plasticity zones hint that plasticity zones might be hot-spots for gaining genetic diversity in *H. pylori* genome.

Slipped strand mispairing during DNA replication also confers great extent of genomic diversity. Many genes encoding outer membrane proteins (OMPs), DNA restriction/modification systems and LPS have shown homopolymeric or dinucleotide sequence repeats. During replication, at these sequences the chances of frame shift mutations are very high due to slipped strand mispairing and these DNA regions with repeat sequences are the hot-spots for mutations and regulate gene expression by switching off or on or by affecting the promoter activity (Alm *et al*., 1999; Henderson *et al*., 1999).

Lack of DNA repair systems in *H. pylori* contributes to genetic diversity. High rate of mutation, especially point mutation in *H. pylori* might be due to limited presence of DNA repair systems. This high rate of point mutation leads to development of antibiotic resistance (Megraud, 2003). RecA is the only reported recombination repair system while other repair systems that are present in other microorganisms are absent in *H. pylori* (Kraft and Suerbaum, 2005).

1.10 Survival strategies of H. pylori

1.10.1 Urease activity and pH homeostasis

The role of urease in nitrogen metabolism, by producing ammonia is well defined but excess ammonia produces toxic effects on gastric epithelium and *H. pylori* produces urease up to 10% of total proteins (Kusters *et al*., 2006). Then why *H. pylori* produce this much urease? And how ammonia, a product of urea, is utilized by bacteria?

On the basis of several investigations, researchers concluded the role of urease enzyme in pH homeostasis inside the high acidic gastric environment. This could be understood by the above proposed model (Figure 1.4) which suggests the use of extracytoplasmic and cytoplasmic urease enzyme to maintain pH homeostasis (Stingl *et al*., 2002; Stingl and De Reuse, 2005).

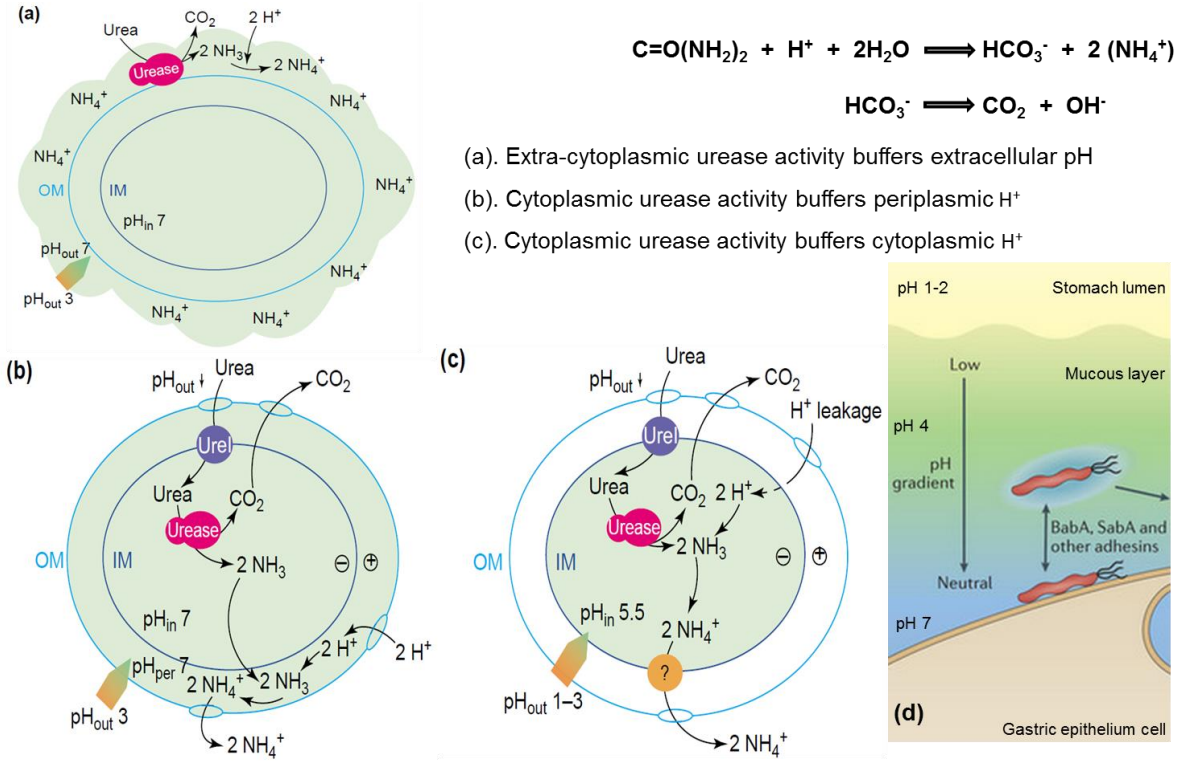

Figure 1.4: Models for Urease dependent pH homeostasis in acidic environment (Stingl K *et al*., 2002 and Salama N *et al*., 2013).

Extracytoplasmic urease is used to neutralize acidic microenvironment outside the bacterial cells by producing ammonia clouds around the bacterial cells. Cytoplasmic urease buffers protons by two ways: first, the ammonium produced by urease is transported to periplasmic space which buffers protons there, thus prevents acidification of the periplasm. Second, the ammonia produced by urease activity in the cytoplasm buffers protons leaking in through the cytoplasmic membrane and charged ammonium ions (NH_4) are extruded from cytoplasm probably through putative ammonium (NH_4) transporter. Thus urease activity maintains pH homeostasis inside and outside the bacterial cells (Stingl *et al.*, 2002; Stingl and De Reuse, 2005)

1.10.2 Evasion of innate immune response

Innate immune response is the first line of defense. It employs various types of cells, i.e., monocytes, neutrophils, NK cells and dendritic cells to recognize and eliminate bacteria from the infection sites. *H. pylori* uses several strategies to evade innate immune system of the human host. Unlike other Gram negative bacteria, *H. pylori* avoids phagocytosis at sub mucosal sites. It is reported that a particular type of professional phagocytes engulf *H. pylori*. Delayed phagocytosis by macrophages has been reported resulting in accumulation of *H. pylori* in 'megasomes' from phagosome fusion. In these phagocytes *H. pylori* induces apoptosis thus enabling escape of bacteria (Allen *et al*., 2000; Chaturvedi *et al*., 2004).

H. pylori employ other important strategies for survival by producing arginase enzyme. They resist the toxic effects of ROS produced by activated neutrophils and monocytes by harnessing catalase and superoxide dismutase activity (Ramarao *et al*., 2000; Seyler *et al*., 2001). The bacterial arginase utilizes L-arginine (at physiological concentration), a common substrate for arginase and iNOS of macrophages and thus limits L-arginine to be utilized by macrophages. The macrophages utilize L-arginine to produce nitric oxide (NO) which is an antimicrobial agent for killing bacterial pathogens (Gobert *et al*., 2001). Thus, arginase of *H. pylori* inhibits production of nitric oxide by macrophages and helps *H. pylori* to evade the essential part of innate immunity.

TLRs are used to recognize Gram negative bacteria by macrophages but *H. pylori* has PAMPs that have evolved to subvert recognition by TLRs expressed on macrophage surface. LPS of *H. pylori*, with only four acyl chains instead of six is 1000 times less active to be recognized by TLR4 (Moran, 1999; Moran *et al*., 1997). Also, flagellin of *H. pylori* is not recognized by TLR5 due to difference in amino acid sequence at TLR5 recognition site (Andersen-Nissen *et al*., 2005). DNA of *H. pylori* is not recognized by TLR9 due to high methylation. It is well known

that TLR9 recognizes unmethylated CpG islands of bacterial and viral DNA (Blaser and Atherton, 2004)

In stress conditions, when rod shaped *H. pylori* is converted to coccoid form, the bacteria use other approaches to escape from host detection. The coccoid form of *H. pylori* is not sensed by NOD1 receptors and also unable to activate NFκB in gastric epithelial cells (Chaput *et al*., 2006; Viala *et al*., 2004).

Dendritic cells (DCs) in the gastrointestinal mucosa are unable to recognize LPS of *H. pylori.* However, in response to *cagA* positive *H. pylori*, DCs produce IL-12 to induce Th1 specific response (Guiney *et al*., 2003). Simultaneously, DCs produce IL-10 in moderate amount inducing Th2 specific response and activate T-regulatory cells (T_{reg}) (Kranzer *et al*., 2004). This, in turn, suppresses T-cell memory response to *H. pylori*. Thus dendritic cells act as bridge between innate and adaptive immune responses.

1.10.3 Evasion of adaptive immune response

Induction of both local and systemic antibiotic responses by *H. pylori* has been reported through induction of IgA, IgM and IgG antibodies (Crabtree *et al*., 1991; Rathbone *et al*., 1986) but elimination of the bacteria in response to these antibodies is ineffective (Ermak *et al*., 1998; Thomas *et al*., 1993). This may be due to phase variability in surface proteins of the bacteria and inaccessibility of the bacteria to the antibodies since bacteria reside in the gastric mucosal protective layer.

Persistent colonization with *H. pylori* causes chronic active gastritis which increases accumulation of $CD4^+$ T_H cells responsible for Th1 response by secreting proinflammatory cytokines IL-12, IL-8 and TNF-α (Tummala *et al*., 2004). This response causes progression of

chronic active gastritis to atrophic gastritis and gastric cancer (Houghton *et al*., 2002) subverting the adaptive immune response of the host. *H. pylori* overcome the adaptive responses in many ways mainly by inducing apoptosis of T cells or by suppressing the cellular responses. *H. pylori* has been reported to induce T cells' apoptosis as one of the ways of immune evasion (Wang *et al*., 2001). Gamma-glutamyl transpeptidase has also been reported to show immunosuppressive effect by inhibiting T cell proliferation (Schmees *et al*., 2007). *cag*PAI-positive *H. pylori* has also shown to trigger DCs mediated increase in Treg cell population which in turn suppress the T_H17 and T_H1 cells, and memory T cell response to *H. pylori* infection (Lundgren *et al*., 2003).

1.11 Bacterial factors

Although the severity of chronic inflammation and gastric disease depends upon interplay of host, bacterial and environmental factors, various bacterial virulence factors play prominent role in the onset of gastric disease. Several studies on these bacterial factors have unraveled their involvement in the infection outcome, severity and progression of the gastric disease (Figure 1.5).

Among these bacterial factors encoded by various clinical *H. pylori* strains, two most studied virulence factors are CagA and VacA. There is ongoing interest in another pathogenicity island, plasticity zone. Genes in this region encode proteins with unknown function. As the data available until now on known bacterial factors of *H. pylori* seem to be inadequate to fully understand the pathogenesis of the gastric disease, researchers believe that investigations on the role of these putative factors, encoded by these genes may shed some light on the pathogenesis of gastric inflammation and gastric cancer. Several outer membrane proteins have also been characterized as virulence factors as they are inevitable for adhesion and expression of the bacterial virulence.

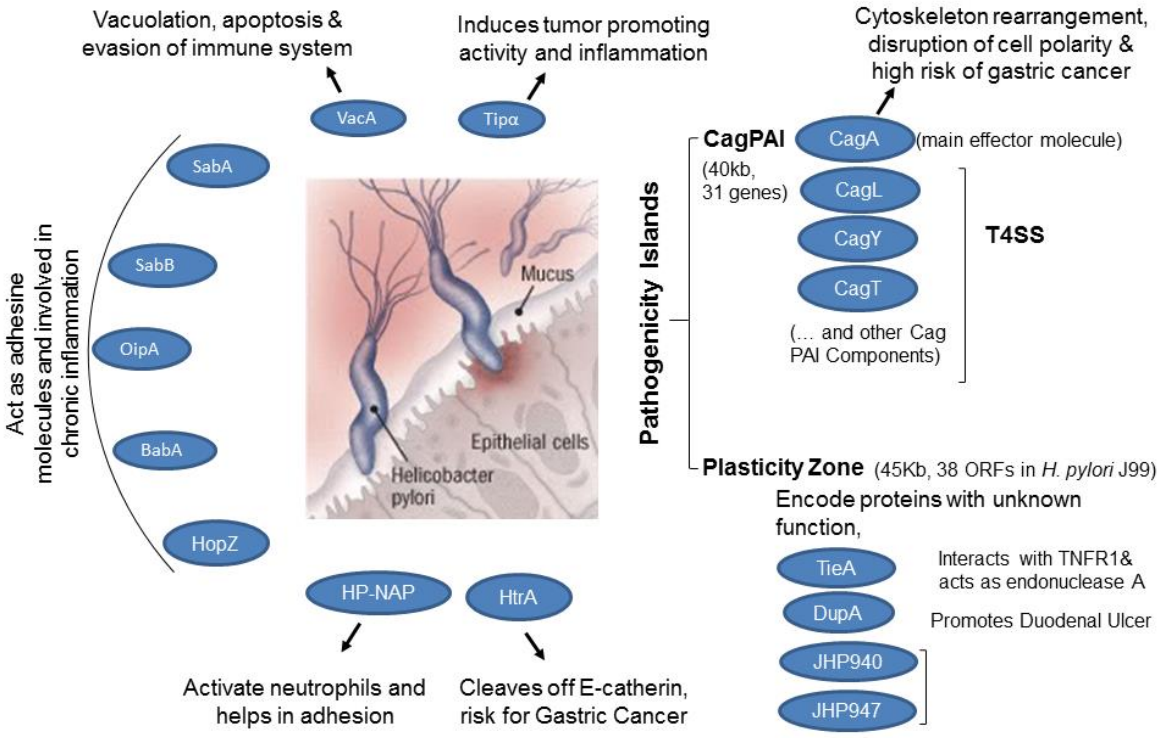

Figure 1.5: Diagrammatic representation of *cag*PAI and virulence factors of *H. pylori.*

1.11.1 CagPAI

Pathogenicity islands are genomic islands in *H. pylori* which encode virulence determinants and are acquired by horizontal gene transfer. In *H. pylori* there are two types of pathogenicity islands; *cag* (cytotoxin-associated gene) pathogenicity island (*cag*PAI) and plasticity zones (PZs). *cag*PAI is a 40 kb genomic island and contains approximately 30 genes (Yamaoka, 2008). *cag*PAI encoded proteins act as building blocks for type IV secretion system which assembles in a syringe like structure facilitating the translocation of CagA, a cardinal effector encoded by this region and probably other macromolecular bacterial factors into the host cells (Christie and Vogel, 2000; Covacci *et al.*, 1993; Fischer *et al.*, 2001).

Mutational analysis of *cag*PAI genes showed that products of most of the *cag*PAI genes are

involved in formation and assembly of secretory apparatus of T4SS and are essential for CagA translocation into the host epithelium. Approximately half of the *cag*PAI encoded proteins induced transcription of IL-8 (Fischer *et al*., 2001; Glocker *et al*., 1998) which is a major chemokine for infiltration of neutrophils at the site of infection causing inflammation of gastric mucosa (Bodger and Crabtree, 1998; Crabtree, 1998). Since mutations in several of *cag*PAI genes affect phosphorylation of CagA at tyr residues and downstream signaling to induce secretion of IL-8 (Censini *et al*., 1996), CagA is considered as a chief effector molecule of *H. pylori*.

1.11.2 CagA

CagA, one of the two most studied bacterial factors is encoded within *cag*PAI and is present in about 60% strains of western countries and is considered as a marker for the presence of a 40 kb *cag*PAI (Hatakeyama, 2004). Clinical and epidemiological studies have described a significant association between *cagA* positive *H. pylori* strains with higher degree of inflammation and epithelial damage in gastric mucosa and enhanced risk for gastric cancer (Nogueira *et al*., 2001).

CagA is translocated into epithelial cells through syringe like structure formed by components of T4SS and it is reported to bind with at least 20 known cellular binding proteins in CagA phosphorylation dependent and independent manner, performing different functions (Backert *et al*., 2010; Selbach *et al*., 2009). Furthermore, CagA is noted for region specific structural variations in EPIYA motifs and varied functions (Figure 1.6). After integrin α5β1 mediated entry through basolateral part of the gastric epithelial cells, CagA is directly phosphorylated by Src and Abl kinases at tyrosine residue in EPIYA motifs which are located within the 3' terminus of CagA (Kwok *et al*., 2007; Selbach *et al*., 2002; Stein *et al*., 2002; Tammer *et al*., 2007). This

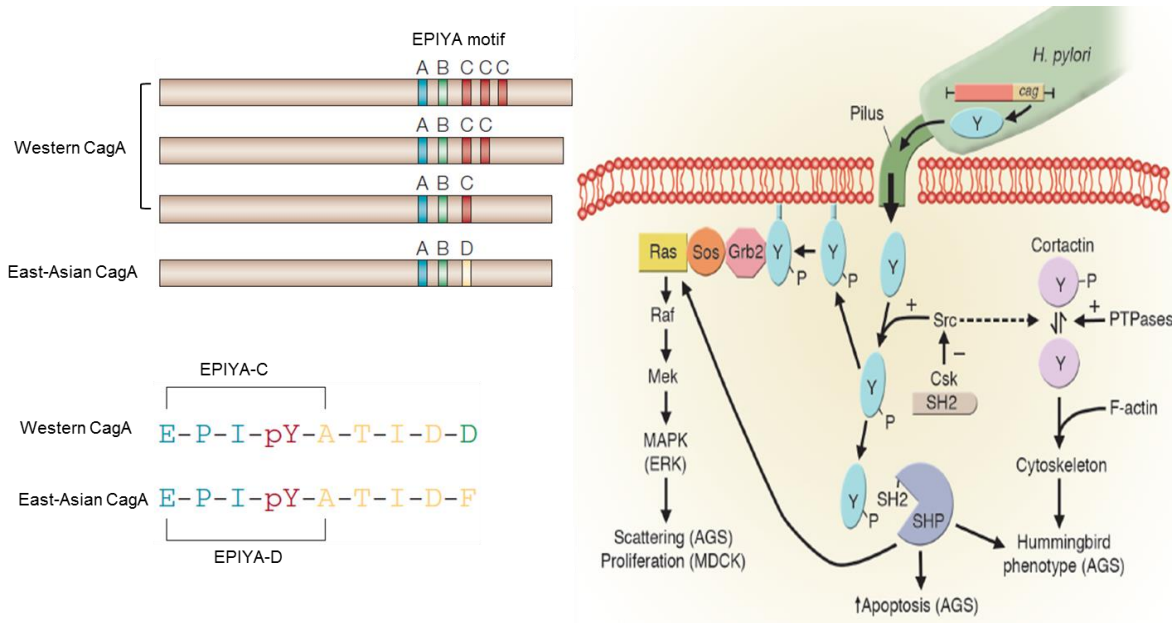

Figure 1.6: CagA structure variation and functions (Hatakeyama M, 2004 and Blaser M *et al*., 2004).

phosphorylated CagA bind to Src homology 2 domain containing phosphatase-2 (SHP2) which results in cytoskeleton rearrangements, cell elongation and motility (Figure 1.8). Moreover, CagA phosphorylation by SRC is controlled by feedback mechanism (Higashi *et al*., 2002). Apart from eliciting the phosphorylation independent signalling pathway, CagA may also undergo self-dimerization which is dependent on CagA multimarization (CM) sequence located within EPIYA-C segment (Ren *et al*., 2006). This CM sequence is also responsible for binding of CagA to PAR1 and binding of Cag-PAR1 (MARK) complex to non-phosphorylated CagA leading to disruption of epithelial cell polarity by inhibiting the kinase activity of PAR1 (Ren *et al*., 2006; Saadat *et al*., 2007).

1.11.3 VacA

Other of the two most studied and genetically diverse bacterial virulence factors is VacA. It is an immunogenic, ~95 kD, multifunctional protein and induces cell vacuolation, membrane channel

formation, disruption of endosomal/lysosomal function, apoptosis, and immune-modulation in gastric epithelial cells (Cover and Blanke, 2005). *vacA* gene is present in almost all *H. pylori* strains. However, approximately 50% *H. pylori* strains secrete the VacA.

Four polymorphic regions have been identified in *vacA*: a signal sequence(s) region (encoding the signal peptide which exists as s1 or s2 genotype), the mid region(m) (encoding the membrane binding site and exists as m1 or m2 genotype), the intermediate(i) region and deletion(d) region. The region between i and m region exists as d1 (no deletion) or d2 (a 69- 81 base pair deletion). It is the mosaic combination of s- and m- genotype which relates with the cytotoxicity of the *vacA*; s1m1 strains have higher cytotoxic activity than s1m2 strains whereas s2m1 and s2m2 strains have no cytotoxicity (Letley *et al*., 2003). The i-region has been described as polymorphic determinant of vacuolation activity in s1m2 strains. A variation in i-region affects the ability of these strains to induce vacuolation (Palframan *et al*., 2012). The presence of d1 is associated with the risk factor for gastric mucosal atrophy in mainly western strains (Ogiwara *et al*., 2009). The s1-m1-i1-d1 genotype of s-m-i-d allelic combination is found to be a risk factor for gastric atrophy than the other combinations (Ogiwara *et al*., 2009).

The Secreted VacA has two functional domains, the N-terminal P33 domain containing hydrophobic sequence involved in pore formation whereas other c-terminal P55 domain is responsible for cell binding activity through proteolytic cleavage (Figure 1.7) (Cover and Blanke, 2005; Palframan *et al*., 2012; Polk and Peek, 2010). VacA by binding to multiple epithelial cell surface components affects cellular processes *via* different routes. It may either bind to cell surface receptors such as epidermal growth factor receptor (EGFR) (Seto *et al*., 1998) or transmembrane protein receptor-type tyrosine protein phosphatase ζ (PTPRZ1) (Fujikawa *et al*., 2003) initiating a proinflammatory response and mitochondrial like mediated apoptosis.

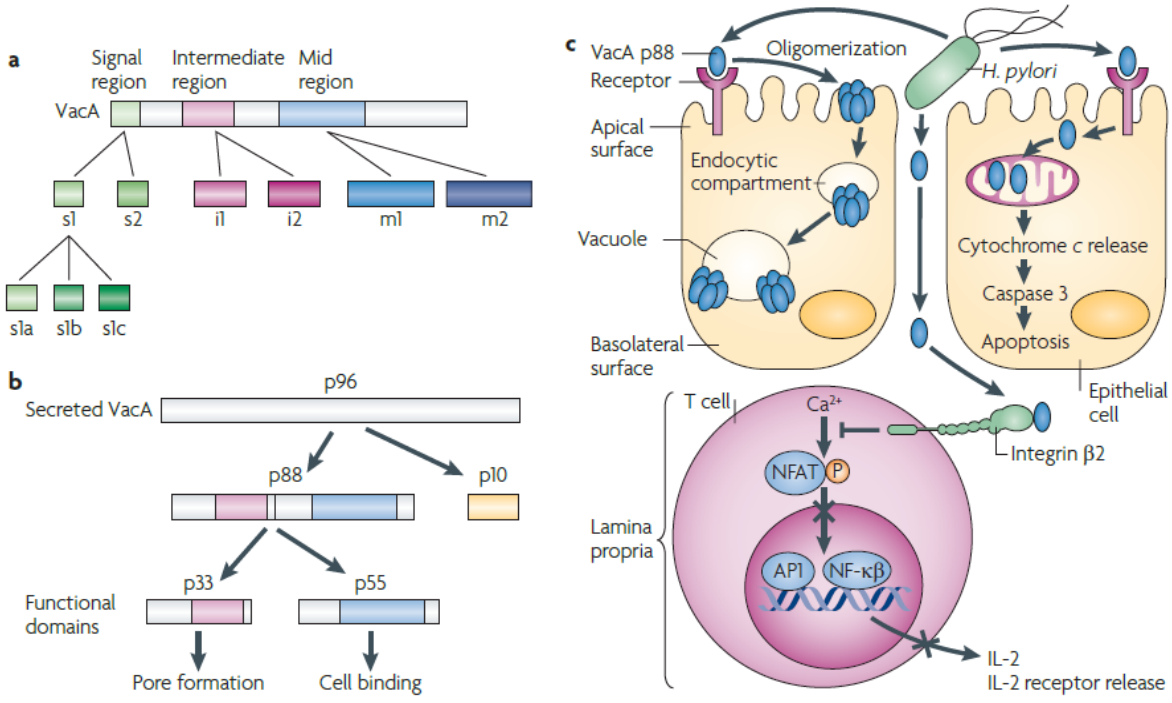

Figure 1.7: Structure and functions of *H. pylori* VacA (Polk DB *et al*., 2012).

VacA oligomerization results in vacuolation by pinocyte like mechanism forming membrane channels in epithelial cells that allow release of ions, nutrients and urea from the host cell (Palframan *et al*., 2012). VacA by binding to CD-18 (integrinß2) ultimately leads to inhibition of primary human T cell (CD 4^{+}) proliferation (Sundrud *et al*., 2004). Furthermore, *H. pylori* blocks the proliferation of $CD4^{+}$ T-cells with the help of VacA and γ-glutamyl transferase (Beigier-Bompadre *et al*., 2011) and inhibition of T lymphocyte activation (Gebert *et al*., 2003) thus opening a gate for *H. pylori* to evade the adaptive immune response.

1.11.4 LPS

Unlike LPS of the other Gram negative bacteria, LPS of *H. pylori* is less toxic and pyrogenic, and less responsive to the innate system of the human host (Muotiala *et al*., 1992). The LPS of *H. pylori* contains O-Lewis antigens (Lewis a, Lewis b, Lewis x and Lewis y). Somehow, these

Lewis antigens are also expressed on the surface of human gastric epithelium. Thus, due to antigenic mimicry, *H. pylori* evades the immune recognition (Appelmelk *et al*., 2000). Furthermore, these Lewis antigens help bacteria to get more gastric exudates and carbon source by inducing inflammation of gastric epithelium. Anti-Le^x (Lewis-x) antibodies produced as a result of Lewis antigen expression by *H. pylori* bind to *H. pylori* and as well as to gastric epithelium causing gastric autoimmune response and enhanced inflammation and injury of the epithelium (Appelmelk *et al*., 2000). Lewis antigens of *H. pylori* have also been shown to undergo phase variation and to play a limited role in adherence of *H. pylori* to the gastric epithelium, thus providing a dynamic adherent nature to the bacteria (Edwards *et al*., 2000; Mahdavi *et al*., 2003).

1.11.5 IceA

iceA is a homologue of putative bacterial restriction endonuclease. There are two clinical variants of iceA; iceA1 and iceA2. iceA1 is a homologue of type II restriction endonuclease of *Neisseria lactamica*. Adherence of *H. pylori* to the gastric epithelium induces the expression of iceA1 (Peek *et al*., 1998) and is associated with enhanced acute mucosal inflammation and peptic ulcer (Peek *et al*., 2000; van Doorn *et al*., 1998). The other allelic variant, iceA2, is also found to be associated with gastric disease and is a predictor of severe histological changes in gastric cancer (Chiurillo *et al*., 2010; Kidd *et al*., 2001). However, the exact role and mechanism of action of iceA is still not clear.

1.11.6 HP-NAP

It is a neutrophil activating protein which activates neutrophils by producing reactive oxygen species and stimulates neutrophils to adhere to endothelium and recruiting them to infected tissue. It was later confirmed as protective antigen and major virulence factor (Evans *et al*., 1995;

Satin *et al*., 2000). It is also reported that HP-NAP translocates through monolayered epithelial cells and stimulates peritoneal mast cells to secrete proinflammatory cytokines (Montemurro *et al*., 2002) which in turn recruit monocytes and neutrophils at the infection site and promote bacterial growth by facilitating the release of nutrients from the inflamed tissue (Montecucco and de Bernard, 2003). Simultaneously, it is also found protective for *H. pylori* DNA damage by oxidative free radicals (Cooksley *et al*., 2003).

The follow up studies on this bacterial factor suggest that HP-NAP activates polymorphonuclear cells (PMNs) and monocytes in addition to mast cells and promotes adherence of PMNs to the endothelium *in vitro*. Further, this factor stimulates leucocytes to recruit at endothelium and secretion of inflammatory cytokines to maintain inflammation (Polenghi *et al*., 2007). HP-NAP has also been shown to promote Th1 immune responses by inducing expression of IL-12 and IL-23 from innate immune cells (neutrophils and monocytes) and modulating the immune response from Th2 to Th1 (Amedei *et al*., 2006). Thus HP-NAP is a proinflammatory and immunomodulatory bacterial factor which helps in survival of the bacteria by providing neutrients and protecting from oxidative DNA damage and immunomodulate Th2 to Th1 response.

1.11.7 Outer Membrane Proteins (OMPs)

Approximately 4 percent of *H. pylori* genome (approx. 1600 genes) is predicted to encode outer membrane proteins (OMPs), some of which are thought to be involved in adhesion of the bacteria to the gastric epithelium. None of the OMPs alone is capable of adherence of *H. pylori* to gastric epithelium but the combined effect of all those identified and unidentified proteins is required. Thus adherence mechanism of *H. pylori* with its outer membrane proteins to the gastric

epithelium is an area of research, although, researchers have focused on some of the important outer membrane proteins e.g., OipA, SabA, BabA, SabB and HopZ etc.

1.11.8 HtrA

HtrA (high temperature requirement A) is a secretory serine protease which cleaves off the extracellular domain of E-cadherin in MKS-28 gastric cancer cells and then processes them into smaller fragments both during the infection and *in vitro* using the recombinant enzymes. HtrA of *H. pylori* acts as a high risk factor for gastric cancer (Hoy *et al*., 2012; Hoy *et al*., 2010) by degrading E-cadherin A loss of E-cadherin's adhesive property is a key step in tumor progression and metastasis (Cavallaro and Christofori, 2004).

1.11.9 Gamma-glutamyl transferase (GGT) and Asparaginase (AnsB)

Recently studied two secreted enzymes, the gamma-glutamyl transpeptidase (GGT) and the asparaginase (AnsB) are common in all *Helicobacter* strains (Leduc *et al*., 2010). GGT is known to induce mitochondria mediated apoptosis, affects proliferation of gastric cells by up-regulating growth factors (Busiello *et al*., 2004; Shibayama *et al*., 2003), and inhibit T-cell proliferation (Schmees *et al*., 2007).

The secreted enzyme asparaginase (AnsB) can deaminate asparagine and are essentially required for full colonization in animal model (Merrell *et al*., 2003). Also, the purified recombinant enzyme (AnsB) was shown to induce cytotoxic effects on cell lines (Cappelletti *et al*., 2008).

Thus, it seems that these two secreted enzymes GGT and AnsB may generate lesions in epithelial cells and have deleterious effects on immune cells by (i) depriving the gastric and immune cells of protective amino acids Gln and Asn and/or (ii) delivering NH_3 directly in the vicinity of

epithelial cells (Leduc *et al*., 2010). It has also been reported that GGT activity is higher in strains isolated from peptic ulcer than those from patients with dyspepsia (Gong *et al*., 2010).

1.11.10 TieA or HP0986

There are increasing evidences on a new novel virulence marker TieA (HP0986). *tieA* has been reported consistently in clinical isolates of gastric diseases from global sources (Alvi et al., 2011). Expression of *tieA* in biopsy samples of *H. pylori* infected individuals and seropositivity of TieA in *H. pylori* positive patients confirmed the expression and presentation of TieA to the immune system of the human host (Devi et al., 2014). The proinflammatory activity of TieA in human macrophages, gastric epithelium cells and mouse macrophages and proapoptotic effect of this factor in human and mouse macrophages suggest that the bacteria might harness the TieA induced inflammatory exudates for carbon and nutrient sources while TNFR1 mediated apoptosis to limit macrophages for immune subversion tactic (Alvi et al., 2011; Ansari et al., 2014; Devi et al., 2014).

1.12 Working Hypothesis and Objectives

Out of several bacterial factors studied so far, only a few have been reported as virulence factors. Most prominently reported are CagA, VacA and urease. CagA plays important roles in inducing peptic ulcer and gastric cancer while VacA is a risk factor for gastric atrophy and induces cell vacuolation, apoptosis and mediates immune evasion by the bacteria (Cover and Blanke, 2005; Ogiwara *et al*., 2009). *H. pylori* uses urease for pH homeostasis in highly acidic gastric environment (Stingl and De Reuse, 2005). Other than these virulence factors several adherent molecules necessary for bacterial adhesion and molecules involved in colonization have been reported. The putative virulence genes of *H. pylori* have been classified into three categories; strain specific genes, phase variable genes (most of the genes encode outer membrane proteins

and show slipped strand mispairing) and genes with different structures/genotypes formed as a result of combination of different genotypes. The *cag*PAI and plasticity region clusters fall in the category of strain specific genes and constitute pathogenicity islands (Yamaoka, 2008).

Being foreign in nature and acquired by horizontal gene transfer, there is an increasing interest, in recent times, in the genes from pathogenicity islands to explore their association with the gastric diseases, disease inducing potential and their cellular function and underlying mechanisms within the host cellular environment. *cag*PAI has been studied in detail from *H. pylori* isolates all over the globe with CagA as a cardinal and effector molecule from this island to induce more severe gastric cancer in most of the populations (Hatakeyama, 2004; Murata-Kamiya *et al*., 2007; Wroblewski *et al*., 2010). Other pathogenicity island, Plasticity Zone (PZ) has been considered important to explore because: 1) PZs from different geographic regions contain different genotypes giving the fitness advantage to the bacteria; 2) many of the genes from this region are linked with different stages of the gastric disease; 3) these genes encode proteins with unknown function and with no homologue in databases and several genes are putative homologues of the components of T4SS. Therefore, functions of genes from this region might play a major role in bacterial pathogenesis inside the host. Exploring the function of these genes may also address issues of long term colonization, genetic heterogeneity, immune evasion, persistent survival and disease inducing potential of *H. pylori*.

Many of the genes from plasticity zone, e.g. *jhp0940*, *jhp0947* and *dupA* from J99 and HP0986 from 26695 strains have been reported to be associated with gastric diseases (de Jonge et al., 2004a; Occhialini et al., 2000; Rizwan et al., 2008; Santos et al., 2003b; Yakoob et al., 2010b), though, there are some contradictory findings. Furthermore, HP0986 has been reported to interact with gastric epithelial AGS cells, found to be proinflammatory in nature in human and

mouse macrophages. Also, this bacterial factor was reported to induce apoptosis in human as well as in mouse macrophags by interacting with TNFR1 receptors (Alvi *et al*., 2011; Ansari *et al*., 2014; Devi *et al*., 2014). These findings further strengthen the idea that genes from plasticity region, despite having no homologue may encode functional bacterial factors to play important roles in bacterial survival and/or disease progression. Under this category, some important ORFs such as *jhp0940* and *jhp0947* have been described in literature to encode potential virulence factors (Rizwan et al., 2008). Having looked at their allelic and protein structures and their predicted functions, it was tempting to study them in detail. We therefore set out to carry out genotyping and population level screening of these ORFs and then functionally characterize them in detail (Tenguria et al., 2014). In this context, we framed the following three objectives:

1. **Screening for plasticity zone genes, *jhp0940* and *jhp0947* in *H. pylori* clinical isolates from different countries**
2. **To characterize function and secretion of JHP0940 and study its effect on mouse macrophage cells**
3. **To study multifaceted kinase activity of JHP0940/CtkA**

Chapter 2

Objective 1: *Screening for plasticity zone genes, jhp0940 and jhp0947 in H. pylori clinical isolates from different countries*

2.1 Introduction

Various bacterial virulence factors have been found to play a key role in *H. pylori* induced gastric disease and its progression, although environmental and host factors are also involved in outcome of the disease. Most of the consistently reported virulence factors are *cag*PAI (Backert *et al*., 2004; Censini *et al*., 1996), VacA (Cover, 1996; Cover and Blanke, 2005) and other outer membrane virulence associated factors like BabA (Boren *et al*., 1993; Rad *et al*., 2002), SabA (Mahdavi *et al*., 2002), SabB (de Jonge *et al*., 2004b), OipA (Yamaoka *et al*., 2000), IceA (Xu *et al*., 2002), DupA (Lu *et al*., 2005a), AlpA and AlpB. Though these virulence factors have been shown to play major role in unfolding the infection outcome and understanding the pathogenesis of *H. pylori* infection but couldn't explain it completely including the fact that why only a fraction of *H. pylori* positive patients develop severe form of the gastric disease and gastric cancer.

With the comparison of complete genome sequences of the two *H. pylori* strains 26695 and J99, a highly variable region with 35% G+C contents against 39%, on an average in *H. pylori* genome has been observed. This change in G+C content suggested the acquisition of this genomic island by horizontal gene transfer from unrelated species in ancient time. This 45kb long region with 38 genes in J99 strain consists approximately 50 per cent of the strain specific genes (Alm *et al*., 1999). Being foreign in nature, plasticity genes were assumed to encode putative virulence factors. Also, there are reports that plasticity zones act as novel transposable elements (Kersulyte *et al*., 2009) and/or they act as integrating conjugative elements (ICEs) with intermediate conjugative specificity (Fischer *et al*., 2014). These findings suggest that *H. pylori* may use plasticity zones for adaptations to changing environmental conditions during long term colonization. Thus the bacteria may use plasticity zone either as transposable elements or as

integrating conjugative elements. It is reported that some of the genes from plasticity zone of J99 strain are homologues of recombinase and/or integrase families, e.g., *jhp0941*and *jhp0951* encode predicted integrase/recombinase of xerCD family. These proteins might control recombination mediated insertions or deletions. Several genes from plasticity zones have been suggested as disease markers, e.g., *jhp0950* for marginal zone MALT-type B-cell lymphoma (Lehours *et al*., 2004) and *dupA* for promotion of duodenal ulcer (Hussein *et al*., 2012; Lu *et al*., 2005a) while other genes are also shown to be associated with gastric disease, e.g., *jhp0945* with duodenal ulcers, gastric ulcers and gastric cancer (Sugimoto *et al*., 2012).

The gene *jhp0940* has been reported to link with gastritis, peptic ulcer and gastric cancer in various regions of the globe, though there are some contradictory reports. However, the protein encoded by this gene has been recently characterized as a proinflammatory protein which acts as an autophosphorylating ser/thr and tyrosine kinase, triggers apoptosis in mouse macrophages, and induces IL-8 and TNF-alpha in human macrophages and increased translocation of NFκB in cultured macrophages (Kim do *et al*., 2010; Rizwan *et al*., 2008; Tenguria *et al*., 2014). These recent reports on the functional role of the protein JHP0940 makes it important to investigate status of the gene in more *H. pylori* isolates from gastric disease patients. Along with *jhp0940* we also checked the status of *jhp0947* gene in these isolates because *jhp0947* is known to be associated with gastritis and gastric cancer (de Jonge et al., 2004a; Santos et al., 2003c; Yakoob et al., 2010a). Moreover, the presence of *jhp0947* was found to be linked with other genes *jhp0945* and *jhp0949* (de Jonge *et al*., 2004a; Sugimoto *et al*., 2012). Therefore in this chapter, we investigated status of *jhp0940* and *jhp0947* in isolates of various stages of the gastric diseases from different geographical regions.

2.2 Materials and methods

2.2.1 Bacterial isolates: We made use of the *H. pylori* DNA collection of Ahmed Lab wherein a total of 215 DNA isolates of *H. pylori* (60 isolates from Ireland, 21 from Peru, 7 from South Africa, 35 from Turkey, 26 from Bangladesh and 66 from India) were available corresponding to different stages of the gastric disease as mentioned in Table 2.2.

2.2.2 Molecular detection of jhp0940 and jhp0947: Primers for *jhp940* and *jhp947* were designed using the sequence of reference strain J99 from PyloriGene web server. Primer sequences and optimised PCR conditions are mentioned in Table 2.1. PCR amplification was carried out in a total volume of 10µl containing 15 to 20 ng of DNA from each sample, 1µl of 10X Taq buffer with KCl (Fermentas, 100mM Tris-HCl, 500mM KCl, 0.8% Nonidate P40), 1.5 mM $MgCl_2$, 200 µM of each dNTP (Fermentas), 0.2µM of each primer, and 0.5 Unit of Dream Taq DNA polymerase (Fermentas) using Master Cycler (Eppendorff) PCR. J99 strain was used as a reference strain for positive control. Amplicons were separated on 1% agarose gel containing ethidium bromide (EtBr).

2.2.3 Status of jhp0940 & jhp0947 in sequenced strains of Helicobacter available in NCBI: The *jhp0940* and *jhp0947* gene sequences were downloaded from PyloriGene database and subjected to BLAST separately with *Helicobacter* genomes available in NCBI database to ascertain the homologies of both the genes (cut off values for query coverage and maximum identity were taken as 95 % and 90 %, respectively as shown in Table 2.3a and 2.3b).

2.3 Results

2.3.1 Prevalence of jhp940 and jhp0947 in gastric disease isolates: Many of the researchers have found paradoxical status of *jhp940* and projected it to be a novel virulence marker (Occhialini et al., 2000; Santos et al., 2003c; Yakoob et al., 2010a). Extending these findings, we

observed in our study (Table 2.2) that the gene failed to amplify in any of the 37 gastritis isolates from Ireland while amplified only in two of the 20 Intestinal Metaplasia isolates. The gene was negative in Peruvian gastric cancer strain PeCan18B, although we cannot ensure its association on the basis of only one isolate which we used but it has been shown that 60 per cent of the Peruvian isolates were positive for *jhp940* (Rizwan *et al*., 2008). The gene was present in all South African gastritis isolates. It is reported in a study that the gene shown 100% prevalence in gastritis isolates from South Africa (Rizwan *et al*., 2008). The ORF was present in only 8 of the 35 (22.85%) isolates (4/21 gastritis, 4/12 duodenal ulcer) from Turkey. In 26 isolates from Bangladesh, *jhp0940* was present only in 3 isolates. These isolates from Bangladesh were from *H. pylori* positive patients but irrespective of disease status. Furthermore, the gene was amplified in only 15.15% (ten out of 66) of gastritis isolates from India.

Another gene that was shown to be associated with gastric disease was *jhp0947*. We checked the gene in a total of 215 isolates from different stages of the gastric disease (Table 2.2). The gene was highly prevalent in Irish and South African strains showing 68 per cent (41/60) presence in Irish isolates and in 4 out of 7 gastritis isolates from South Africa. The gene was prevalent in 9 of the 20 (45%) gastritis isolates from Peru while it was absent in all 35 isolates from Turkey. In case of strains from Bangladesh and India, the gene was present in only 1 and 5 out of 26 and 66 gastritis isolates, respectively, which seems of no significance.

In the three sequenced strains 908, 2017 and 2018 of a duodenal ulcer patient from France, the gene was absent. Furthermore, the same status was also confirmed by microarray based comparative genomic hybridization by our laboratory.

2.3.2 Status of jhp940 in genomes of Helicobacter strains: In the published genomes and

plasticity zone sequences of *H. pylori*, *jhp940* was found in twelve genomes of different *H. pylori* strains (Table 2.3a) isolated from Asian gastric cancer (*H. pylori* F32, *H. pylori* 83) and gastritis (*H. pylori* Puno120, *H. pylori* SJM180 and *H. pylori* Puno135) patients. Furthermore, the presence of the gene in strains *H. cetorum* (host- Beluga Whale) MIT-00-7128 and in *H. cetorum* MIT 99-5656 (host- Atlantic white sided dolphin) with 100 per cent query coverage (in both), respectively, indicated simultaneous evolution of J99 strain in the Beluga whale and Atlantic white sided dolphin in the ancient times. Furthermore, a strong association of *jhp0940* with African isolates and its presence in Beluga whale open the possibility to explore J99 strain as an ancient strain and to explain basis for weak association of *jhp0940* with Asian and European geographical regions.

2.3.3 Status of jhp947 in genomes of Helicobacter strains: As shown in Table 2.3 and 2.4, *jhp0947* gene was found in gastritis, intestinal metaplasia and gastric cancer from different parts of the world. *jhp0947* has been found in 16 sequenced strains of *H. pylori* with cut off values of 100 % query coverage and 95 % maximum identity (Table 2.3b). The strains are from different geographical region and various stages of the gastric disease indicating that *jhp0947* is associated with *H. pylori* strains isolated representing gastric diseases of varied spectrum.

2.4 Discussion

To date, the function of most of the genes from plasticity zone is unknown. Since the plasticity zone has been obtained by horizontal gene transfer (Alm and Trust, 1999) from unrelated organisms and about half of the strain specific genes are present in this island hence these genes are being suggested to function as disease markers which may play role in development of the gastric disease and in virulence or pathogenesis of *H. pylori* infection. Some of the genes from

plasticity zone, for example, *dupA*, *jhp940*, *jhp947* have been envisaged to be virulence markers associated with certain stages of the gastric disease but in most of the cases prevalence of these genes varied geographically. For instance, *jhp0940* and *jhp0947* were found to be prevalent in gastric cancer patients from Costa Rica (Occhialini *et al*., 2000). Furthermore, in a study of 200 Brazilian strains of duodenal ulcer, gastric cancer and gastritis, it was found that *jhp0947* but not *jhp0940* was associated with gastric cancer and duodenal ulcer (Santos et al., 2003c) and *jhp0947* was envisaged to be a novel virulence marker candidate of chronic gastric disease.

The gene *jhp0940* was absent in gastritis and intestinal metaplasia isolates from Ireland, it was present in all gastritis isolates from South Africa. Also, the gene was absent in gastric cancer isolates from Costa Rica (Figure 2.1). Furthermore, 30% (6/20) of gastritis isolates but not gastric cancer isolate PeCan18B from Peru possess the gene. The sequenced *H. pylori* gastric cancer strain from Mongolian gerbil lacks the gene. There are reports that evidenced the deletion of some genes from *H. pylori* strains isolated from advanced stages of the gastric disease when chronic atrophic gastritis progressed to gastric cancer in the same patient over a time of four years (Oh *et al*., 2006) and it is possible that *jhp0940*, which was present in gastritis stage got deleted on progression of gastritis to gastric cancer. From these observations (Table 2.2 and Table 2.3a) we noticed that *jhp0940* showed geographic region specific distribution and inconsistent association, and is strongly associated with gastritis in South African strains while weak and arbitrarily associated in strains from other parts of the world. Also, there are reports from several geographical regions for the prevalence of *jhp0940* as shown in Table 2.4a (Table 2.4a: updated literary view on *jhp0940*).

This inconsistent and geographical region specific association of *jhp0940* with the gastric disease might be due to: 1) different migration patterns and admixture of human populations and various

types of genomic rearrangements in *H. pylori* genome, 2) some genes in the plasticity zone e.g., *jhp941* and *jhp0951* encode *xer*T recombinases for recombination dependent insertion and/or deletion and 3) plasticity zones of *H. pylori* act as novel transposable elements, for example in strains HP26695 and HPJ99 are complex mosaics of novel transposable element remnants which are formed by multiple transposable element insertion and spontaneous and transposable element mediated deletions. This suggests that plasticity zones are highly unstable which undergo frequent transpositions mediated by *Xer*T recombinases, rearranging the genes within the plasticity zone and giving instability to *jhp940* and probably to other genes of plasticity zone during the course of evolution (Kersulyte *et al*., 2009). However, functional studies on JHP940 have shown that the protein encoded by gene *jhp0940* induces TNF-alpha and secretion of interleukin 8 by human macrophages and enhanced translocation of transcription factor NF-kB complex in cultured macrophages (Rizwan *et al*., 2008). Recently, it was shown that the protein acts as cell translocating kinase and enhances the translocation of NF-κB by indirectly phosphorylating the p65 subunit at ser276 acting as ser/thr kinase (Kim do *et al*., 2010). Furthermore, the protein was recently reported to act also as tyrosine kinase and inducing apoptosis in mouse macrophage cells (Tenguria *et al*., 2014).

In several studies from various geographical regions of the world, *jhp947* was reported to be significantly associated with different stages of the gastric disease (Table 2.4b) (de Jonge *et al*., 2004a; Kersulyte *et al*., 2009; Modena *et al*., 2007; Romo-Gonzalez *et al*., 2009) but direct functional role of the gene has not been identified yet. However, in a study by de Jong *et al*., it has been shown that the disruption of *jhp0945-jhp0947-jhp0949* locus decreases the production of IL-12 in THP1 cells significantly in *in vitro* conditions. Indeed, it is not direct evidence of functionality of *jhp0947* and hints to explore it more in isolates from various geographic

populations. Therefore, we checked the status of *jhp0947* in other geographical regions as mentioned in Table 2.2. In gastritis and intestinal metaplasia patients from Ireland, *jhp0947* was present in 78.4 (29/37) and 50 (10/20)%, respectively. However, the gene was present in one of two duodenitis isolates we analyzed. In isolates from Peru and South Africa, the gastritis patients' isolates were found to be 45 (9/20) and 57 (4/7)% positive for the gene. Thus, prevalence of this gene in gastric patients from different countries (Table 2.4b, an updated view of *jhp0947* gene) points towards a virulence marker for the gastric disease but simultaneously a very low prevalence in isolates from India and Bangladesh and absence in isolates from Turkey gastric patients leaves the debate open.

The varying prevalence of these genes in populations across the world raises a question that whether these genes can really be considered as virulence markers? It has been proposed that an ideal *H. pylori* virulence factor should meet the following criteria: 1), it should have a disease or other *in vivo* correlation, 2), it should be epidemiologically consistent across populations and geographical regions and 3). should be biologically plausible and the effect should be reduced or eliminated by gene deletion and be restored by complementation (Lu *et al*., 2005b). Most of the genes studied till date showed paradoxical status on the first and second criteria and leaving the debate open on the role of these genes as virulence markers. In summary, *jhp0940* shows geographical region specific distribution pattern which still need to be confirmed amidst its functional role as cell translocating ser/thr kinase as well as tyrosine kinase and apoptotic and proinflammatory nature. *jhp0947* showed random association to different stages of the disease from gastritis to gastric cancer. It is intriguing to precisely determine the role of *jhp940* and *jhp0947* until we get the products of the plasticity zone genes and know their interplay.

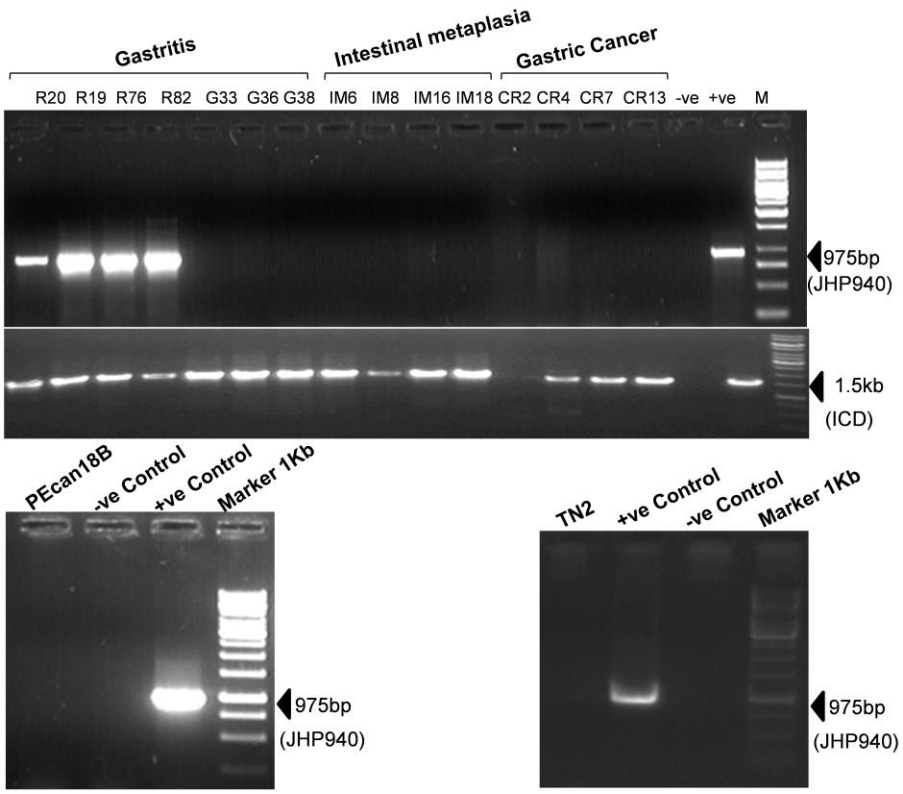

Figure 2.1: Upper panel: Distribution pattern of *jhp0940* in isolates from different geographical regions as shown by PCR. From left, lane R20-R82: South African gastritis, lane G33-G38: Irish gastritis, lane IM6-18: Irish intestinal metaplasia, lane CR2-CR13: Costa Rican gastric cancer, lane -ve: negative control, lane +ve: positive control with J99 DNA, lane M: 1kb marker. 1.5kb band of ICD (isocitrate dehydrogenase of H. pylori) shows clonality and integrity of genomic DNA. Lower panel: status of *jhp0940* in Pecan18B, a Peruvian gastric cancer, sequenced strain and TN2: a Mongolian gerbil gastric cancer strain.

Table 2.1: PCR primers and thermal cycle parameters for amplification of the genes

Gene	Nucleotide position	Sequence	PCR parameters	Product Size
jhp0940	1-24 956-978	ATGCCAACCATTGATTTTACTTTT TTATCGTCTACGCTTAGGTGTG	95^0C / 5 min 35 cycles 94^0C / 30 sec 54^0C / 1 min 72^0 C / 1 min 72^0C / 10 min (final extension)	978 bp
jhp0947	1 – 19 1380 – 1398	TCCCCATGGATGAAAGCGTTGAAGACTT ACCCTCGAGATACGGATTAAACGACTG	95^0C / 4 min 35 cycles 94^0C / 1 min 53^0C / 20 sec 72^0C / 1.2 min 72^0C / 10 min (final extension)	1398 bp

Table 2.2: Prevalence of *jhp0940* & *jhp947* in isolates from different countries (Ireland, Peru, S. Africa, Turkey, Bangladesh and India)

Country	Disease status	No. Of Isolates	Status of *jhp0940*	Status of *jhp947*
Ireland	Intestinal metaplasia	20	2	10
	Duodenitis	2	1	1
	Gastritis	37	0	29
	Duodenal ulcer	1	0	1
Peru	Gastric Cancer	1	0	0
	Gastritis	20	6	9
S. Africa	Gastritis	7	7	4
Turkey	Gastritis	21	4	0
	Duodenal ulcer	12	4	0
	Gastric ulcer	2	0	0
Bangladesh	Irrespective of disease status	26	3	1
India	Gastritis	66	10	5
Total		**215**	**37**	**60**

Table 2.3a: Presence of *jhp0940* (978 bp) gene in genomes of *H. pylori* strains

***H. pylori* strains**	**Query coverage**	**Max Identity**	**Disease Status (source)**	**Geographical Location**
***H. pylori* J99**	100	100	Duodenal ulcer	USA
***H. pylori* F32**	100	99	Gastric cancer	East Asia
***H. pylori* BM013B**	100	99	Asymptomatic infected human	Australia
***H. pylori* BM013A**	100	99	Asymptomatic infected human	Australia
***H. pylori* UM066**	100	99	Peptic ulcer	Malaysia
***H. pylori* OK310**	100	99		Japan
***H. pylori* 83**	100	99	Gastric cancer	Japan
***H. pylori* Puno120**	100	94	Gastritis	Peru
***H. pylori* SJM180**	99	94	Gastritis	Peru
***H. cetorum* MIT 00-7128**	100	93	Stomach of beluga whale	USA
***H. pylori* Puno135**	95	94	Gastritis	Peru
***H. cetorum* MIT 99-5656**	100	92	Stomach of Atlantic white sided dolphin	Atlantic ocean

Table 2.3b: Presence of *jhp0947* (1398) gene in genomes of *H. pylori* strains

***H. pylori* strains**	**Query coverage**	**Max Identity**	**Disease Status (source)**	**Geographical Location**
***H. pylori* J99**	100	100	Duodenal ulcer	USA
***H. pylori* PeCan4**	100	99	Gastric cancer	Peru
***H. pylori* Gambia94/24**	100	98		Gambia
***H. pylori* strain6**	100	97		
***H. pylori* strain1**	100	97		
***H. pylori* SJM180**	99	94	Gastritis	Peru
***H. pylori* strain166**	100	97		
***H. pylori* B8**	100	96	Peptic ulcer	USA
***H. pylori* oki112**	100	96	Gastric atrophy	Japan
***H. pylori* oki102**	100	96	Gastric atrophy	Japan
***H. pylori* strain175**	100	96		
***H. pylori* oki898**	100	96	Duodenal ulcer	Japan
***H. pylori* oki422**	100	96	Gastric atrophy	Japan
H. pylori P12	100	96	Duodenal ulcer	Germany
***H. pylori* SouthAfrica7**	100	96		South Africa
***H. pylori* UM037**	100	98	Peptic ulcer	Malaysia

Table 2.4a: Updated review status of *jhp940* gene

Country/ Geographical region	Total isolates	+ve for *jhp940* (%)	Disease status	Isolates/ disease type	Reference	Product size
Costa Rica	43	7 (14.8)	Gas GC	0/26 7/17	(Occhialini et al., 2000)	591
Brazil	200	3 (1.5)	Gas DU GC	1/68 1/53 1/79	(Santos et al., 2003c)	591
Pakistan	114	71 (62.2)	Gas GU GC DU	14/36 17/22 22/29 18/27	(Yakoob et al., 2010a)	591
United States of America	200	35 (17.32)	Gas GU DU GC	106 32 43 21		
Columbia	94	16 (17.02)	Gas GU DU GC	32 0 25 37	(Sugimoto et al., 2012)	591
S. Korea	105	27 (25.71)	Gas GU DU GC	32 19 18 36	(Sugimoto et al., 2012)	591
Japan	112	24 (21.42)	Gas GU DU GC	27 30 26 29	(Sugimoto et al., 2012)	591
Ireland	70	3 (4.28)	IM Gas Duo PU	2/23 0/43 1/3 0/1	This study	978
Bangladesh	26	3 (11.53)	IDS	3/26	This study	978
India	66	10 (15.15)	Gas	10/66	This study	978
S. Africa	7	7 (100)	Gas	7/7	This study	978
Turkey	35	8 (22.85)	Gas DU GU	4/22 4/12 0/2	This study	978
Peru	21	6 (28.57)	Gas GC	6/20 0/1	This study	978

Gas: gastritis, GC: gastric cancer, DU: duodenal ulcer, GU: gastric ulcer, IM: Intestinal metaplasia, Duo: duodenitis, IDS: irrespective of disease state.

Country/ Geographical region	Total isolate	+ve for *jhp947* (%)	Disease status	Isolates/ disease type	Reference	Product size
Costa Rica	43	20 (46.5)	Gas GC	9/26 11/17	(Occhialini et al., 2000)	
Brazil	200	140 (70)	Gas DU GC	30/68 42/53 68/79	(Santos et al., 2003c)	611
Dutch	45	15 (33.3)	Gas DU	5/26 10/19	(de Jonge et al., 2004a)	
Brazil	85	30 (35.3)	Gas GU DU	11/39 15/24 14/22	(Modena et al., 2007)	
Pakistan	114	66 (57.89)	Gas GU GC DU	13/36 8/22 22/29 23/27	(Yakoob et al., 2010a)	611
Iran	143	83 (58.0)	Gas	83/143	(Siavoshi et al., 2011)	
United States of America	202	76 (37.6)	Gas GU DU GC	106 32 43 21		611
Columbia	94	52 (55.3)	Gas GU DU GC	32 0 25 37	(Sugimoto et al., 2012)	611
S. Korea	105	9 (8.6)	Gas GU DU GC	32 19 18 36	(Sugimoto et al., 2012)	611
Japan	112	3 (2.7)	Gas GU DU GC	27 30 26 29	(Sugimoto et al., 2012)	611
Ireland	60	41 (68.3)	Gas IM Duo DU	29/37 10/20 1/2 1/1	This study	1398
Turkey	35	0	Gas DU GU	0/22 0/12 0/2	This study	1398
Bangladesh	26	1 (3.8)	IDS	1/26	This study	1398
India	125	5 (4)	Gas	5/125	This study	1398
Peru	23	9 (39.1)	Gas GC	9/23 0/1	This study	1398
S. Africa	12	7 (58.3)	Gas	7/12	This study	1398

Chapter 3

Objective 2: *To characterize function and secretion of JHP0940 and its effect on mouse macrophage cells*

Objective 3: *To study multifaceted kinase activity of JHP0940/CtkA*

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3.1 Introduction

Even 30 years after the discovery of *Helicobacter pylori* (Marshall and Warren, 1984) and establishment of its role in gastric diseases, more than 80 % of the population in developing countries (Hunt et al., 2011) as compared to 30-50 % in developed countries (Covacci et al., 1999) is infected with the bacterium. However, the prevalence of *H. pylori* infection varies from geographic region to region throughout the world (Ferlay et al., 2010) which is more likely correlated with socioeconomic status of the population (Malaty and Graham, 1994) and sex; because in many populations males have 20-30 % higher rates of infection than females (Replogle et al., 1995). Since *H. pylori* is the main cause of gastric cancer, WHO has classified the bacterium as a class I carcinogen in 1994 and severity of the disease in the present scenario is so high that gastric cancer is the second leading cause of cancer related deaths worldwide and constitutes the fourth most common cancer type (Ferlay et al., 2010).

H. pylori causes infection of the gastric mucosa (Marshall and Warren, 1984) and is found to be the most successful colonizer of the human stomach causing chronic gastritis, duodenal ulcer, intestinal metaplasia and gastric cancer as a consequence of long term colonization (Graham, 1997), (Uemura et al., 2001). Although the severity of chronic inflammation depends on host genetic factors, environmental parameters and bacterial factors play an important role in the onset of gastric diseases. Among the bacterial factors encoded by various clinical *H. pylori* strains, one of the two most studied proteins is CagA which is encoded within the cytotoxin-associated genes (*cag*) pathogenicity island (PAI) and is about 40 kb in size (Covacci et al., 1993). It has been established that most of the genes from the *cag*PAI act as components of a type IV secretion system (T4SS) forming a syringe like structure that facilitates translocation of CagA and probably other macromolecular bacterial factors into the host cells (Backert et al.,

2010). Moreover, the strains that harbor the *cag*PAI are associated with more severe gastric inflammation and overt outcomes as compared to *cag*PAI-negative strains. The other most studied *H. pylori* virulence factor is VacA (vacuolating cytotoxin A) which induces cytoplasmic vacuolation by forming membrane channels in epithelial membranes *in vitro* (Cover and Blaser, 1992). Besides this activity, it has a multifunctional role in colonization, virulence and immunomodulation (Cover and Blanke, 2005; Pachathundikandi et al., 2013). Apart from these two cardinal effectors, virulence genes such as BabA, SabA, SabB, OipA etc. (Basso et al., 2010; Odenbreit et al., 2009) code for the other important factors known which facilitate the adhesion of the bacterium to the gastric epithelium. In addition, IceA that carries a restriction endonuclease activity was also found to be associated with gastritis and duodenal ulcer (Xu et al., 2002). There is an on-going interest in another putative virulence factor, DupA, on account of its association and envisaged role in gastric diseases (Hussein et al., 2010), (Shiota et al., 2010), (Schmidt et al., 2009). Furthermore, findings on some other *H. pylori* gene products such as protease HtrA (Hoy et al., 2012), AnsB (asparginase), GGT (γ-glutamyl transpeptidase) (McGovern et al., 2001), HP0986, HP-NAP (Satin et al., 2000) and HorB (Snelling et al., 2007) revealed their association with inflammation and pointed to their role in gastric diseases. Given this, the data available about these new generation bacterial factors do not seem to be adequate to fully understand pathogenesis of gastric inflammation and cancer. Therefore, it seems prudent to focus on the discovery of novel virulence factors and their biological roles relevant to the development and progression of gastric diseases. JHP0940 or CtkA is one such potential virulence factor that has been discovered recently and was partly characterized (Kim do et al., 2010).

In the present study, we attempted to investigate the functional roles of *jhp0940* which is

located in a plasticity region gene cluster of the chromosome. The plasticity region is a highly variable zone that was identified by comparison of complete genome sequences of the two *H. pylori* strains (26695 and J99) that contain approximately 50 % strain specific genes (Alm et al., 1999). Some of the genes in this region are thought to be acquired by *H. pylori* through horizontal gene transfer from some environmental bacteria (Datta et al., 2003b). Thus, the plasticity zone genes provide an assumption that the encoded proteins encoded by them may contribute to virulence or modulation of host immune responses. The association of *jhp940* gene with gastric cancer (Occhialini et al., 2000), (Yakoob et al., 2010c), expression of JHP940 protein in response to interaction of *H. pylori* with the gastric mucosa in Mongolian gerbils *in vivo* (Graham et al., 2002) and the high antigenicity potential of JHP0940 protein *in vitro* prompted us to look at its virulence properties in more detail. Previous observations indicated that JHP940 can induce the pro-inflammatory cytokines IL-8 and TNFα and increases translocation of transcription factor NF-κB in the human macrophage cell line Thp1 (Rizwan et al., 2008). Consequently, it was reported that JHP0940 also acts as a cell-translocating Ser/Thr Kinase and could indirectly upregulate the phosphorylation of NF-κB at serine residue 276 in human gastric epithelial cells (Kim do et al., 2010).

In the present study, we further extend the functional acumen of the JHP0940 protein and report its interaction with the host immune apparatus as evident from serum antibody titers present in infections with JHP0940-positive *H. pylori*. We also found that JHP940 acts as an auto-phosphorylating tyrosine kinase. This is the first described tyrosine kinase in *H. pylori* and we can show here that it induces strong pro-inflammatory responses as well as Fas-mediated apoptosis in murine macrophage cells.

3.2 Material and methods

3.2.1 Bacterial strains: The *H. pylori* wild-type strains J99 (Alm et al., 1999), SJM180 (Kersulyte et al., 2003) and P1 (Mueller et al., 2012) were used in the study. *H. pylori* was grown in thin layers on horse serum GC agar plates supplemented with vancomycin (10 μg/mL), nystatin (1 μg/mL), and trimethoprim (5 μg/mL) as described previously (Tegtmeyer et al., 2013). All antibiotics were obtained from Sigma-Aldrich Sigma-Aldrich (St. Louis, MO, USA). Bacteria were grown at 37°C for 2 days in an anaerobic jar containing a Campygen gas mix of 5% O_2, 10% CO_2, and 85% N_2 (Oxoid, Wesel, Germany) (Tegtmeyer et al., 2011).

3.2.2 JHP0940 (Ctk) secretion assays: Bacterial wild-type strains were grown in BHI broth medium supplemented with 10% FCS for 12 hours starting with an OD_{600nm} ~0.2. The supernatant and the cell pellets were separated by centrifugation at 4,000 rpm, and the supernatant was further purified from remaining bacterial cells by passage through a 0.21 μm sterile filter (Sigma Aldrich). The resulting bacterial pellets and supernatants were analysed by immunoblotting (Boehm et al., 2012). Absence of live bacteria in the supernatant was also confirmed by incubation on agar plates showing no growth.

3.2.3 Cell culture: Mouse macrophage RAW264.7 cell line was obtained from National Centre for Cell Science (Pune, India) and maintained in RPMI 1640 medium (Hyclone, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37°C. The cells were trypsinized and seeded for the experiments.

3.2.4 Expression and purification of recombinant JHP0940 protein: The *jhp0940* gene was cloned in pRSET-A expression vector and the construct (recombinant plasmid-*jhp940* cloned into pRSET-A expression vector) was used to transform *Escherichia coli* BL21(DE3) cells, and

the recombinant colonies were picked up against ampicillin selection marker. Transformed colonies were taken for culture growth and induced with 0.1mM IPTG when cells were grown to an OD_{600} of 0.4 to 0.6. After five hours of incubation cells were centrifuged at 6,000 rpm for 5min and pellet was lysed in 20mM Tris-Cl (pH 8.0) and 200mM NaCl (lysis buffer) by sonication. The resultant lysate was centrifuged at 12,000 rpm for 45 min at 4°C, and its supernatant was loaded onto a Cobalt based Talon® resin (Clontech) to purify the His-tagged recombinant protein. The column was washed with buffer (lysis buffer with 20mM imidazole), and the expressed His-tagged protein was eluted using elution buffer (Lysis buffer with 250mM imidazole). The homogeneity of the protein in elusions was confirmed by SDS-PAGE (12%) and the amount of protein was estimated with BCA Assay.

3.2.5 Decontamination of the protein from LPS: The purified protein JHP0940 was treated with polymyxin B beads for 4 hours and separated by centrifugation at 10,000 rpm for 10 minutes. LPS contamination in polymyxin B treated protein was quantitated by *Limulus* amebocyte lysate assay using Chromogenic Endotoxin Quantitation Kit (Pierce Thermo Scientific).

3.2.6 Treatment of cells with JHP0940 and preparation of cell lysate: RAW264.7 cells were cultured in 60 mm culture dish at a density of 4×10^6 per plate and treated with JHP0940 protein. Before harvesting, the cells were washed in cold PBS and scraped by cold plastic cell scraper to cold microfuge tube and lysed with ice cold lysis buffer (20mM Tris HCl pH 7.4, 137 mM NaCl, 10 % Glycerol, 1% NP-40, 2 mM EDTA, PI cocktail 1µl/1×10^6 cells, 1 mM PMSF, 1 mM DTT, 1 mM Na_3VO_4,) by intermittent vortex of 40-60 seconds 5 times after interval of 10 minutes followed by centrifugation at 14000 rpm for 10 minutes at 4°C. Supernatant was collected and quantified by BCA method for western blotting.

3.2.7 In silico prediction of JHP0940/CtkA: The antigenic regions and surface probability of the protein was predicted using off line tool DNASTAR. This software predicted alpha and beta regions, regions for surface probability and antigenic index. Kinase activity of the protein was predicted using KinasePhos2 online tool while NetPhos2 was used for prediction of phosphorylation sites in JHP0940 while.

3.2.8 Production of anti-JHP0940 polyclonal antibody in rabbit: A New Zealand white rabbit was given prime dose of 500 µg of JHP0940 intramuscularly after acclimatization. First intra-dermal booster of JHP0940 (300 µg) was given 21 days after the prime dose. Second intramuscular booster dose of the protein (300 µg) was followed by third intramuscular dose of JHP0940 (300 µg) 14 days after the second dose. Titre was checked 14 days after each booster dose. Serum was obtained by removing 20 ml blood from marginal ear vein of the rabbit. Serum was used in Western blot experiments to detect JHP0940.

3.2.9 Humoral response: Twenty human sera samples from each group of *Helicobacter pylori* infected patients and healthy people were obtained from Prof. Francis Megraud, Bordeaux (France) and used for performing ELISA. 0.5 µg/well JHP0940 protein was coated in bicarbonate coating buffer in 96 well plate followed by overnight incubation at 4°C, unbound surface was blocked with 1% BSA for 2 hours at 37°C. 1:50 dilution of sera (primary antibody) were used and incubated 2 hours at 37°C. Anti-human HRP secondary antibody was used in 1:1500 dilution followed by 1 hour incubation at 37°C. At each step, 3 times washing was done with PBST. This was followed by development of water soluble coloured reaction product with o-phenylenediamine dihydrocloride (OPD) substrate. The reaction was stopped by adding 100 µl/well 3N H_2SO_4 and the plate was read by ELISA plate reader at 492 nm. *P* value was

calculated by two tailed Mann Whitney U test between *H. pylori* infected and non-infected groups considering replicates of three independent experiments in each case.

3.2.10 ELISA for cytokine quantification: Murine macrophage RAW264.7 cells were cultured in the absence or presence of the endotoxin free JHP0940 protein for 24 hours in a humidified atmosphere with 5% CO_2 at 37°C. Culture supernatants were harvested for the estimation of various cytokines (TNF-α, IL-1β and IL-6) by two-site sandwich ELISA as described earlier (Khan et al., 2006; Kumar and Kishore, 2013). In brief, the 96-well polyvinyl chloride microtiter plates were coated with purified anti-TNF- or anti-IL-6 or anti- IL-1β antibody at 1:250 dilutions in coating buffer (0.1 M carbonate buffer, pH 9.5) and were incubated for overnight at 4°C. The plates were washed with wash buffer (PBS with 0.05% Tween-20) and blocked with 1% BSA in PBS followed by incubation with the test samples for overnight at 4°C. After washing, plates were incubated with biotin conjugated anti-TNF- or anti-IL-6 or anti- IL-1β antibody followed by incubation with streptavidin coupled to HRP. The HRP activity was detected using a chromogenic substance o-phenylenediamine tetrahydrochloride (Sigma) in citrate-phosphate buffer (pH 5.4) and H_2O_2. The reaction was terminated using 1 N H_2SO_4, and the absorbance values was measured at 492 nm. Standard curve for the cytokine was obtained using the recombinant standard protein provided by the manufacturer.

3.2.11 Real Time PCR: RAW264.7 cells were cultured in the absence or presence of the JHP0940 protein in a humidified atmosphere with 5% CO_2 at 37°C for 6 hrs. Following incubation cells were harvested. For RT-PCR of cytokines, mRNA was extracted from the stimulated cells by RNeasy Mini Kit according to the manufacturer's protocol. Reverse transcription were performed with SuperScript III reverse transcriptase, oligo-dT (Invitrogen) according to manufacturer's recommendation. Real-time PCR were performed on a MyiQ

Icycler, BioRad), using SYBR Green SuperMix. Quantification of various cytokine levels were performed by the Ct method, normalized by β-2 microglobulin level.

3.2.12 Cell viability assay: MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay was carried out for checking cell viability of mouse macrophage RAW cells in 96 well culture plate using different concentration of JHP0940 protein. The cells were seeded at a density of 5×10^3 cells per well in 200 µl of complete medium followed by protein treatment for 24 hours. 20 µl of 5mg/ml MTT per well was added followed by 3 hours incubation in dark or until the colour of the formazen product developed. Culture media was removed and 50 µl/well DMSO was added followed by 5 minutes incubation in dark. The plate was measured at 570 nm wavelength.

3.2.13 Ladder assay: Ladder assay was performed according to method described by Gong et al with minor modifications (Gong et al., 1994). The one million of mouse macrophage cell line RAW 264.7 cells were treated with JHP0940 protein (10 µg/ml) for 48 hours. The cells were trypsinized and suspended in HBSS (Hank's Buffer Salt Saline) solution and prefixed in ice cold 70% ethanol, stored in -20 for 24 hours before assay. After fixing, the cells were centrifuged at 800 x g for 5 minutes to remove ethanol and DNA was extracted with 0.2 M phosphate-citrate buffer (PCB) at pH 7.8 by suspending the pellet in 40µl of PCB and incubating at room temperature for 30minutes. Under these conditions, the partially degraded, oligonucleosomal DNA is extracted quite selectively from the cells whereas the higher molecular weight DNA stays associated with the nuclei. The extract (supernatant) was centrifuged at 1000 x g for 5minutes and were treated with 1% Nonide NP40 (in water), 3µl RNase A (5 mg/ml in water) and incubated at 37°C for 30 minutes. 6µl Proteinase K was added and incubated at 37°C for 30

minutes. DNA loading dye was added to these samples and loaded to the 1.2 % agarose gel with 0.5% EtBr. The gel was run at 4V/ cm & the bands were observed.

3.2.14 Confocal Microscopy: Confocal microscopy was used for visualization of DAPI stained nucleus of apoptotic cells. RAW264.7 cells were seeded at a density of 0.1×10^6/well on autoclaved coverslips in 24 well culture plate followed by protein treatment for 48 hours. The cells were fixed in 4 % paraformaldehyde and incubated for 15 minutes. After 3 wash with PBS the cells were permeabilized with pre-chilled 0.2% Triton-X followed by 15 minutes incubation at room temperature. DAPI with anti-fade reagent was added and the cells were mounted on slide followed by confocal microscopy for observing degraded nucleus.

For TUNEL Assay 0.05×10^6 cells/well were seeded followed by protein treatment for 48 hours. The cells were fixed and permeabilized as mentioned above. After washing, the cells were equilibrated for 10 minutes at room temperature with equilibration buffer provided with ApoAlert DNA Fragmentation Assay Kit. This was followed by tailing reaction in TdT incubation buffer according to the manufacturer's protocol. Tailing reaction was terminated followed by washing with PBS. Cells were stained using DAPI with anti-fade reagent followed by observation under confocal microscope.

3.2.15 Flow Cytometry (Expression of Fas receptors on RAW264.7 cells): To determine expression of Fas receptors on RAW264.7 cells, cells were seeded followed by treatment with JHP0940 for 24 hours. Cells were harvested by trypsinization very carefully to avoid loss of receptors, washed in PBS and suspended in wash buffer (PBS + 0.5% BSA) followed by incubation with FITC-conjugated anti-mouse CD-95 monoclonal antibody for 30 minutes at 4°C. For isotype control, Cells incubated with FITC-conjugated mouseIgG1 were used. The

incubation was followed by washing of the cells with the wash buffer. The cells expressing Fas receptor were detected by FACS Canto II (BD Biosciences) and analysed by FlowJo software.

3.2.16 Western Blotting: Equivalent amount of proteins were separated on SDS-PAGE and transferred electrophoretically to PVDF membranes. The blots were blocked with 4% BSA followed by overnight incubation with primary antibodies such as anti-CagA (Conradi et al., 2012) and various commercial antibodies (NEB and Santa Cruz) at 4°C in TBST (0.1% v/v) and 2% (w/v) BSA. The blots were washed and incubated with secondary HRP antibody in 2 % (w/v) BSA for 1 hour at room temperature. After washing with TBST the blots were developed with enhanced chemiluminescence regent and exposed to x-ray film.

3.2.17 In vitro tyrosine kinase assay: Kinase reactions were carried out in 25 µl of volume consisting of 5 µl 2X Assay buffer (2mM DTT, 2mM $MnCl_2$, Na_3VO_4), 5 µl of 250 µM ATP solution, 5 µl of Src substrate (1mg/ml), a synthetic peptide with sequence-KVEKIGEGTYGVVYK and 1 µl Src or c-Abl enzymes (NEB) at 30°C for 15 minutes as described (Lind et al., 2014). Both Src substrate and Src enzyme were replaced by JHP0940 in the test reactions for showing auto phosphorylation activity of the JHP0940. Then 25 µl ADP-Glo Reagent was added to deplete unused remaining ATP, plate was shaken and followed by 40 minute incubation at RT. 50 µl kinase detection reagent was added and incubated for 30 minute at RT followed by reading in luminometer (Promega).

3.2.18 Statistical Analysis: Two tailed Mann-Whitney U test was performed for the humoral response in *H. pylori* infected patient v/s healthy control cohorts and p values were calculated at 95 per cent confidence level. Student's t-test was performed for the analysis of results wherever

required. The data were expressed as the mean of triplicates ± SEM unless or otherwise mentioned. P< 0.05 was considered as significant.

3.3 Results

3.3.1 In silico prediction of JHP0940: Prediction with DNASTAR shows the presence of alpha and beta regions in enough ratios of 4:6 or vice versa that predict possibility of stability of the protein. The antigenic index of the protein as shown by the presence of several epitope regions supports the probability of being the protein as immunogenic.

3.3.2 Expression and purification of JHP0940: The protein JHP0940 was expressed and purified according to procedure mentioned in material and methods. The homogeneity of the protein was checked on 12% SDS-PAGE by coomassie blue and silevr staining. The expression of the protein was confirmed by probing with anti-His primary antibodies and antimouse-HRP secondary antibodies (Figure 3.2).

3.3.3 JHP0940 is presented to the innate immune system in H. pylori infected patients: Earlier observations of our group showed that *jhp0940* is associated with gastric disease in patients from different regions of the world and that the encoded protein enhanced the activation of NF-κB occurs in cultured macrophages (Rizwan et al., 2008). This prompted us to investigate if the protein is expressed by *H. pylori* in gastric environment and/or presented to the immune system. Since *H. pylori* colonizes the stomach gastric epithelium, the bacterium should express some of its proteins/factors for establishing persistence, while modulating the immune responses of the host. In this context, expression and exposure of factors to the immune system becomes important. To test this idea, purified JHP0940 was used to performed ELISA experiments by immobilizing JHP0940 protein and probing it with human serum samples of *H. pylori* infected

patients as compared to that from non-infected healthy individuals. A significant difference in *p-value* (0.0004) between *H. pylori* infected patients versus healthy group was found (Fig. 3.3). This suggests that the protein was indeed expressed and presented to the immune system leading to the occurrence of anti-JHP0940 antibodies in the host.

3.3.4 JHP0940 is expressed in a subset of H. pylori strains and is secreted into the culture supernatant: Before being presented to the immune system, we aimed to investigate if the Ctk protein is secreted into the extracellular environment. We tested this idea by fractionation studies of various wild-type *H. pylori* isolates including *ctk* gene-positive (J99 and SJM180) and *ctk*-negative (P1) strains, which were incubated for 12 hours in liquid broth. Western blotting experiments showed that Ctk protein is expressed in strains J99 and SJM180, but not P1 (Figure. 3.4A/B). In addition, bands for Ctk were also found in the culture supernatant fraction. As a control, the type IV secretion effector protein CagA, which has been used as loading control for the *H. pylori* cell pellets (Figure 3.4A) is not found in the supernatant as expected (Figure 3.4B), thereby excluding artificial lysis of bacteria in our experiments. Therefore our results hint at the secretory nature of the protein, allowing us to study how JHP0940 may affect signal transduction events during interaction with macrophages.

3.3.5 JHP0940 induces pro-inflammatory cytokines in RAW264.7 macrophage cells: RAW264.7 cells were grown and treated with the JHP0940 protein for 6 hours, cells harvested and total RNA was isolated using RNeasy and performed real time quantitative PCR as detailed in material and methods to investigate inflammatory nature of JHP0940. qRT-PCR results shown up-regulation of TNF-α, IL-1β and IL-6 cytokines (Figure. 3.5). Furthermore, to confirm the pro-inflammatory properties of JHP0940, ELISA was performed. For this, the macrophage cells were treated with recombinant JHP0940 protein at a concentration of 10 ng to 1,000 ng/ml for 24

hours followed by ELISA-based quantitation of cytokines in the culture supernatant. We found that the cytokines TNF-α, IL-1β and IL-6 were upregulated in a dose dependent manner (Figure 3.6). Our results from ELISA corroborated with the qRT-PCR results confirming the upregulation of the above cytokines (Figure 3.6).

H. pylori infection is recognized by the presence of chronic gastric inflammation and there is increasing evidence that caspase-1 is involved in the activation of the two important pro-inflammatory cytokines (IL-1β and IL 18), which normally mediates bacterial clearance after infection (Hitzler et al., 2012) (van de Veerdonk et al., 2011). In this regard, we investigated the involvement of JHP0940 in caspase-1 induction. We found that caspase-1 expression was induced upon treatment of RAW264.7 cells with recombinant JHP0940. We observed that JHP0940 induces caspase-1 processing and activation in a dose dependent manner (Figure 3.7B). We also observed that active caspase-1 is secreted into the cell culture supernatant (Figure 3.7A). This observation partly corroborates with the above observation related to IL-1β secretion by murine macrophage cells that were treated with JHP040. Given these findings, we espouse the hypothesis that JHP0940 could most likely contribute to *H. pylori* pathogenesis by producing chronic gastric inflammatory responses and consequently reduces *H. pylori* colonization. In our experiments, every time before treating the cells, JHP0940 protein was incubated with polymyxin B for four hours to remove putative endotoxin contamination and to rule out the effect of bacterial endotoxin. Furthermore, we used proteinase K treated JHP0940 as control to ensure that the effect was not due to bacterial endotoxins.

3.3.6 JHP0940 decreases viability of RAW264.7 cells by inducing apoptosis through Fas receptors: Persistence of *H. pylori* in the gastric environment is a hallmark of this pathogen, not allowing the immune system to clear the infection. The role of putative bacterial proteins/factors

in immune evasion and related processes, however, is not yet fully clear. We therefore hypothesized that JHP0940 could induce caspase-1 activation to trigger macrophage apoptosis, thus permitting enhanced bacterial survival. We therefore investigated the viability of RAW264.7 cells in response to exposure with JHP0940 protein. We observed that the protein induced cell death in these cells in a dose dependent manner leaving 56 % of viable cells at 24 hours upon treatment with 10 μg/ml of JHP0940. The cell viability was accessed by MTT assay for 48 hours in a dose dependent manner (Figure 3.8A). Cell viability assays performed with RAW264.7 cells intrigued us to identify the cause of cell death and therefore, we performed the ladder assay. We observed that JHP0940 induced DNA fragmentation similar to the staurosporin control, explaining its role in apoptosis (Figure 3.8B).

To confirm these observations, we investigated the nuclear morphology of RAW264.7 cells by staining with DAPI using confocal microscopy. Our results revealed that JHP0940 induced degradation of chromosomal DNA in RAW264.7 cells (Figure 3.9). Furthermore, these observations were corroborated by TUNEL assay results (Figure 3.10). TUNEL assay revealed that most of the cells undergo apoptosis.

These results were further confirmed by expression of Fas receptor on the surface of JHP0940-treated RAW264.7 cells in a time- and dose-dependent manner (Figure 3.11). The expression of Fas receptors on murine macrophages intrigued us to test if JHP0940 acts as non-receptor tyrosine kinase. There are evidences where c-Abl non-receptor tyrosine kinases are involved in expression of Fas receptors (Gut et al., 2012).

3.3.7 JHP0940 acts as an auto-phosphorylating tyrosine kinase: In a previous study, it was shown that JHP0940 acts as cell-translocating ser/thr kinase (CtkA) and leads to phosphorylation

at serine residue 276 residue of p65 subunit of NF-κB in cultured AGS gastric epithelial cells (Kim do et al., 2010). But, the association of the gene *jhp0940* with gastric cancer (Santos et al., 2003a) intrigued us to check if it can also act as a tyrosine kinase and hence we performed *in vitro* tyrosine kinase assay. In our results, JHP0940 was found to be an auto phosphorylating tyrosine kinase apart from its described serine/threonine kinase activity on western blot analysis (Figure 3.13 & 3.14).

In an *in vitro* tyrosine kinase assay, JHP0940 was used in enzyme range concentrations in the kinase reaction and when it was used along with the enzyme provided with the kit, it has shown cumulative enzymatic activity (Figure 3.15).

3.4 Discussion

Putative virulence-associated genes of *H. pylori* have been categorized into three groups: strain specific genes, phase variable genes and genes with different structures and phenotypes (Yamaoka, 2008). The strain specific genes are further sub-classified into those encoded by two pathogenicity islands Firstly, there is the *cag*PAI, which encodes various virulence factors along with its T4SS and the effector protein CagA and plasticity region clusters (called plasticity zones) encoding nearly half of the strain-specific genes including another T4SS, called *tfs3* (Kersulyte et al., 2003). These genes within the plasticity region were acquired extraneously and are of utmost interest because of their association with the gastric disease (Yamaoka, 2008). In previous studies, various genes of the plasticity region i.e., *dupA*, *jhp*0940, *jhp0945*, *jhp0947* and *jhp0949* have been shown to be associated with various stages of the gastric disease (Yamaoka, 2008). The first characterized member from this region, JHP0940 or CtkA, intrigued us to explore it in detail due to pre-existing observations such as its association with gastritis and gastric cancer in different geographical regions, high antigenicity index and *in vivo* expression in

gastric mucosa of Mongolian gerbils. Furthermore, the mechanism of secretion of JHP0940 from *H. pylori* and its transfer to the gastric epithelial cells is not fully clear. It has also not been confirmed if JHP0940 uses T4SS-mediated injection mechanism like CagA or it harnesses some other secretory mechanism similar to other Gram-negative bacteria. However, our ELISA results portray it as a secretory protein (Figure 3.1). The cell-translocating activity of the protein has been shown using GFP-fused full length recombinant protein in HeLa cells (Kim do et al., 2010). Regarding the pro-inflammatory nature of the protein, we do not rule out the involvement of kinase activity of JHP940 in IL-1β secretion since there are reports where death associated protein kinase, a type of serine/threonine kinases is required for IL-1β production (Chuang et al., 2011). But, at the same time, non-receptor tyrosine kinase members of the c-Abl family are also shown to involve in T-cell mediated inflammation (Gu et al., 2012).

It is well established that IL-1β plays a crucial role in inflammation and maintenance of infection (Dinarello, 2009). Subsequent to NF-κB activation, pro-IL-1β is synthesized in its pro-form, which is then cleaved by the action of caspase-1 to produce mature IL-1β. It is reported that the activation of caspase-1 is dependent on formation of multi-protein complexes, called inflammasome (Martinon et al., 2009), (Dinarello, 2009), (von Moltke et al., 2013). Recent studies identified the bacterial *cag* pathogenicity island and the cooperative interaction among host innate receptors TLR2, NOD2, and NLRP3 as important regulators of IL-1β production in *H. pylori* infected murine dendritic cells (Kim et al., 2013). Here, our study by Western blot analysis of caspase-1 shows its involvement in secretion of IL-1β (Figure 3.3) and therefore, we propose a strong pro-inflammatory role for another *H. pylori* protein CtkA, with the involvement of inflammasome components, at least in murine macrophage cell lines.

In order to examine the effects of recombinant JHP940 on mouse macrophage cells in more

detail, we observed decreased viability of host cells by MTT assay. This was followed by the visualization of apoptotic bodies in the DAPI-stained nucleus. Furthermore, the TUNEL assay confirmed occurrence of apoptosis in response to treatment of the cells with JHP940 protein. In a previous *in vitro* study, translocation of GFP-tagged JHP940 into to HeLa cells was documented, indicating its cell-translocating behaviour (Kim do et al., 2010). The authors also found that transiently expressed protein translocates into the nucleus in a time dependent manner in AGS cells (Rizwan et al., 2008), although the protein doesn't contain a nuclear localization signal (NLS). These observations together with our findings based on an *in vitro* non receptor tyrosine kinase assay and its apoptotic effect on mouse macrophage cell line through Fas expression (death receptor) revealed that the activity of JHP0940 mimics the function of a non-receptor tyrosine kinase such as c-Abl. Upon caspase activation, c-Abl retained tyrosine kinase activity and triggered Fas induced apoptosis (Barila et al., 2003). The Fas receptor activated by specific ligand binding triggers assembly of a complex called as Death Inducing Signalling Complex (DISC) leading to the cleavage and activation of more downstream executive caspases and thus apoptosis (Raina et al., 2005). Moreover, epithelial and endothelial non-receptor tyrosine kinases (Etk) mediate apoptosis in small cell lung cancer cells (Cheng et al., 2010). We also found the enhanced expression of Fas receptors in the mouse macrophage cell line in a time and dose dependent manner (Figure 3.6).

Somehow, JHP940 does not share any sequence homology with c-Abl tyrosine kinases in any of the databases but it is predicted as cytoplasmic protein (score of 8.96 out of 10) by PSORTb v3.0.2 prediction tool (Gardy et al., 2003). The protein was found to have one SH2 domain when searched for domains in Scansite Motif Scanner database (http://scansite.mit.edu/motifscan_seq.phtml). Also, it revealed homology with one of the SH2

domains of CrkII. This SH2 domain of CrkII serves as binding site for SH3 domain of c-Abl mediated by Crk phosphopeptide and thus regulates biological functions (Donaldson et al., 2002). Despite the homology of SH2 domain with CrkII, there are no experimental evidences to show that JHP0940 acts as an adaptor molecule like CrkII.

Other encoded proteins from the plasticity region of *H. pylori* strain 26695, HP0986 (TieA), showed the same pro-inflammatory and pro-apoptotic behaviour but when tested for kinase activity, it was negative for that. On the other hand, HP0986 showed TNFR1 interacting and endonuclease activity postulating the potential virulent role of proteins/factors encoded by plasticity region genes (Alvi et al., 2011; Devi et al., 2014).

It will not be surprising that JHP0940, acting as an auto-phosphorylating tyrosine kinase and by translocating into the nucleus activates transcription factor(s) apart from NF-κB to modulate signalling in the host cells. It hints that JHP0940 may be a secretory protein, though the secretory mechanism of the protein is not established, of which bacteria harness for changing the signalling responses in the host cells. Thus, on the basis of our above observations and results, we assume that on one hand JHP0940 helps *H. pylori* to orchestrate inflammation as a survival mechanism and on the other hand it averts the immune recognition by limiting macrophages through Fas mediated apoptosis. Further, it will be pertinent to investigate if JHP0940 acts as dual specific kinase or it acts as serine/threonine or tyrosine kinase in certain specific conditions and or cellular environment to help *H. pylori* in progression of gastric disease.

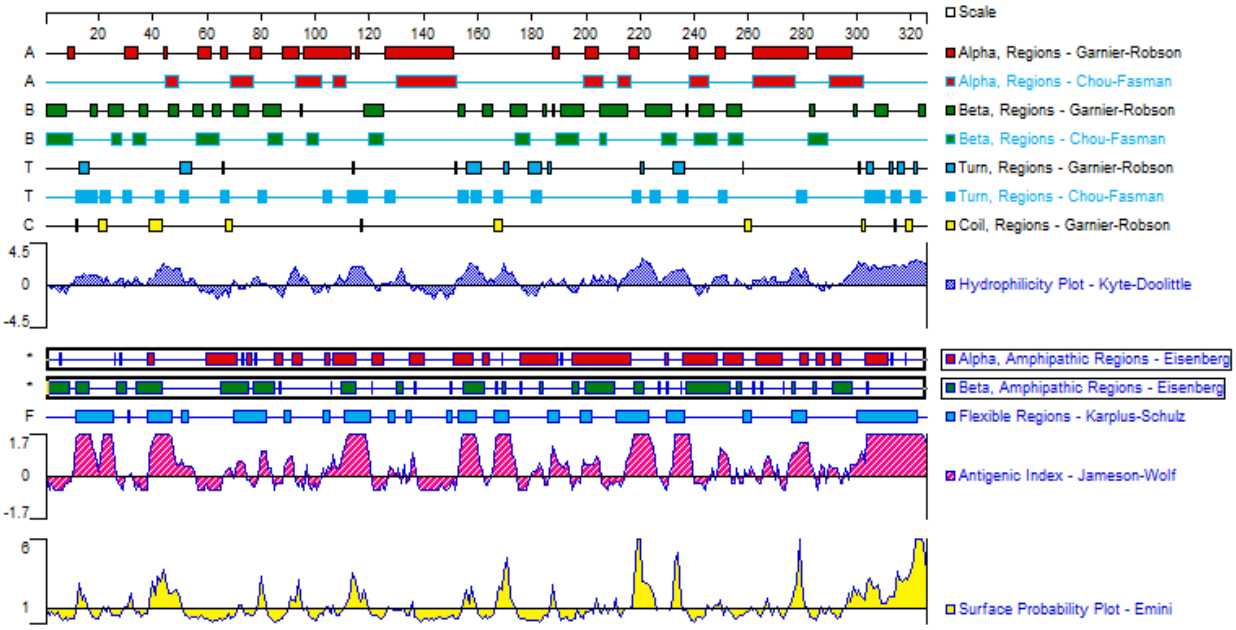

Figure 3.1: prediction of JHP0940 protein using DNASTAR off line tool. Alpha and beta regions in enough ratios of 4:6 or vice versa predict the stability of the protein. The antigenic index of the protein as shown by the presence of several epitope regions supports the probability of being the protein as immunogenic.

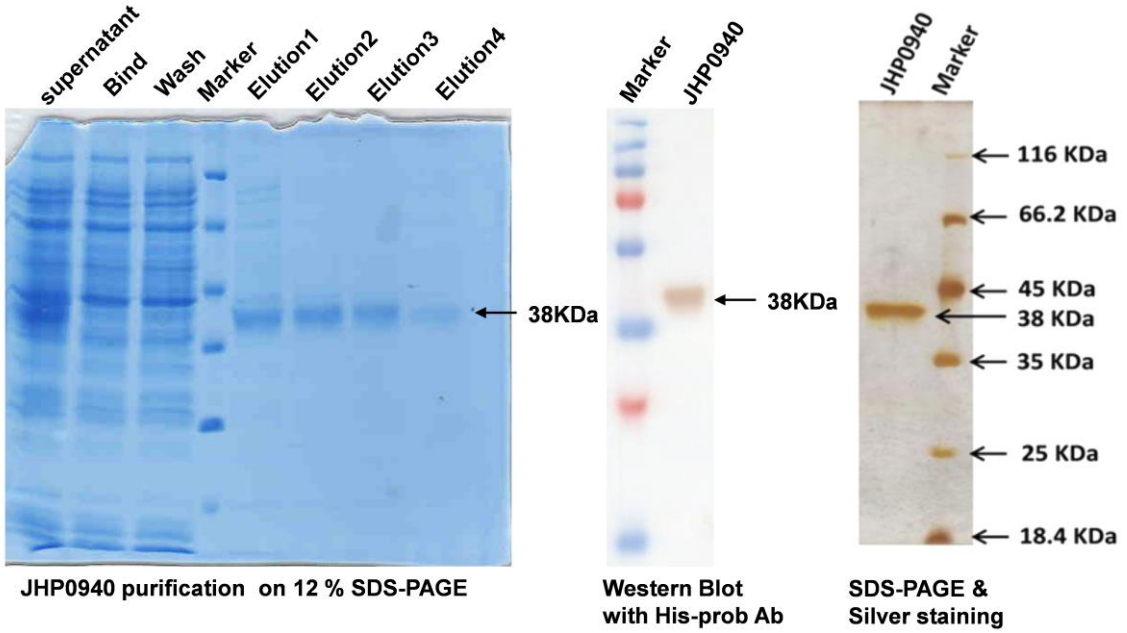

Figure 3.2: Left- Coomassie staining of JHP0940 loaded on 12% SDS-PAGE, middle- JHP0940 was probed with anti-His antibody, Right- Silver staining of JHP0940 loaded on 12% SDS-PAGEs

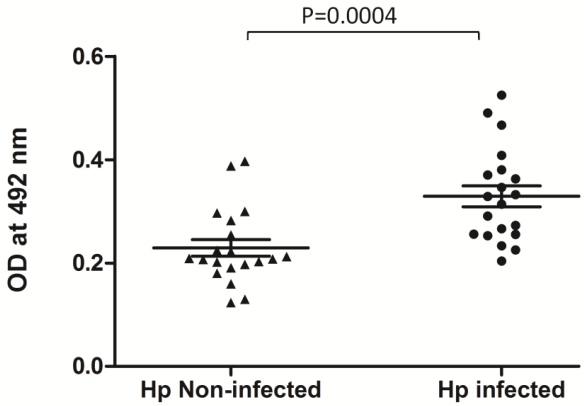

Figure 3.3: ELISA of JHP0940 in two groups of human sera samples of 20 *H. pylori* infected and 20 healthy individuals. Statistically significant difference in *P* value (0.0004) was determined by two tailed Mann Whitney U test between healthy and *H. pylori* infected individuals. The data shown here represent mean ± SEM of triplicates of three independent experiments.

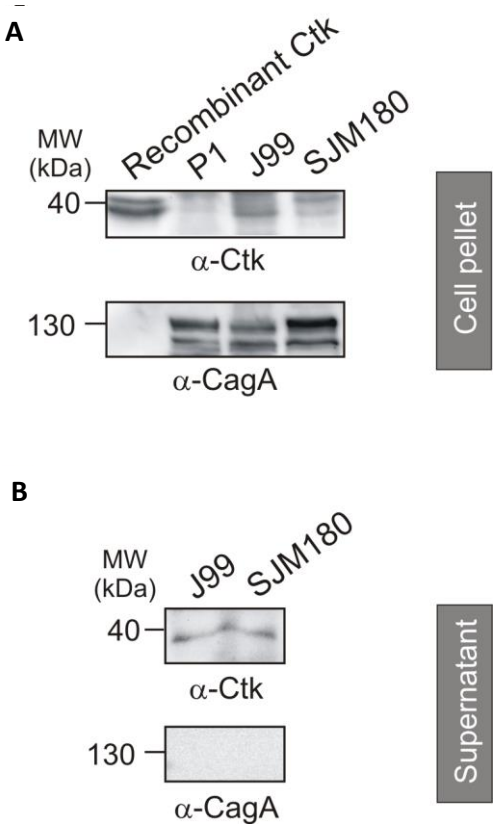

Figure 3.4: (A) Total cell lysates of *H. pylori* wild-type strains J99, SJM180 and P1 were investigated for the expression of Ctk. Equal amounts of protein per sample was confirmed by immunoblotting using an α-CagA antibody as control. (B) Ctk secretion assay in BHI medium with 10% FCS. The above *H. pylori* wild-type strains were grown for 12 hours at 37°C. Bacterial supernatants were filtered and prepared as described in Materials & Methods. The presence of secreted Ctk proteins in the supernatant was investigated by immunoblotting using the α-Ctk antibody and as control with α-CagA antibody.

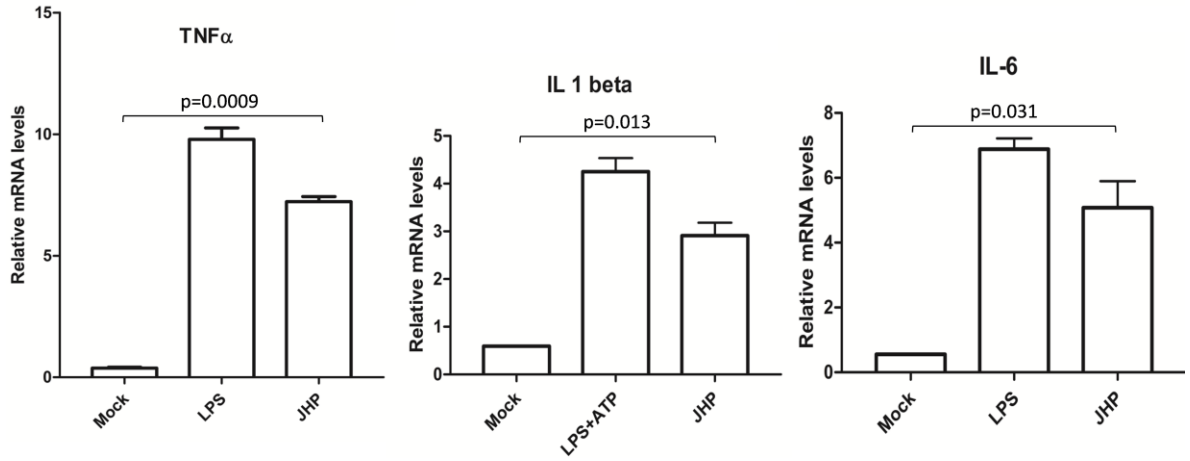

Figure 3.5: Real time analysis of relative expression of TNF- α, IL-6 and IL-1β after 6 h of JHP0940 protein treatment (1μg/ml) of RAW264.7 cells indicated increased relative expression of above cytokines when compared to mock. β-2 microglobulin was used as endogenous control for normalization. The data represent Mean ± S.E. of duplicates (n=3). *P* value for TNF- α, IL-6 and IL-1β was calculated separately by comparing with corresponding mock control. $P < 0.05$ was considered as

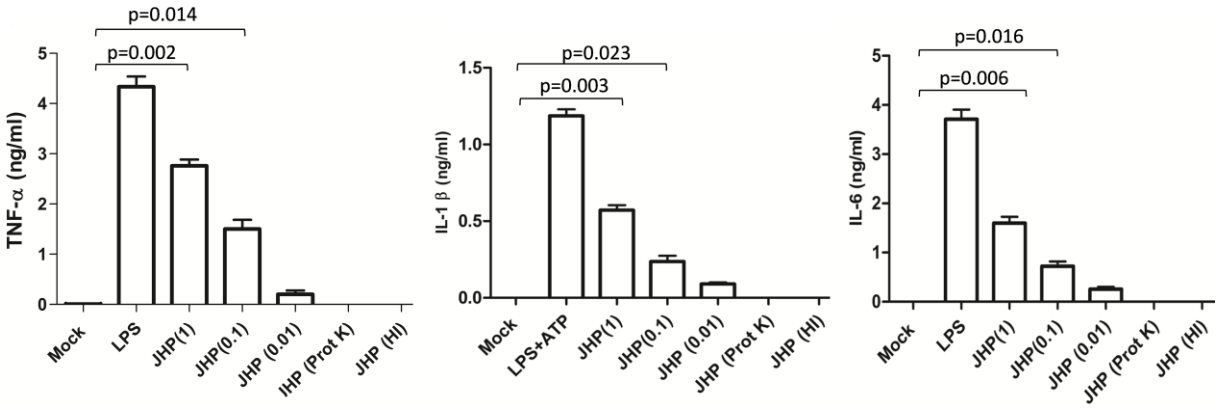

Figure 3.6: Treatment with JHP0940 protein of varying concentrations (0.01, 0.1 and 1 μg/ml) induced secretion of pro-inflammatory cytokines in mouse macrophage cell line (RAW264.7): TNF-α and IL-6 were measured at 24 h post treatment. For IL-1β analysis, the cells were treated (primed) with either JHP0940 (CtkA) or LPS (as positive control) for 3 h followed by 20 min of incubation with 5mM ATP to activate the release of mature IL-1β. The cells treated with LPS (0.1 μg/ml), heat inactivated JHP0940 (100°C for 10 min) and Proteinase K were taken as controls. The data represents Means ± S.E. (n=3) and *P* value for TNF- α, IL-6 and IL-1β was calculated separately by comparing with corresponding mock control.

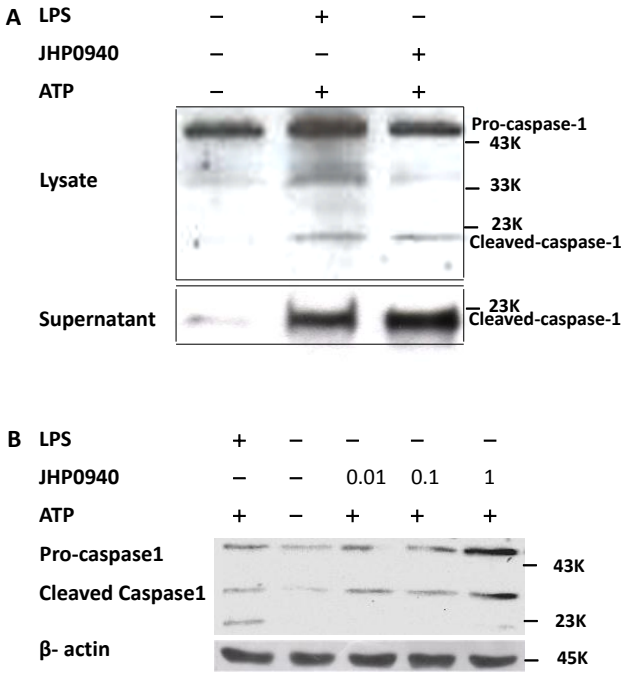

Figure 3.7: Induction of caspase-1 in mouse macrophage RAW264.7 cells on treatment with JHP0940: (A) Activation of caspase-1 was detected in whole cell lysate and in culture supernatants after priming either with JHP0940 (1µg/ml) or LPS (as positive control) for 3 h followed by activation with 5mM ATP for 20 minutes. (B) Dose dependent activation of caspase-1 in the cells pre-treated with JHP0940 and LPS for 3 h followed by 5µM ATP treatment for 20 min. JHP0940 was used in varied doses of 0.01, 0.1 and 1 µg/ml for 3 h. LPS (positive control) was used at 100 ng/ml concentration. (K: molecular weight in kilodaltons).

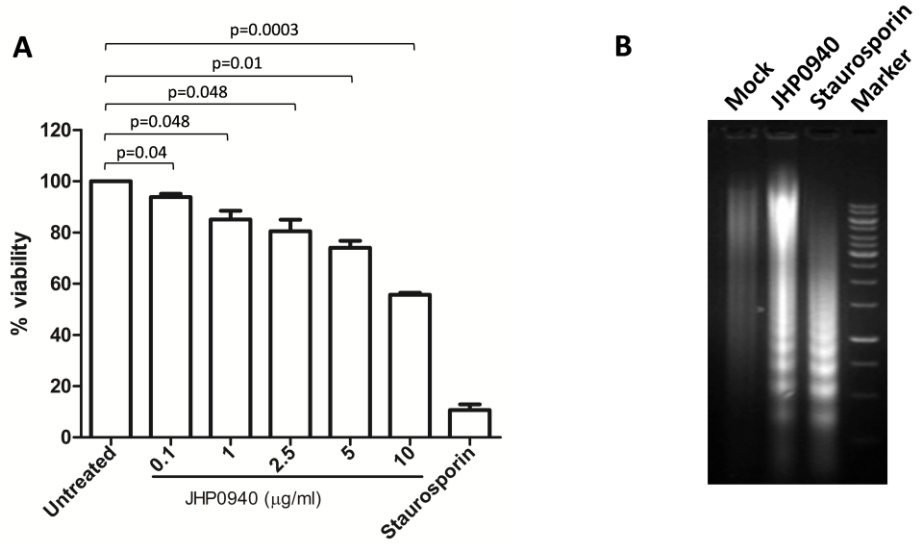

Figure 3.8: (A) MTT assay of RAW264.7 cells after 24 h of treatment with different concentration of JHP0940 indicated dose dependent decreases in viability. The data are represented as Means ± S.E. (n=3). *P* value for different treatment experiments was calculated by comparing with untreated control. $P<0.05$ was considered as significant and $P<0.01$ as highly significant. (B) Ladder assay: The fragmentation of DNA of RAW264.7 cells on treatment with JHP0940 (10 µg/ml) was found similar to RAW264.7 cells treated with staurosporin as positive control. In mock (untreated RAW264.7 cells), DNA fragmentation was not observed. (Marker: 1Kb DNA ladder).

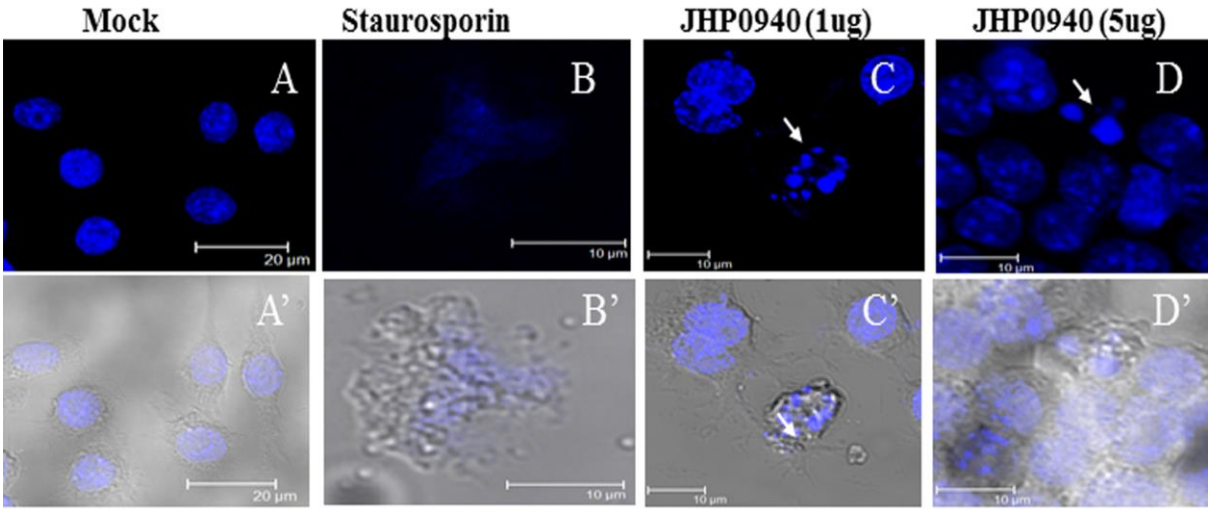

Figure 3.9: Confocal microscopy images indicating apoptosis of RAW264.7 cells on treatment with JHP0940 protein, after 48 hours. Arrows in the images show apoptotic bodies. The RAW264.7 cells upon treatment with 1µg/ml and 5 µg/ml of JHP0940 protein resulted in apoptosis as observed to the cells treated with staurosporin (0.1 µg/ml) as positive control. No apoptosis was observed in the Mock (untreated cells).

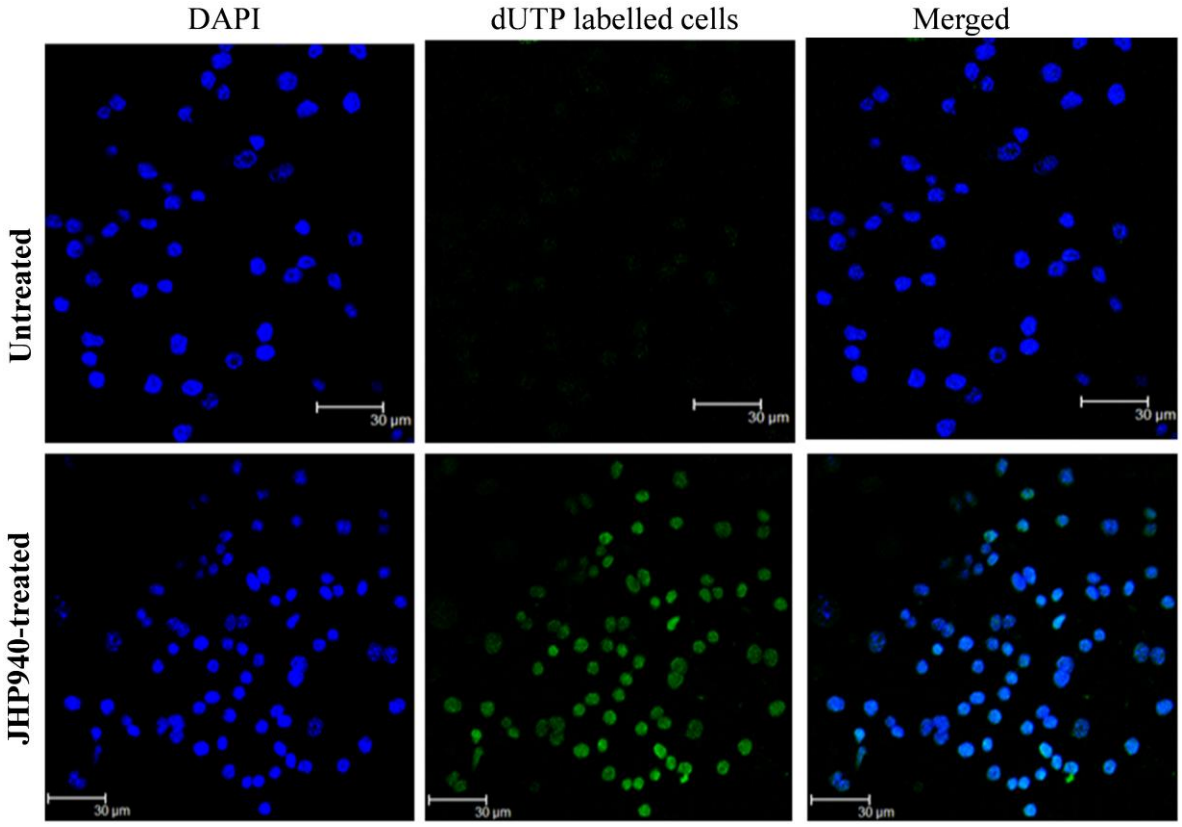

Figure 3.10: TUNEL assay showing apoptosis in RAW264.7 cells upon treatment with JHP0940 protein after 48 hours. Fluorescence-labeled dUTP in the fragmented DNA of cells undergoing apoptosis was observed in cells treated with JHP0940 but such fluorescence was not observed in mock (untreated cells). The figures shown are representative of three independent experiments.

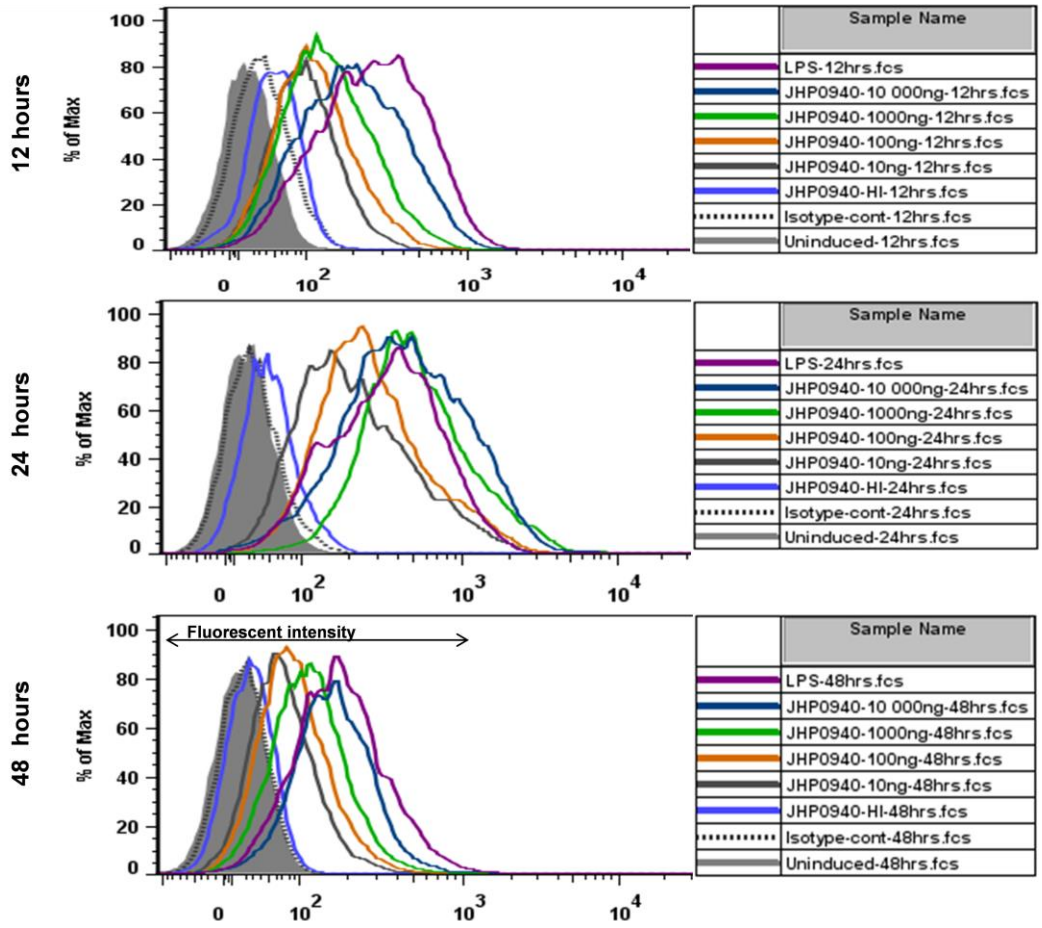

Figure 3.11: Flow cytometry analysis showing expression of Fas receptors on RAW264.7 cells treated with JHP0940 in a dose dependent manner at 12, 24 and 48 hours. The shift in the histogram peaks was observed in accordance with increase in concentration of JHP0940. RAW264.7 cells treated with LPS also indicated shift in histogram peak as compared to untreated. The shift in histogram peaks indicates increased expression of Fas receptors with respect to untreated.

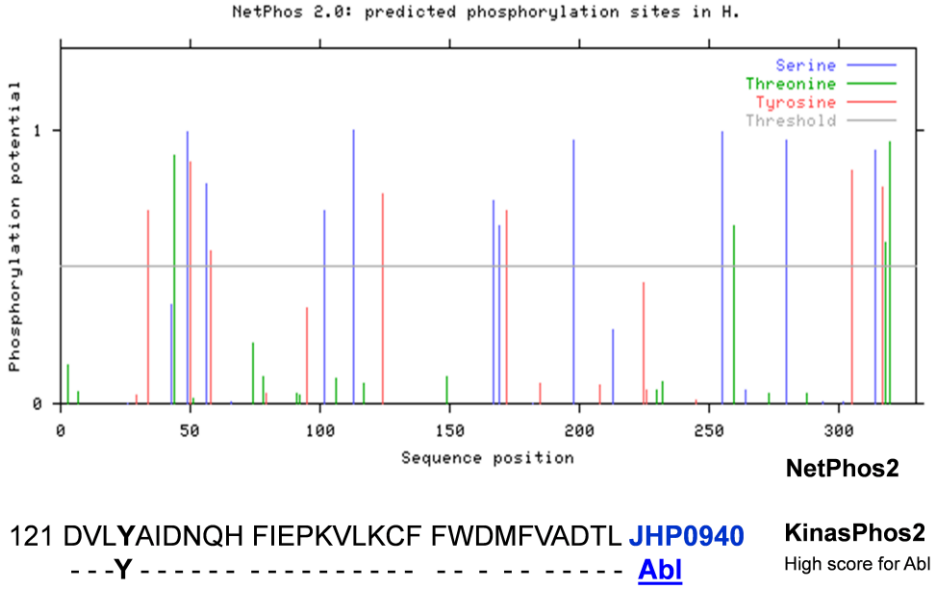

Figure 3.12: JHP0940 was predicted for phosphorylation sites within itself by online tool NetPhos2 and for kinase activity by KinasePhos2 which showed homology with Abl kinase.

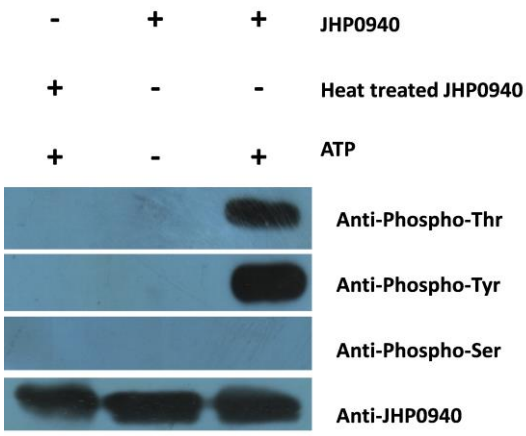

Figure 3.13: JHP0940 acts as auto phosphorylating kinase. *In vitro* kinase reactions were performed and phosphorylation at tyrosine and threonine residues was determined by the respective anti-tyrosine and anti-threonine antibodies.

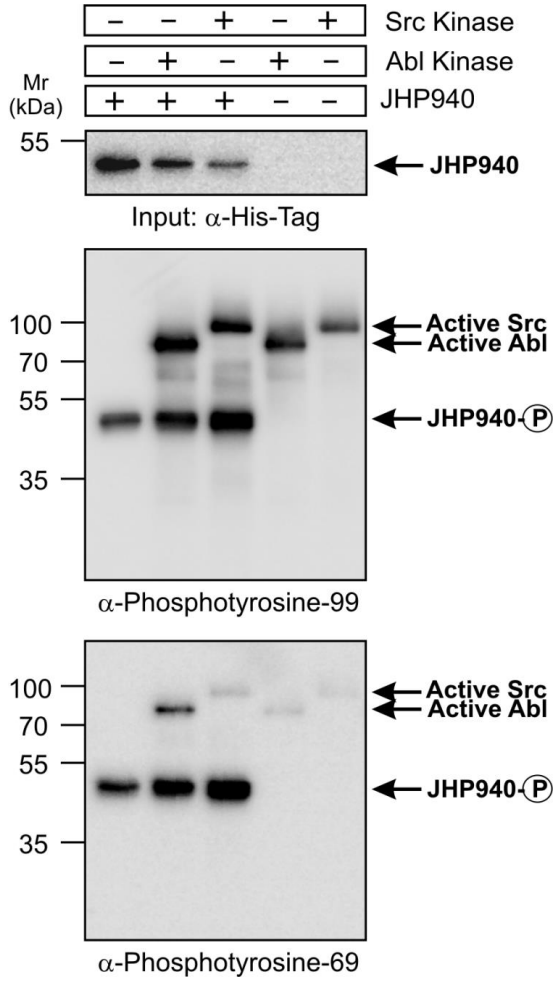

Figure 3.14: Western blot showing auto-tyrosine phosphorylation of JHP0940. Lane 1, from left represents kinase reaction with only JHP0940, lane 2 and 3 represent kinase reactions using JHP0940 along with Abl and Src kinase respectively. Lane 4 and 5 represent in vitro kinase reactions only with Abl and Src kinase respectively. Phosphorylation at tyrosine residues was probed using α-phosphotyrosine-99 and α-phosphotyrosine-69 antibodies. JHP0940 was probed with His-tag antibody.

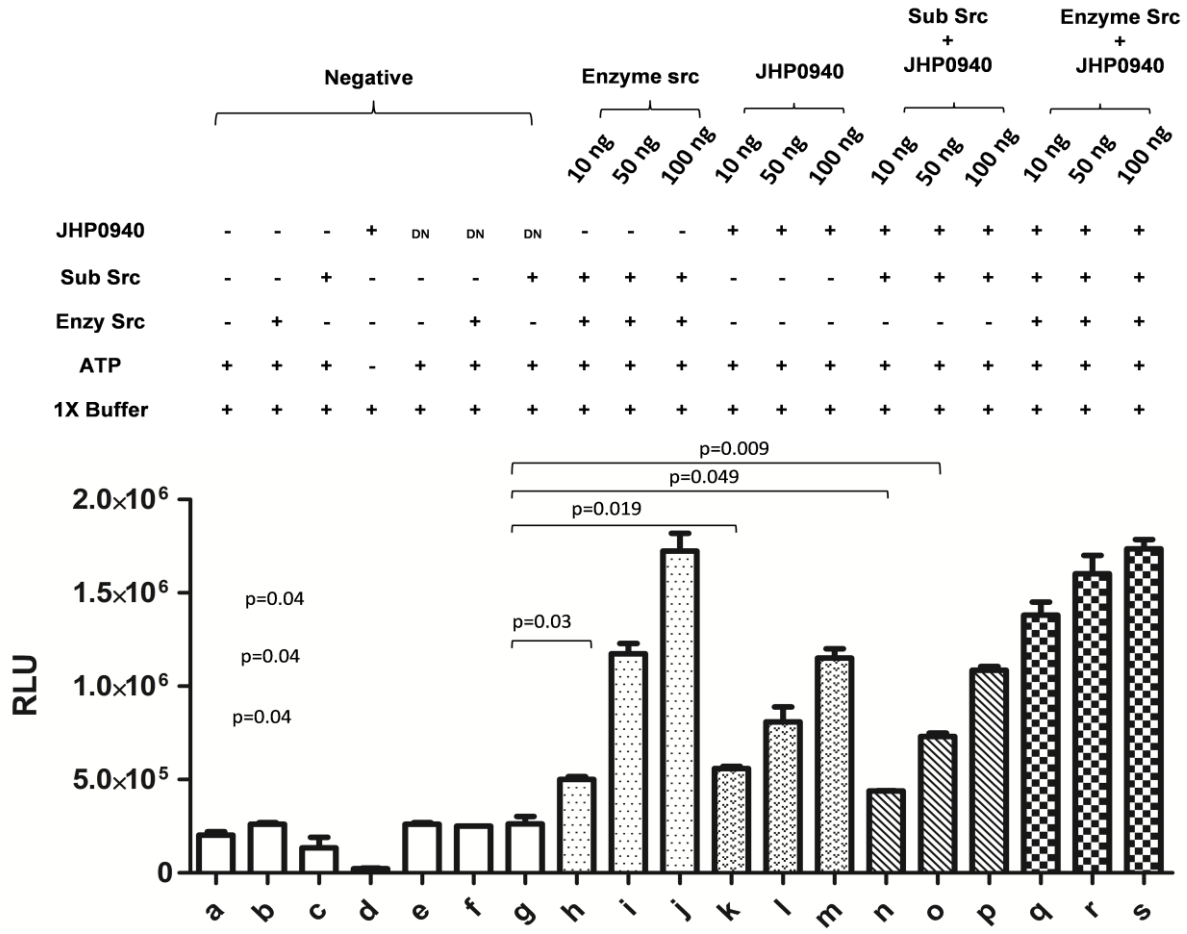

Figure 3.15: *In vitro* tyrosine kinase assay. On X-axis, a to g are negative controls while h, i and j are reactions with increasing concentrations of Src enzyme (10, 50 and 100 ng) serving as positive controls. k, l and m are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) only. n, o and p are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src substrate (5 µg/reaction, the same concentration was maintained throughout the experiment). q, r and s are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src enzyme (50 ng/reaction). Y- axis represents Relative Light Units (RLU). The data is represented as Mean ± S.E. of duplicates of three independent experiments. DN represents denatured protein JHP0940. *P* values have been indicated in comparison with test and control samples.

Chapter 4

Summary of the observations

In our work, we focused on studies of the two genes *jhp0940* and *jhp0947* which were associated with different stages of the gastric disease. Our study using 215 DNA samples representing the isolates from gastric diseases from different countries and updated reviews on the prevalence of *jhp0940* and *jhp0947* suggest that *jhp0940* could reveal geographic region specific distribution and strongly associate with gastritis in some countries. However, it is inconsistently associated with other gastric disease types from other parts of the world. The gene was found absent in most of the gastric cancer isolates including Mongolian gerbil adapted gastric cancer strain. The absence of *jhp0940* was supported by a probability of deletion because there are reports showing deletion of some genes from evolved *H. pylori* strains isolated from advanced stages of gastric diseases when chronic atrophic gastritis progressed to gastric cancer in the same patient over a time of four years (Oh *et al*., 2006). Thus it is possible that due to high rate of evolution of the bacteria, *jhp0940* might get deleted from *H. pylori* strains during progression of gastritis to gastric cancer. However, there are no reports on how the bacteria modulate these types of deletions within a single host during disease progression and to justify such rapid evolution.

Decreased production of IL-12 in THP1 cells in response to disruption of *jhp0945-jhp0947-jhp0949* locus and several other reports on *jhp0947* together with our study suggest that *jhp0947* can be considered as a virulence marker for gastric disease. However, very low prevalence of the gene in gastric isolates from India and Bangladesh and absence in gastric isolates from Turkey somewhat dampened our interest in pushing this gene further.

Earlier, our group reported that jhp0940 induced secretion of TNFα and IL-1β in human macrophages and Peripheral Blood Mononuclear Cells (PBMCs), and also enhanced translocation of NFκB in macrophages (Rizwan et al., 2008). These effects on human macrophages could be significant only if the JHP0940 is secreted by the bacteria to interact with

human immune cells. Therefore, as our second objective, we investigated if JHP0940 is a secretory protein and presented to the immune system of the host. We found that *H. pylori* strains possessing *jhp0940* gene express and secrete the protein. This was done by growing *H. pylori* in brain heart infusion broth medium for 12 hours and fractionating the culture into supernatant and pellet. The pellets and supernatants were probed with anti-JHP0940 antibody on western blot analysis. The presence of JHP0940 in culture supernatant indicated expression and secretion of JHP0940 in J99 and SJM180 bacterial strains (these strains possess *jhp0940* gene) but not in *jhp0940* gene negative bacterial strain P1. In these experiments, CagA was taken as control. *H. pylori* expresses effector molecules and injects them into epithelial cells only if bacteria are in contact with epithelial cells *via* the type IV secretions system (T4SS). Absence of CagA in filtered culture supernatants ruled out artificial lysis of the bacteria in our experiments. Thus, presence of JHP0940 in culture supernatants hints that *jhp0940* is expressed in a subset of *H. pylori* strains and secreted into the culture supernatant and also suggests that JHP0940 is secreted through a secretory system other than T4SS.

These results, pointing at the secretory nature of the protein along with previous findings by our group that JHP0940 induces enhanced translocation of NFκB in human macrophages (Rizwan *et al*., 2008) intrigued us to investigate if this bacterial factor is expressed by *H. pylori* in human gastric environment and/or presented to the immune system of the host. For this, we performed ELISA in a cohort of human sera samples in each group of *H. pylori* infected patients and healthy people by immobilizing purified JHP0940 protein. A significant p value difference of 0.0004 suggested that the protein JHP0940 was expressed and presented to the immune system of the human host and induced the production of anti-JHP0940 antibodies in *H. pylori* infected patients.

H. pylori infection is recognized by the presence of chronic gastric inflammation wherein IL-1β plays cardinal role in *H. pylori* associated gastric diseases (El-Omar, 2001; Tu *et al*., 2008). Hence, we checked for proinflammatory response by ELISA based quantitation of cytokines (IL-1β, TNF-α and IL-6) in culture supernatants 24 hours after the treatment of mouse macrophages with the protein in dose dependent manner. Furthermore, these ELISA results were corroborated with relative expression of the cytokines IL-1β, TNF-α and IL-6 by real time quantitative PCR after 6 hours of JHP0940 protein treatments. Here, we used mouse macrophages so as to investigate the generic response of mammalian macrophages (proinflammatory response of human macrophages upon treatment with JHP0940 has already been established earlier by our group) in response to JHP0940 treatments. The generic proinflammatory response hints towards the usefulness of mouse as an animal model for further *in vivo* studies.

There is increasing evidence that caspase-1 is involved in activation of the two important proinflammatory cytokines IL-1β and IL-18 that normally mediate bacterial clearance after infection (Hitzler *et al*., 2012; van de Veerdonk *et al*., 2011). In this connection, we investigated involvement of JHP0940 in caspase-1 induction and found that expression of caspase-1 was induced in mouse macrophage cells upon treatment with JHP0940 (chapter 3).

Persistence of *H. pylori* in the gastric environment is the hallmark of this pathogen, not allowing the immune system to clear the infection. The role of putative bacterial proteins/factors in immune evasion and related processes, however, is not yet fully clear. We therefore hypothesized that JHP0940 could induce caspase-1 activation to trigger macrophage apoptosis, thus allowing enhanced bacterial survival. Furthermore, most of the studies on caspase-1 have been done to explore its role in inflammation (by inducing IL-1 family cytokines:IL-1beta, IL-18 and IL33), and its role in inflammasome induction. However, there are reports for the

involvement of caspase-1 in cell death process by executing several effector molecules (caspase-2, 3, 7, Bid, XIAP, Calpastatin) (Denes *et al*., 2012) and also capase-1 has been reported in Fas mediated apoptosis (Enari et al., 1995). Therefore, we investigated if JHP0940 can induce apoptosis. We performed ladder assay for preliminary testing of apoptosis. Laddering (fragmentation) of DNA in JHP0940 treated cells gave hints that the protein induced apoptosis in mouse macrophage cells which was confirmed by DAPI stained nuclear morphology of these cells using confocal microscopy. These observations were further corroborated by TUNEL assay results that showed that treatment of these cells with JHP0940 induced apoptosis.

Furthermore, to investigate the pathways underlying the apoptosis induced by JHP0940 in mouse macrophage cells, and based on existing findings (that caspase-1 induces apoptosis through Fas mediated apoptosis) we explored the involvement of Fas receptor in cell death process and found the expression of Fas receptor on the surface of JHP0940-treated RAW264.7 cells in a time- and dose-dependent manner. Thus confirming apoptosis *via* Fas mediated pathway (chapter 3).

In a previous study, it was shown that JHP0940 acted as cell-translocating ser/thr kinase (CtkA) and leads to phosphorylation at Serine276 residue of p65 subunit of NF-κB in cultured AGS cells (Kim do *et al*., 2010). But, the association of the gene *jhp0940* with *H. pylori* isolates obtained from gastric cancer patients (Santos et al., 2003a) and Fas mediated apoptosis of mouse macrophages prompted us to test if JHP0940 acts as non-receptor tyrosine kinase. Therefore, we performed *in vitro* tyrosine kinase assay and found that JHP0940 acted as an auto phosphorylating tyrosine kinase apart from its envisaged serine/threonine kinase activity (chapter 3). In these assays, JHP0940 was used in enzyme range concentrations in the kinase reaction and worked robustly and also showed cumulative enzymatic activity when used along with the enzyme provided in the kit. These observations together with our findings based on an *in vitro*

non receptor tyrosine kinase assay and its apoptotic effect on mouse macrophage cell line through Fas expression (death receptor) revealed that the activity of JHP0940 mimics the function of a non-receptor tyrosine kinase such as c-Abl.

Thus, on the basis of our observations and results, we assume that on one hand JHP0940 helps *H. pylori* to orchestrate inflammation as a survival mechanism and on the other hand it averts the immune recognition by limiting macrophages through Fas mediated apoptosis, although this needs functional validation with *in vivo* studies.

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Helicobacter pylori cell translocating kinase (CtkA/JHP0940) is pro-apoptotic in mouse macrophages and acts as auto-phosphorylating tyrosine kinase

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ABSTRACT

The *Helicobacter pylori* gene JHP0940 has been shown to encode a serine/threonine kinase which can induce cytokines in gastric epithelial cells relevant to chronic gastric inflammation. Here we demonstrate that JHP0940 can be secreted by the bacteria, triggers apoptosis in cultured mouse macrophages and acts as an auto-phosphorylating tyrosine kinase.

Recombinant JHP0940 protein was found to decrease the viability of RAW264.7 cells (a mouse macrophage cell line) up to 55% within 24 h of co-incubation. The decreased cellular viability was due to apoptosis, which was confirmed by TUNEL assay and Fas expression analysis by flow-cytometry. Further, we found that caspase-1 and IL-1beta were activated upon treatment with JHP0940. These results point towards possible action through the host inflammasome. Our *in vitro* studies using tyrosine kinase assays further demonstrated that JHP0940 acts as auto-phosphorylating tyrosine kinase and induces pro-inflammatory cytokines in RAW264.7 cells. Upon exposure with JHP0940, these cells secreted IL-1beta, TNF-alpha and IL-6, in a dose- and time-dependent manner, as detected by ELISA and transcript profiling by q-RT-PCR.

The pro-inflammatory, pro-apoptotic and other regulatory responses triggered by JHP0940 lead to the assumption of its possible role in inducing chronic inflammation for enhanced bacterial persistence and escape from host innate immune responses by apoptosis of macrophages.

Introduction

Even 30 years after the discovery of *Helicobacter pylori* (Marshall and Warren, 1984) and establishment of its role in gastric diseases, more than 80% of the population in developing countries (Hunt et al., 2011) as compared to 30–50% in developed countries (Covacci et al., 1999) is infected with the bacterium. However, the prevalence of *H. pylori* infection varies from geographic region to region throughout the world (Ferlay et al., 2010) which is more likely correlated with socioeconomic status of the population (Malaty and Graham, 1994) and sex; because in many populations males have 20–30% higher rates of infection than females (Replogle et al., 1995). Since *H. pylori* is the main cause of gastric cancer, WHO has classified the bacterium as a class I carcinogen in 1994 and severity of the disease in the present scenario is so high that gastric cancer is the second leading cause of cancer related deaths worldwide and constitutes the fourth most common cancer type (Ferlay et al., 2010).

H. pylori causes infection of the gastric mucosa (Marshall and Warren, 1984) and is found to be the most successful colonizer of the human stomach causing chronic gastritis, duodenal ulcer, intestinal metaplasia and gastric cancer as a consequence of long term colonization (Graham, 1997; Uemura et al., 2001). Although the severity of chronic inflammation depends on host genetic factors, environmental parameters and bacterial factors play an important role in the onset of gastric diseases. Among the bacterial factors encoded by various clinical *H. pylori* strains, one of the two most studied proteins is CagA which is encoded within the cytotoxin-associated genes (*cag*) pathogenicity island (PAI) and is about 40 kb in size (Covacci et al., 1993). It has been established that most of the genes from the *cag*PAI act as components of a type IV

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secretion system (T4SS) forming a syringe like structure that facilitates translocation of CagA and probably other macromolecular bacterial factors into the host cells (Backert et al., 2010). Moreover, the strains that harbour the *cag*PAI are associated with more severe gastric inflammation and overt outcomes as compared to *cag*PAI-negative strains. The other most studied *H. pylori* virulence factor is VacA (vacuolating cytotoxin A) which induces cytoplasmic vacuolation by forming membrane channels in epithelial membranes *in vitro* (Cover and Blaser, 1992). Besides this activity, it has a multifunctional role in colonization, virulence and immunomodulation (Cover and Blanke, 2005; Pachathundikandi et al., 2013). Apart from these two cardinal effectors, virulence genes such as BabA, SabA, SabB, OipA etc. (Basso et al., 2010; Odenbreit et al., 2009) code for the other important factors which facilitate adhesion of the bacterium to the gastric epithelium. In addition, IceA that carries a restriction endonuclease activity was also found to be associated with gastritis and duodenal ulcer (Xu et al., 2002). There is an on-going interest in another putative virulence factor, DupA, on account of its association and envisaged role in gastric diseases (Hussein et al., 2010; Shiota et al., 2010; Schmidt et al., 2009). Furthermore, findings on some other *H. pylori* gene products such as protease HtrA (Hoy et al., 2012), AnsB (asparginase), GGT (γ-glutamyl transpeptidase) (McGovern et al., 2001), HP0986 (Alvi et al., 2011), HP-NAP (Satin et al., 2000) and HorB (Snelling et al., 2007) revealed their association with inflammation and pointed to their role in gastric diseases. Given this, the data available about these new generation bacterial factors do not seem to be adequate to fully understand pathogenesis of gastric inflammation and cancer. Therefore, it seems prudent to focus on the discovery of novel virulence factors and their biological roles relevant to the development and progression of gastric diseases. JHP0940 or CtkA is one such potential virulence factor that has been discovered and partly characterized (Rizwan et al., 2008) and its crystal structure has been solved (Kim do et al., 2010).

In the present study, we attempted to investigate the functional roles of JHP0940 which is located in a plasticity region gene cluster of the chromosome. The plasticity region is a highly variable zone that was identified by comparison of complete genome sequences of the two *H. pylori* strains (26695 and J99) that contain approximately 50% strain specific genes (Alm et al., 1999). Some of the genes in this region are thought to be acquired by *H. pylori* through horizontal gene transfer from some environmental bacteria (Datta et al., 2003). Thus, the plasticity zone genes provide an assumption that the proteins encoded by them may contribute to virulence or modulation of host immune responses. The association of jhp940 gene with gastric cancer (Occhialini et al., 2000; Yakoob et al., 2010), expression of JHP940 protein in response to interaction of *H. pylori* with the gastric mucosa in Mongolian gerbils *in vivo* (Graham et al., 2002) and its antigenicity profile prompted us to look at its virulence properties in more detail. Previous observations indicated that JHP940 can induce pro-inflammatory cytokines IL-8 and TNFα and increases translocation of transcription factor NF-κB in the human macrophage cell line Thp1 (Rizwan et al., 2008). Consequently, it was reported that JHP0940 also acts as a cell-translocating Ser/Thr Kinase and could indirectly upregulate the phosphorylation of NF-κB at serine residue 276 in human gastric epithelial cells (Kim do et al., 2010).

In the present study, we further extend the functional acumen of the JHP0940 protein and report its interaction with the host immune apparatus as evident from serum antibody titres present in patients infected with JHP0940-positive *H. pylori*. We also found that JHP940 acts as an auto-phosphorylating tyrosine kinase. This is perhaps the first described tyrosine kinase in *H. pylori* and we could demonstrate that it induces strong pro-inflammatory responses as well as Fas-mediated apoptosis in murine macrophage cells.

Materials and methods

Bacterial strains

The *H. pylori* wild-type strains J99 (Alm et al., 1999), SJM180 (Kersulyte et al., 2003) and P1 (Mueller et al., 2012) were used in the study. *H. pylori* was grown in thin layers on horse serum GC agar plates supplemented with vancomycin (10 μg/ml), nystatin (1 μg/ml), and trimethoprim (5 μg/ml) as described previously (Tegtmeyer et al., 2013). All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA). Bacteria were grown at 37 °C for 2 days in an anaerobic jar containing a Campygen gas mix of 5% O_2, 10% CO_2, and 85% N_2 (Oxoid, Wesel, Germany) (Tegtmeyer et al., 2011).

JHP0940 (Ctk) secretion assays

Bacterial wild-type strains were grown in BHI broth medium supplemented with 10% FCS for 12 h starting with an $OD_{600nm} \sim 0.2$. The supernatant and the cell pellets were separated by centrifugation at 4000 rpm, and the supernatant was further purified from remaining bacterial cells by passage through a 0.21 μm sterile filter (Sigma Aldrich). The resulting bacterial pellets and supernatants were analysed by immunoblotting (Boehm et al., 2012). Absence of live bacteria in the supernatant was also confirmed by incubation on agar plates showing no growth.

Cell culture

Mouse macrophage RAW264.7 cell line was obtained from National Centre for Cell Science (Pune, India) and maintained in RPMI 1640 medium (Hyclone, USA) supplemented with 10% heat inactivated fetal bovine serum (Gibco, USA), 100 U/ml penicillin and 100 μg/ml streptomycin, in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were trypsinized and seeded for the experiments.

Expression and purification of recombinant JHP0940 protein

The *jhp0940* gene was cloned in pRSET-A expression vector and the protein was expressed in *Escherichia coli* BL21 (DE3) cells, induced with 0.1 mM IPTG. His-tagged JHP0940 was purified as described earlier (Rizwan et al., 2008) with minor modifications, using Cobalt based Talon® resin (Clontech, USA). The homogeneity of the protein was confirmed by SDS-PAGE (12%) and the amount of protein estimated by bicinchoninic acid assay (BCA). The purified protein JHP0940 was treated with polymyxin B beads for 4 h and separated by centrifugation at 10,000 rpm for 10 min. LPS contamination in polymyxin B treated protein was quantitated by *Limulus* amebocyte lysate assay using Chromogenic Endotoxin Quantitation Kit (Pierce Thermo Scientific).

Treatment of cells with JHP0940 and preparation of cell lysate

RAW264.7 cells were cultured in 60 mm culture dish at a density of 4×10^6 per plate and treated with JHP0940 protein. Before harvesting, the cells were washed in cold PBS, scraped by cold plastic cell scraper, transferred to cold microfuge tube and lysed with ice cold lysis buffer (20 mM Tris HCl pH 7.4, 137 mM NaCl, 10% Glycerol, 1% NP-40, 2 mM EDTA, PI cocktail 1 μl/1×10^6 cells, 1 mM PMSF, 1 mM DTT, 1 mM Na_3VO_4) by intermittent vortexing for 40–60 s and repeated 5 times after an interval of 10 min and followed by centrifugation at 14,000 rpm for 10 min at 4 °C. Supernatant was collected and quantified by BCA method for western blotting.

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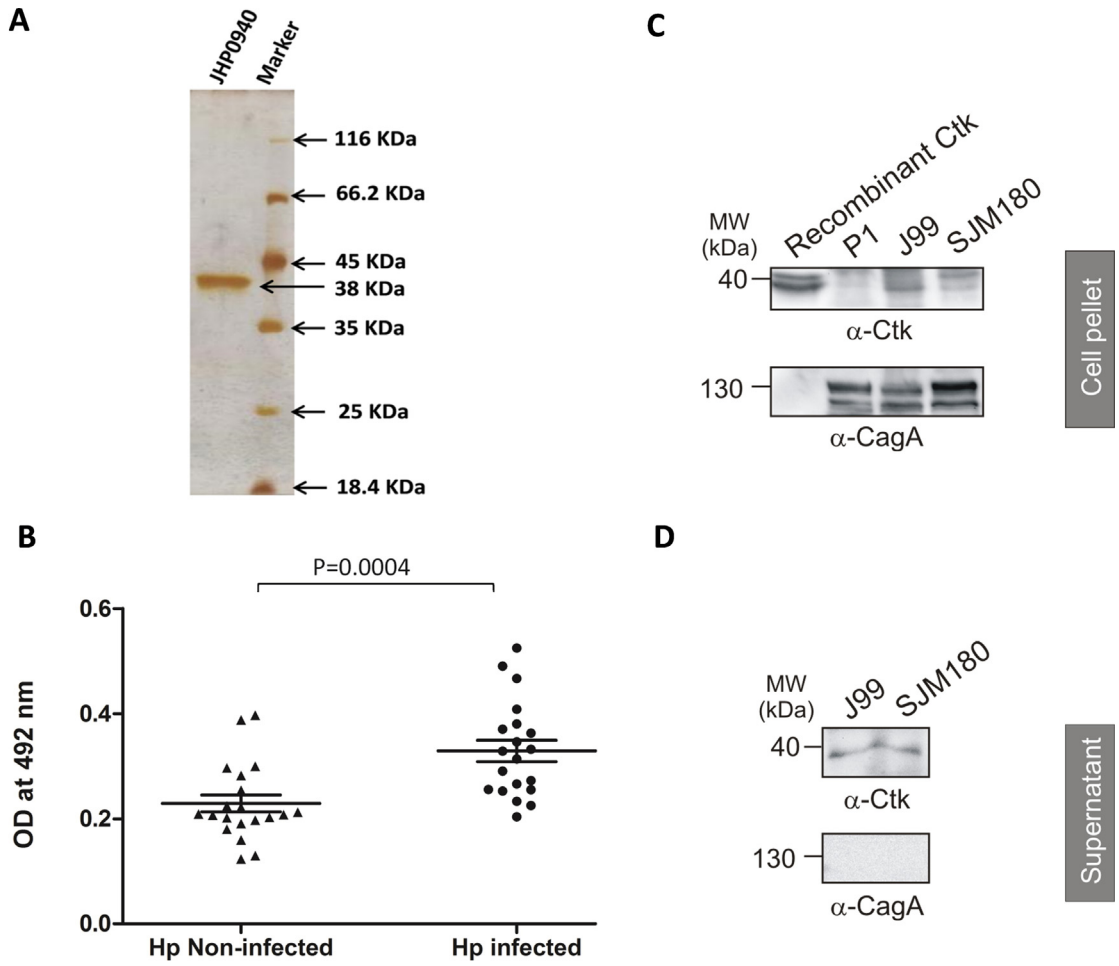

Fig. 1. (A) Purified JHP0940 in silver stained SDS gel. (B) ELISA of JHP0940 in two groups of human sera samples of 20 *H. pylori* infected and 20 healthy individuals. Statistically significant difference in *P* value (0.0004) was determined by two tailed Mann Whitney *U* test between healthy and *H. pylori* infected individuals. The data shown here represent mean ± SEM of three independent experiments. (C) Total cell lysates of *H. pylori* wild-type strains J99, SJM180 and P1 were investigated for the expression of Ctk. Equal amounts of protein per sample was confirmed by immunoblotting using an α-CagA antibody as control. (D) Ctk secretion assay in BHI medium with 10% FCS. The above *H. pylori* wild-type strains were grown for 12 h at 37 °C. Bacterial supernatants were filtered and prepared as described in the Materials and Methods section. The presence of secreted Ctk proteins in the supernatant was investigated by immunoblotting using the α-Ctk antibody and as control with α-CagA antibody. (OD: optical density; KDa: molecular weight in kilodalton).

Production of anti-JHP0940 polyclonal antibody in rabbit

A New Zealand white rabbit was given prime dose of 500 μg of JHP0940 intramuscularly after acclimatization. First intra-dermal booster of JHP0940 (300 μg) was given 21 days after the prime dose. Second intramuscular booster dose of the protein (300 μg) was followed by third intramuscular dose of JHP0940 (300 μg) 14 days after the second dose. Titre was checked 14 days after each booster dose. Serum was obtained by removing 20 ml blood from marginal ear vein of the rabbit. Serum was used in Western blot experiments to detect JHP0940.

Humoural response

Twenty human sera samples from each group of *H. pylori* infected patients and healthy subjects were obtained from the sera collection of Prof. Francis Megraud, INSERM-U853, Bordeaux (France) and used for performing ELISA. JHP0940 protein was coated in bicarbonate coating buffer@ 0.5 μg/well in 96 well plates followed by overnight incubation at 4 °C; unbound surface was blocked with 1% BSA for 2 h at 37 °C. 1:50 dilution of sera (primary antibody) were used and incubated for 2 h at 37 °C. Anti-human HRP secondary antibody was used in 1:1500 dilution followed by 1 h incubation at 37 °C. At each step, washing was done 3 times with PBST. This was followed by development of water soluble coloured reaction product with o-phenylenediamine dihydrocloride (OPD) substrate. The reaction was stopped by 3N H_2SO_4 added @ 100 μl/well and the plate was read by ELISA plate reader at 492 nm. *P* value was calculated by two tailed Mann Whitney *U* test between *H. pylori* infected and non-infected groups considering replicates of three independent experiments in each case.

ELISA for cytokine quantification

Murine macrophage RAW264.7 cells were cultured in the absence or presence of the endotoxin free JHP0940 protein for 24 h in a humidified atmosphere with 5% CO_2 at 37 °C. Culture supernatants were harvested for the estimation of various cytokines (TNF-α, IL-1β and IL-6) by two-site sandwich ELISA as described earlier (Khan et al., 2006; Kumar et al., 2013). In brief, the 96-well polyvinyl chloride microtitre plates were coated with purified anti-TNF- or anti-IL-6 or anti- IL-1β antibody at 1:250 dilutions in coating buffer (0.1 M carbonate buffer, pH 9.5) and were incubated for overnight at 4 °C. The plates were washed with wash buffer (PBS with 0.05% Tween-20) and blocked with 1% BSA in PBS followed by incubation with the test samples for overnight at 4 °C. After

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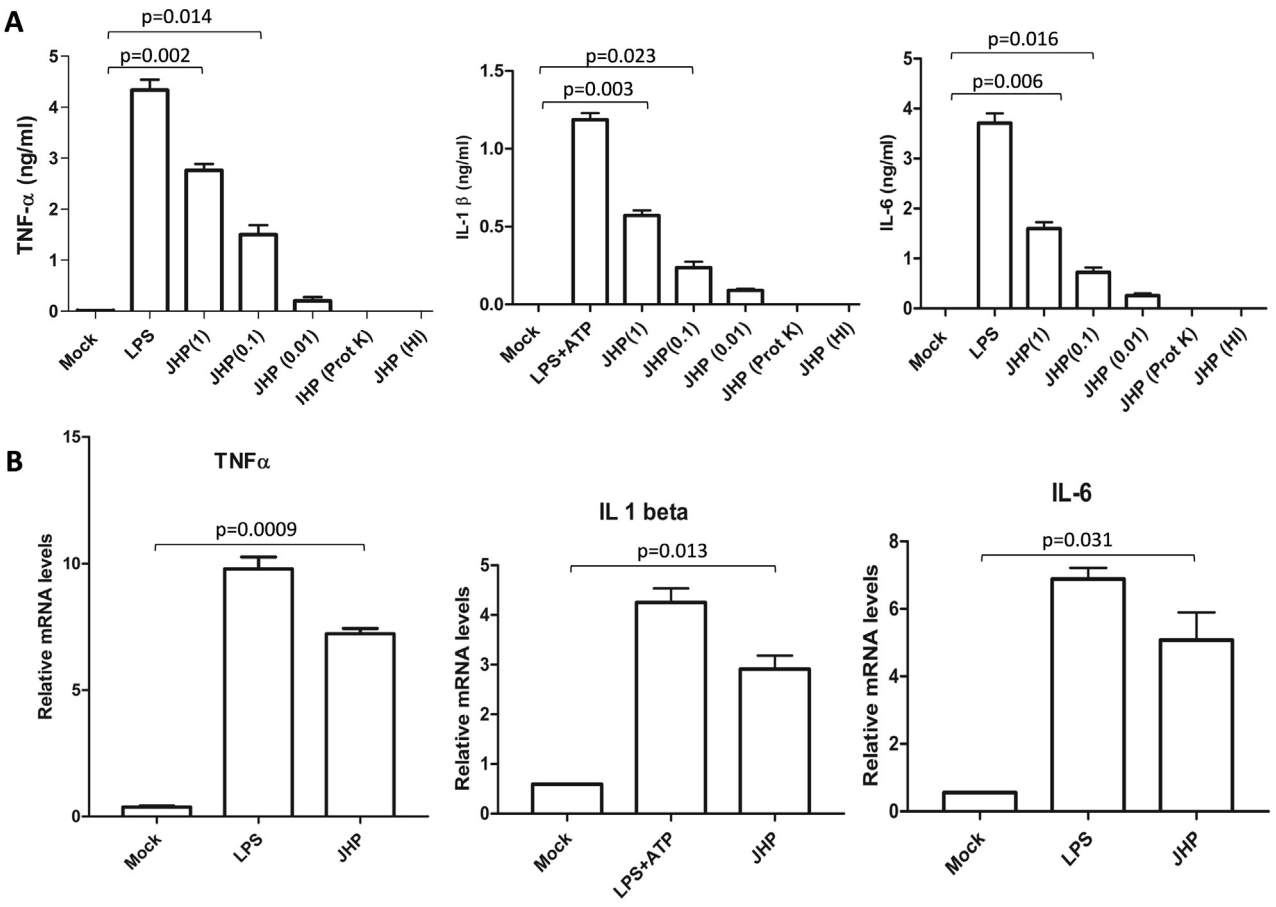

Fig. 2. (A) Treatment with JHP0940 protein in varying concentrations (0.01, 0.1 and 1 μg/ml) induced secretion of pro-inflammatory cytokines in mouse macrophage cell line (RAW264.7): TNF-α and IL-6 were measured at 24 h post treatment. For IL-1β analysis, the cells were treated (primed) with either JHP0940 (CtkA) or LPS (as positive control) for 3 h followed by 20 min of incubation with 5 mM ATP to activate the release of mature IL-1β. The cells treated with LPS (0.1 μg/ml), heat inactivated JHP0940 (100 °C for 10 min) and Proteinase K were taken as controls. The data represent means ± S.E. ($n = 3$) and P value for TNF-α, IL-6 and IL-1β was calculated separately by comparing with corresponding mock control. (B) Real time analysis of relative expression of TNF- α, IL-6 and IL-1β after 6 h of JHP0940 protein treatment (1 μg/ml) of RAW264.7 cells indicated increased relative expression of above cytokines when compared to mock. β-2 microglobulin was used as endogenous control for normalization. The data represent mean ± S.E. of duplicates ($n = 3$). P value for TNF-α, IL-6 and IL-1β was calculated separately by comparing with corresponding mock control. $P < 0.05$ was considered as significant.

washing, plates were incubated with biotin conjugated anti-TNF- or anti-IL-6 or anti-IL-1β antibody followed by incubation with streptavidin coupled to HRP. The HRP activity was detected using a chromogenic substance *o*-phenylenediamine tetrahydrochloride (Sigma) in citrate-phosphate buffer (pH 5.4) and H_2O_2. The reaction was terminated using 1 N H_2SO_4, and the absorbance values was measured at 492 nm. Standard curve for the cytokine was obtained using the recombinant standard protein provided by the manufacturer.

Real time PCR

RAW264.7 cells were cultured in the absence or presence of the JHP0940 protein in a humidified atmosphere with 5% CO_2 at 37 °C for 6 h. Following incubation, cells were harvested. For RT-PCR of cytokines, mRNA was extracted from the stimulated cells by RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Reverse transcription was performed with SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's recommendation. Real-time PCR was performed on a MyiQ Icycler, BioRad), using SYBR Green SuperMix. Quantification of various cytokine levels were performed by the Ct method and normalized by β-2 microglobulin level.

Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out for checking cell viability of mouse macrophage RAW cells in 96 well culture plate using different concentration of JHP0940 protein. The cells were seeded at a density of 5×10^3 cells per well in 200 μl of complete medium followed by protein treatment for 24 h. 20 μl of 5 mg/ml MTT per well was added followed by 3 h incubation in dark or until the colour of the formazen product developed. Culture medium was removed and 50 μl/well DMSO was added followed by 5 min incubation in dark. The plate was measured at 570 nm.

Ladder assay

Ladder assay was performed according to method described by Gong et al. (1994) with minor modifications. Approximately one million RAW 264.7 cells were treated with JHP0940 protein (10 μg/ml) for 48 h. The cells were trypsinized and suspended in HBSS (Hank's buffer salt saline) solution and prefixed in ice cold 70% ethanol, stored in deep freezer for 24 h before the assay. After fixing, the cells were centrifuged at $800 \times g$ for 5 min to remove ethanol and DNA was extracted with 0.2 M phosphate-citrate buffer

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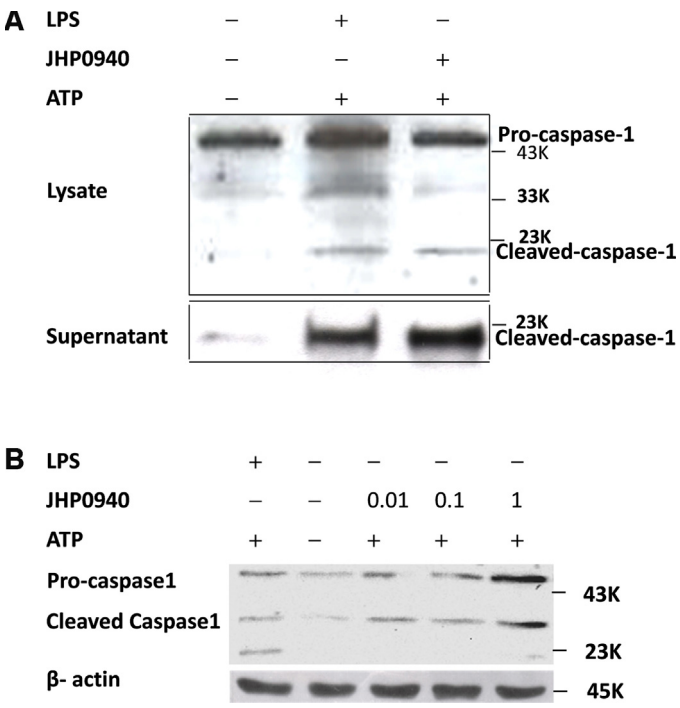

Fig. 3. Induction of caspase-1 in mouse macrophage RAW264.7 cell line on treatment with JHP0940: (A) Activation of caspase 1 was detected in whole cell lysate and in culture supernatants after priming either with JHP0940 (1 μg/ml) or LPS (as positive control) for 3 h followed by activation with 5 mM ATP for 20 min. (B) Dose dependent activation of caspase-1 in the cells pre-treated with JHP0940 and LPS for 3 h followed by 5 μM ATP treatment for 20 min. JHP0940 was used in varied doses of 0.01, 0.1 and 1 μg/ml for 3 h. LPS (positive control) was used at 100 ng/ml concentration. (K: molecular weight in kilodaltons).

(PCB) at pH 7.8 by suspending the pellet in 40 μl of PCB and incubating at room temperature for 30 min. Under these conditions, the partially degraded, oligonucleosomal DNA could be extracted selectively from the cells whereas the higher molecular weight DNA stayed associated with the nuclei. The extract (supernatant) was centrifuged at 1000 × *g* for 5 min and were treated with 1% Nonide NP40 (in water), 3 μl RNase A (5 mg/ml in water) and incubated at 37 °C for 30 min. 6 μl Proteinase K was added to it and incubated at 37 °C for 30 min. DNA loading dye was added to these samples and loaded to the 1.2% agarose gel with 0.5% EtBr. The gel was run at 4 V/cm and the bands were visualized.

Confocal microscopy

Confocal microscopy was used for visualization of DAPI stained nucleus of apoptotic cells. RAW264.7 cells were seeded at a density of 0.1×10^6/well on autoclaved coverslips in 24 well culture plate followed by protein treatment for 48 h. The cells were fixed in 4% paraformaldehyde and incubated for 15 min. After 3 washes with PBS the cells were permeabilized with pre-chilled 0.2% Triton-X followed by 15 min incubation at room temperature. DAPI with anti-fade reagent was added and the cells were mounted on slide followed by confocal microscopy for observing degraded nucleus.

For TUNEL assay, 0.05×10^6 cells/well were seeded followed by protein treatment for 48 h. The cells were fixed and permeabilized as mentioned above. After washing, the cells were equilibrated for 10 min at room temperature with equilibration buffer provided with ApoAlert DNA Fragmentation Assay Kit. (Clontech) This was followed by tailing reaction in TdT incubation buffer according to the manufacturer's protocol. Tailing reaction was terminated followed by washing with PBS. Cells were stained using DAPI with anti-fade reagent followed by observation under confocal microscope.

Flow cytometry (expression of fas receptors on RAW264.7 cells)

To determine expression of Fas receptors on RAW264.7 cells, cells were seeded followed by treatment with JHP0940 for different time intervals and in different doses. Cells were harvested by trypsinization very carefully to avoid loss of receptors, washed in PBS and suspended in wash buffer (PBS + 0.5% BSA) followed by incubation with FITC-conjugated anti-mouse CD-95 monoclonal antibody for 30 min at 4 °C. For isotype control, cells incubated with FITC-conjugated mouseIgG1 were used. The incubation was followed by washing of the cells with the wash buffer. The cells expressing Fas receptor were detected by flow cytometry analysis on BD FACS Canto II (BD Biosciences) and analysed by FlowJo software (FlowJo).

Western blotting

Equivalent amount of proteins were separated on SDS-PAGE and transferred electrophoretically to PVDF membranes. The blots were blocked with 4% BSA followed by overnight incubation with primary antibodies such as anti-CagA (Conradi et al., 2012) and various commercial antibodies (NEB and Santa Cruz) at 4 °C in TBST (0.1% v/v) and 2% (w/v) BSA. The blots were washed and incubated with secondary HRP antibody in 2% (w/v) BSA for 1 h at room temperature. After washing with TBST the blots were developed with enhanced chemiluminescence regent and exposed to X-ray film.

In vitro tyrosine kinase assay

Kinase reactions were carried out in 25 μl of volume consisting of 5 μl 2× Assay buffer (2 mM DTT, 2 mM $MnCl_2$, Na_3VO_4), 5 μl of 250 μM ATP solution, 5 μl of Src substrate (1 mg/ml), a synthetic peptide with sequence-KVEKIGEGTYGVVYK and 1 μl Src or c-Abl enzymes (NEB) at 30 °C for 15 min as described (Lind et al., 2014). Both Src substrate and Src enzyme were replaced by JHP0940 in the test reactions for showing auto phosphorylation activity of the JHP0940. Then 25 μl ADP-Glo reagent was added to deplete unused ATP, plate was shaken and allolwed 40 min incubation at room temperature 50 μl kinase detection reagent was added and incubated for 30 min at room temperature followed by reading in luminometer (Promega).

Statistical analysis

Two tailed Mann–Whitney *U* test was performed for the humoural response in *H. pylori* infected patient v/s healthy control cohorts and *P* values were calculated at 95 per cent confidence level. Student's *t*-test was performed for the analysis of results wherever required. The data were expressed as the mean of triplicates ± SEM unless or otherwise mentioned. $P < 0.05$ was considered as significant.

Results

JHP0940 is likely presented to the immune system in H. pylori infected patients

Earlier observations of our group showed that JHP0940 is associated with gastric disease in patients from different regions of the world and that enhanced activation of NF-κB occurs in cultured macrophages (Rizwan et al., 2008). This prompted us to investigate if the protein is expressed by *H. pylori* in gastric

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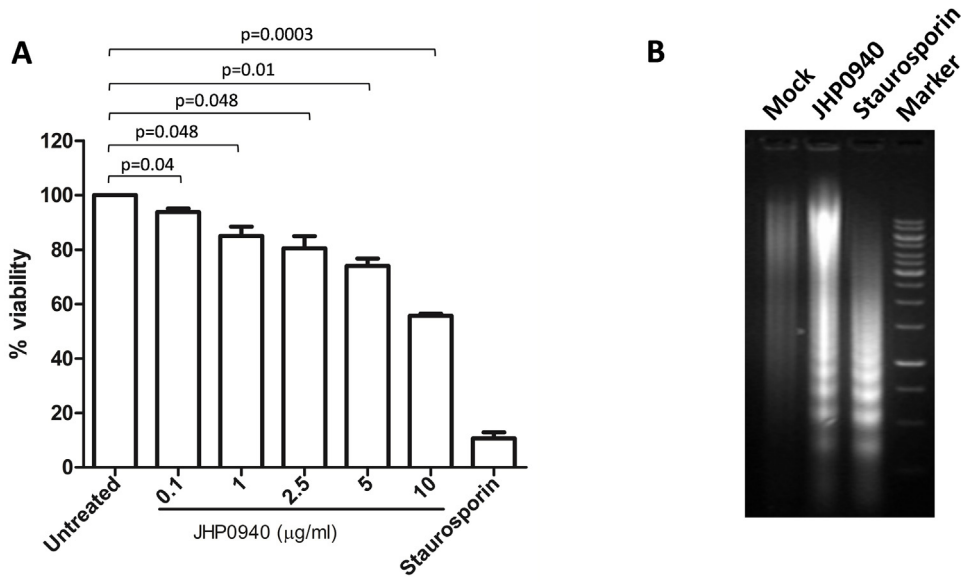

Fig. 4. (A) MTT assay of RAW264.7 cells after 24 h of treatment with different concentration of JHP0940 indicated dose dependent decrease in viability. The data are represented as means ± S.E. ($n = 3$). P value for different treatment experiments was calculated by comparing with untreated control. $P < 0.05$ was considered as significant and $P < 0.01$ as highly significant. (B) Ladder assay: The fragmentation of DNA of RAW264.7 cells on treatment with JHP0940 (10 μg/ml) was found similar to RAW264.7 cells treated with staurosporin as positive control. In mock (untreated RAW264.7 cells), DNA fragmentation was not observed. (Marker: 1Kb DNA ladder).

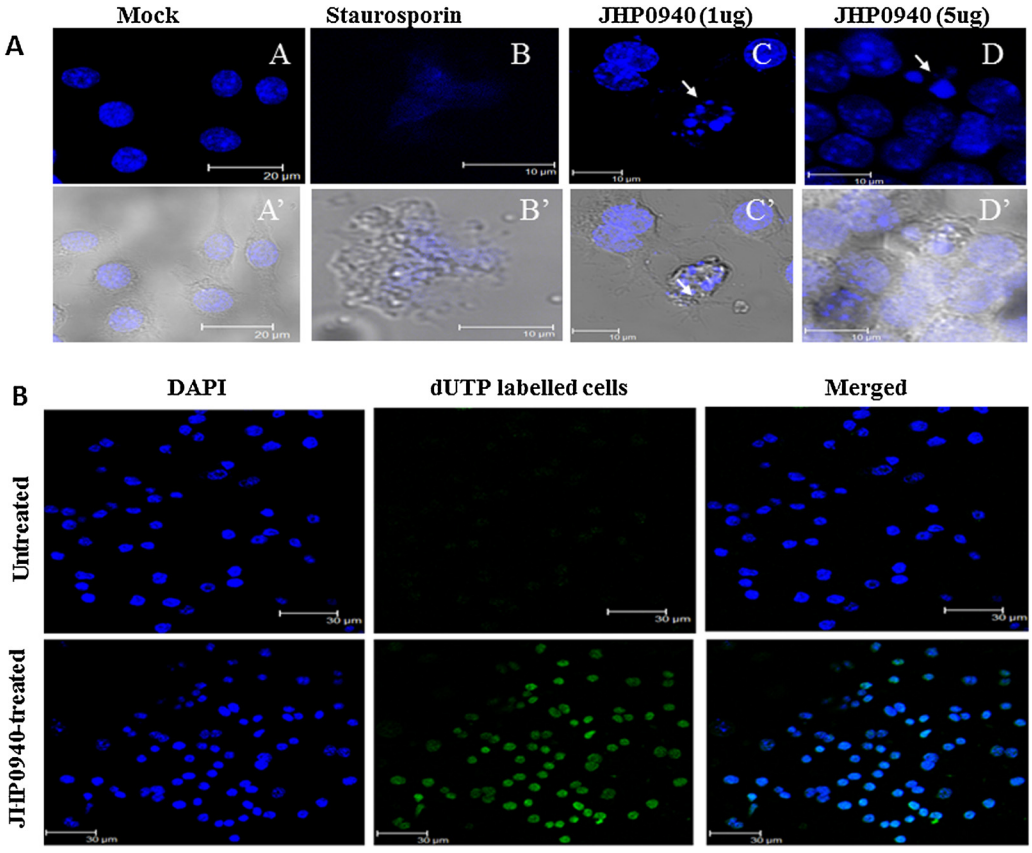

Fig. 5. (A) Confocal microscopy images indicating apoptosis of RAW264.7 cells on treatment with JHP0940 protein, after 48 h. Arrows in the images show apoptotic bodies. The RAW264.7 cells upon treatment with 1 μg/ml and 5 μg/ml of JHP0940 protein resulted in apoptosis as compared to the cells treated with staurosporin (0.1 μg/ml) used as positive control. No apoptosis was observed in the mock experiment (untreated cells). (B) TUNEL assay showing apoptosis in RAW264.7 cells upon treatment with JHP0940 protein after 48 h. Fluorescence-labelled dUTP in the fragmented DNA of cells undergoing apoptosis was observed in cells treated with JHP0940 but such fluorescence was not observed in mock (untreated cells). The figures shown are representative of three independent experiments.

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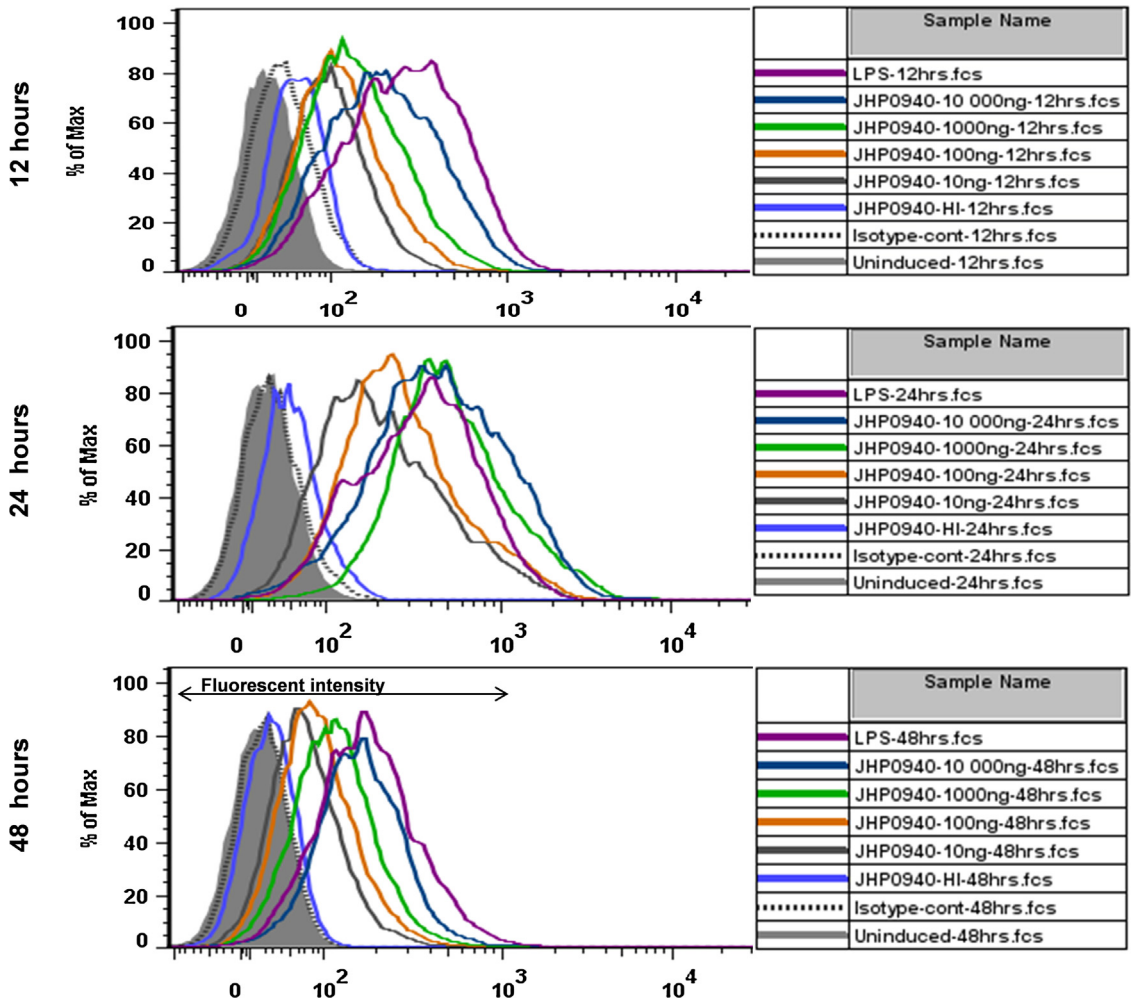

Fig. 6. Flow cytometry analysis showing expression of Fas receptors on RAW264.7 cells treated with JHP0940 in a dose dependent manner at 12, 24 and 48 h. The shift in the histogram peaks was observed in accordance with increase in concentration of JHP0940. RAW264.7 cells treated with LPS also indicated shift in histogram peak as compared to untreated. The shift in histogram peaks indicates increased expression of Fas receptors with respect to untreated.

environment and/or presented to the immune system. Since *H. pylori* colonizes the gastric epithelium, the bacterium should express some of its proteins/factors for gaining persistence while modulating the immune responses of the host. In this context, expression and exposure of factors to the immune system becomes important. To test this idea, we expressed and purified JHP0940 to homogeneity (Fig. 1A) and performed ELISA experiments with immobilized JHP0940 protein and human serum samples of *H. pylori* infected patients as compared to sera from non-infected healthy individuals. A significant difference in *P* value (0.0004) between *H. pylori* infected patients *versus* healthy group was found (Fig. 1B). This suggests that the protein expresses and presented to the immune system leading to the presence of anti-JHP0940 antibodies in the host.

JHP0940 is expressed in a subset of H. pylori strains and is secreted into the culture supernatant

Before its being presented to the immune system, we aimed to investigate if the Ctk protein is secreted into the extracellular environment. We tested this idea by fractionation studies of various wild-type *H. pylori* isolates including *ctk* gene-positive (J99 and SJM180) and *ctk*-negative (P1) strains, which were incubated for 12 h in liquid broth. Western blotting experiments showed that Ctk protein is expressed in strains J99 and SJM180, except P1 (Fig. 1C or D). In addition, bands for Ctk were also found in the culture supernatant fraction. As a control, the type IV secretion effector protein CagA, used as loading control for the *H. pylori* cell pellets (Fig. 1C) was not found in the supernatant as expected (Fig. 1D), thereby excluding artificial lysis of bacteria during experiments. Given this, our results hint at the secretory nature of the protein, allowing us to study how JHP0940 might affect signal transduction events during interaction with macrophages.

JHP0940 induces pro-inflammatory cytokines in RAW264.7 macrophage cells

RAW264.7 cells were grown and treated with recombinant JHP0940 protein at a concentration of 10 ng to 1000 ng/ml for 24 h followed by ELISA-based quantitation of cytokines in the culture supernatant. We found that the cytokines TNF-α, IL-1β and IL-6 were upregulated in a dose dependent manner (Fig. 2A). Furthermore, to confirm the pro-inflammatory properties of JHP0940, qRT-PCR was performed for mRNA profiling of the above cytokines (Fig. 2B). Our results from qRT-PCR corroborated with the ELISA results confirming the upregulation of the above cytokines (Fig. 2 B). *H. pylori* infection is recognized by the presence of chronic gastric inflammation and there is increasing evidence that caspase-1 is involved in the activation of the two important pro-inflammatory cytokines (IL-1β and IL 18), which normally mediates bacterial

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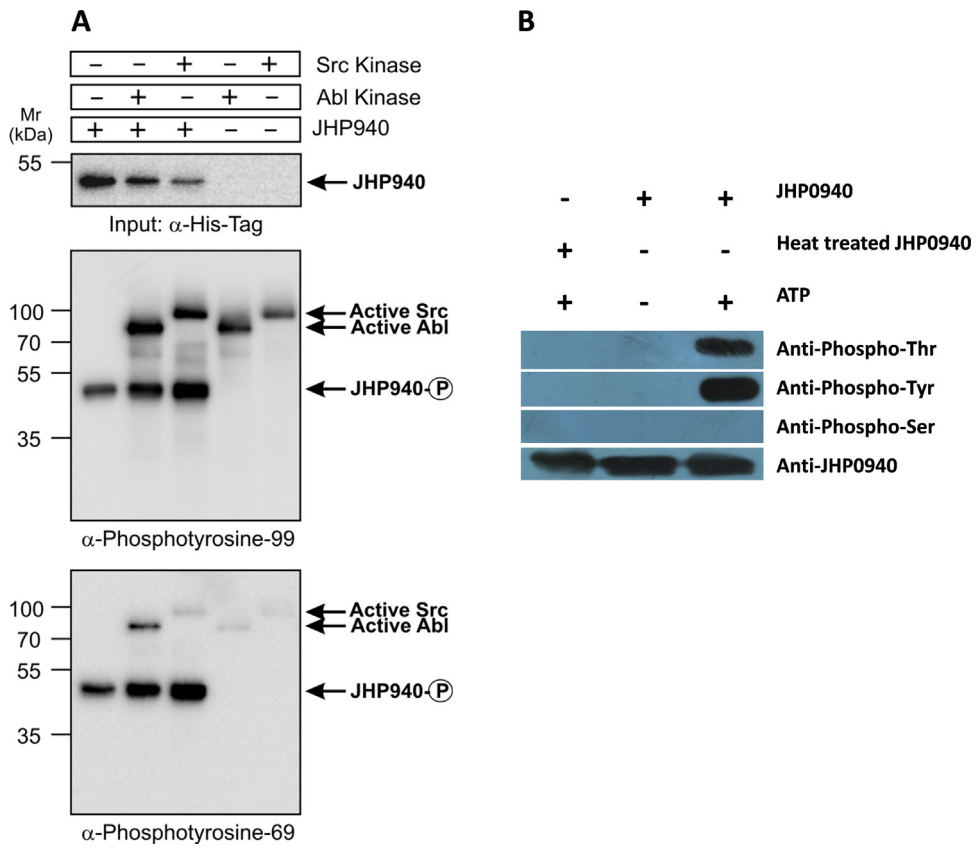

Fig. 7. (A) Western blot showing auto-tyrosine phosphorylation of JHP0940. Lane 1, from left represents kinase reaction with only JHP0940, lane 2 and 3 represent kinase reactions using JHP0940 along with Abl and Src kinase respectively. Lane 4 and 5 represent *in vitro* kinase reactions only with Abl and Src kinase respectively. Phosphorylation at tyrosine residues was probed using α-phosphotyrosine-99 and α-phosphotyrosine-69 antibodies. JHP0940 was probed with anti-His-tag antibody. (B). JHP0940 acts as auto phosphorylating kinase. *In vitro* kinase reactions were performed and phosphorylation at tyrosine and threonine residues was determined by respective anti-tyrosine and anti-threonine antibodies.

clearance after infection (Hitzler et al., 2012; van de Veerdonk et al., 2011). In this regard, we investigated the involvement of JHP0940 in caspase-1 induction. We found that caspase-1 expression was induced upon treatment of RAW264.7 cells with recombinant JHP0940. We observed that JHP0940 induces caspase-1 processing and activation in a dose dependent manner (Fig. 3B). We also observed that active caspase-1 is secreted into the cell culture supernatant (Fig. 3A). This observation partly corroborates with the above observation related to IL-1β secretion by murine macrophage cells that were treated with JHP040. Given these findings, we espouse the hypothesis that JHP0940 could most likely contribute to *H. pylori* pathogenesis by producing chronic gastric inflammatory responses and consequently ensuring *H. pylori* colonization. In our experiments, every time before treating the cells, JHP0940 protein was incubated with polymyxin B for 4 h to remove putative endotoxin contamination and to rule out the effect of bacterial endotoxin. Furthermore, we used proteinase K treated JHP0940 as control to ensure that the effect was not due to bacterial endotoxins.

JHP0940 decreases viability of RAW264.7 cells by inducing apoptosis through Fas receptors

Persistence of *H. pylori* in the gastric environment is the hallmark of this pathogen, not allowing the immune system to clear infection. The role of putative bacterial proteins/factors in immune evasion and related processes, however, is not yet fully clear. We therefore hypothesized that JHP0940 could induce caspase-1 activation to trigger macrophage apoptosis, thus facilitating enhanced bacterial survival. We therefore investigated the viability of RAW264.7 cells in response to exposure with JHP0940 protein. We observed that the protein induced cell death in these cells in a dose dependent manner leaving 56% of viable cells at 24 h upon treatment with 10 μg/ml of JHP0940. The cell viability was accessed by MTT assay for 24 h in a dose dependent manner (Fig. 4A).

Cell viability assays performed with RAW264.7 cells intrigued us to identify the cause of cell death and therefore, we performed the ladder assay. We observed that JHP0940 induced DNA fragmentation similar to the staurosporin control, explaining its role in apoptosis (Fig. 4B). To confirm these observations, we investigated the nuclear morphology of RAW264.7 cells by staining with DAPI using confocal microscopy. Our results revealed that JHP0940 induced degradation of chromosomal DNA in RAW264.7 cells (Fig. 5A). Furthermore, these observations were corroborated by TUNEL assay results (Fig. 5B). TUNEL assay revealed that most of the cells undergo apoptosis. These results were further confirmed by expression of Fas receptor on the surface of JHP0940-treated RAW264.7 cells in a time- and dose-dependent manner (Fig. 6). The expression of Fas receptors on murine macrophages intrigued us to test if JHP0940 acts as non-receptor tyrosine kinase. There are evidences where c-Abl non-receptor tyrosine kinases are involved in expression of Fas receptors (Gu et al., 2012).

JHP0940 acts as an auto-phosphorylating tyrosine kinase

In a previous study, it was shown that JHP0940 acts as cell-translocating ser/thr kinase (CtkA) and leads to phosphorylation

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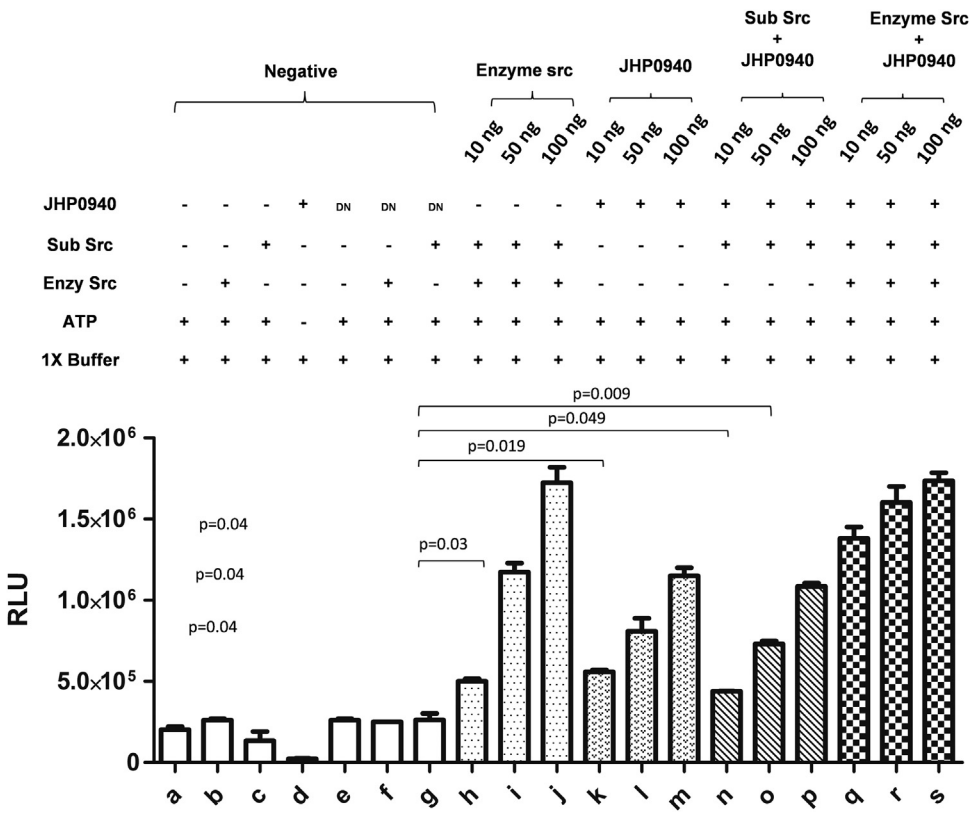

Fig. 8. *In vitro* tyrosine kinase assay. On X-axis, a to g are negative controls while *h*, *i* and *j* are reactions with increasing concentrations of Src enzyme (10, 50 and 100 ng) serving as positive controls. *k*, *l* and *m* are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) only. *n*, *o* and *p* are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src substrate (5 μg/reaction, the same concentration was maintained throughout the experiment). *q*, *r* and *s* are reactions with increasing concentrations of JHP0940 (10, 50 and 100 ng) along with Src enzyme (50 ng/reaction). *Y*-axis represents relative light units (RLU). The data are represented as mean ± S.E. of three independent experiments. DN represents denatured protein JHP0940. *P* values have been indicated in comparison with test and control samples.

at serine residue 276 residue of p65 subunit of NF-κB in cultured AGS gastric epithelial cells (Kim do et al., 2010). But, the association of the gene jhp0940 with gastric cancer (Santos et al., 2003) intrigued us to check if it can also act as a tyrosine kinase and hence we performed *in vitro* tyrosine kinase assay. In our results, JHP0940 was found to be an auto phosphorylating tyrosine kinase apart from its described serine/threonine kinase activity on western blot analysis (Fig. 7). In an *in vitro* tyrosine kinase assay, JHP0940 was used in enzyme range concentrations in the kinase reaction and when it was used along with the enzyme provided with the kit, it revealed cumulative enzymatic activity (Fig. 8).

Discussion

Putative virulence-associated genes of *H. pylori* have been categorized into three groups: strain specific genes, phase variable genes and genes with different structures and phenotypes (Yamaoka, 2008). The strain specific genes are further sub-classified into those encoded by two pathogenicity islands, the *cag*PAI, which encodes various virulence factors along with its T4SS and the effector protein CagA and plasticity region clusters (called plasticity zones) encoding nearly half of the strain-specific genes including another T4SS, called *tfs3* (Kersulyte et al., 2003; Fernandez-Gonzalez et al., 2014). These genes within the plasticity region are seemingly acquired extraneously and are of utmost interest because of their association with the gastric disease (Yamaoka, 2008). In previous studies, various genes of the plasticity region *i.e.*, *dupA*, *jhp*0940, *jhp0945*, *jhp0947* and *jhp0949* have been shown to be associated with various stages of gastric disease (Yamaoka, 2008). The first characterized member from this region, JHP0940 or CtkA, intrigued us to explore it in detail due to pre-existing observations such as its association with gastritis and gastric cancer in different geographical regions, high antigenicity index and *in vivo* expression in gastric mucosa of Mongolian gerbils (Graham et al., 2002). Furthermore, the mechanism of secretion of JHP0940 from *H. pylori* and its transfer to the gastric epithelial cells is not fully clear. It has also not been confirmed if JHP0940 uses T4SS-mediated injection mechanism like CagA or it harnesses some other secretory mechanism similar to other Gram-negative bacteria. However, our ELISA results portray it as a secretory protein (Fig. 1). The cell-translocating activity of the protein has been shown using GFP-fused full length recombinant protein in HeLa cells (Kim do et al., 2010). Regarding the pro-inflammatory nature of the protein, we do not rule out the involvement of kinase activity of JHP940 in IL-1β secretion since there are reports where death associated protein kinase, a type of serine/threonine kinases is required for IL-1β production (Chuang et al., 2011). But, at the same time, non-receptor tyrosine kinase members of the c-Abl family are also shown to involve in T-cell mediated inflammation (Gu et al., 2012).

It is well established that IL-1β plays a crucial role in inflammation and maintenance of infection (Dinarello, 2009). Subsequent to NF-κB activation, pro-IL-1β is synthesized in its pro-form, which is then cleaved by the action of caspase-1 to produce mature IL-1β. It is reported that the activation of caspase-1 is dependent on formation of multi-protein complexes, called inflammasome (Martinon et al., 2009; Dinarello, 2009; von Moltke et al., 2013). Recent studies identified the bacterial *cag* pathogenicity island and the cooperative interaction among host innate receptors TLR2, NOD2, and NLRP3

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as important regulators of IL-1β production in *H. pylori* infected murine dendritic cells (Kim et al., 2013). Here, our study by Western blot analysis of caspase-1 revealed its involvement in secretion of IL-1β (Fig. 3) and therefore, we propose a strong pro-inflammatory role for Ctk, with the involvement of inflammasome components, at least in murine macrophage cells.

In order to examine the effects of recombinant JHP940 on mouse macrophage cells in more detail, we observed decreased viability of host cells by MTT assay. This was followed by the visualization of apoptotic bodies in the DAPI-stained nucleus. Furthermore, the TUNEL assay confirmed occurrence of apoptosis in response to treatment of the cells with JHP940 protein. In a previous *in vitro* study, translocation of GFP-tagged JHP940 into HeLa cells was documented, indicating its cell-translocating behaviour (Kim do et al., 2010). The authors also found that transiently expressed protein translocates into the nucleus in a time dependent manner in AGS cells, although the protein doesn't contain a nuclear localization signal (NLS). These observations together with our findings based on an *in vitro* non receptor tyrosine kinase assay and its apoptotic effect on mouse macrophage cell line through Fas expression (death receptor) revealed that the activity of JHP0940 mimics the function of a non-receptor tyrosine kinase such as c-Abl. Upon caspase activation, c-Abl retained tyrosine kinase activity and triggered Fas induced apoptosis as previously shown (Barila et al., 2003). The Fas receptor activated by specific ligands, triggers assembly of a complex called as Death Inducing Signalling Complex (DISC) leading to the cleavage and activation of more downstream executive caspases and thus apoptosis (Raina et al., 2005). Moreover, epithelial and endothelial non-receptor tyrosine kinases (Etk) mediate apoptosis in small cell lung cancer cells (Cheng et al., 2010). We also found enhanced expression of Fas receptors in the mouse macrophage cell line in a time and dose dependent manner (Fig. 6).

Somehow, JHP940 does not share any sequence homology with c-Abl tyrosine kinases in any of the databases but it is predicted as cytoplasmic protein (score of 8.96 out of 10) by PSORTb v3.0.2 prediction tool (Gardy et al., 2003). The protein was found to have one SH2 domain when searched for domains in Scansite Motif Scanner database (http://scansite.mit.edu/motifscan_seq.phtml). Also, it revealed homology with one of the SH2 domains of CrkII. This SH2 domain of CrkII serves as binding site for SH3 domain of c-Abl mediated by Crk phosphopeptide and thus regulates biological functions (Donaldson et al., 2002). Despite homology with SH2 domain of CrkII, there is no experimental evidence to show that JHP0940 acts as an adaptor molecule like CrkII.

Other encoded protein from the plasticity region of *H. pylori* strain 26695, HP0986 (TieA), showed the same pro-inflammatory and pro-apoptotic behaviour but tested negative for kinase activity. However, HP0986 interacted with TNFR1, also having an endonuclease activity hinting at the potential pathobiological role of proteins/factors encoded by plasticity region genes (Alvi et al., 2011; Devi et al., 2014).

It will not be surprising that JHP0940, acting as an auto-phosphorylating tyrosine kinase and by translocating into the nucleus activates transcription factor(s) apart from NF-κB to modulate signalling in the host cells. It hints that JHP0940 may be a secretory protein, though the secretory mechanism of the protein is not established, which bacteria could use in modifying the signalling responses of the host cells. Thus, on the basis of our above observations and results, we assume that on one hand JHP0940 helps *H. pylori* to orchestrate inflammation as a survival mechanism and on the other hand it averts immune recognition by limiting macrophages through Fas mediated apoptosis. Further, it will be pertinent to investigate if JHP0940 acts as dual specific kinase or it acts as serine/threonine or tyrosine kinase in certain specific conditions and or cellular environment to help *H. pylori* in progression of gastric disease.

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Helicobacter pylori protein HP0986 (TieA) interacts with mouse TNFR1 and triggers proinflammatory and proapoptotic signaling pathways in cultured macrophage cells (RAW 264.7)

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ABSTRACT

HP0986 protein of *Helicobacter pylori* has been shown to trigger induction of proinflammatory cytokines (IL-8 and TNF-α) through the activation of NF-κB and also to induce Fas mediated apoptosis of human macrophage cells (THP-1). In this study, we unravel mechanistic details of the biological effects of this protein in a murine macrophage environment. Up regulation of MCP-1 and TNF-α in HP0986-induced RAW 264.7 cells occurred subsequent to the activation and translocation of NF-κB to the cell nucleus. Further, HP0986 induced apoptosis of RAW 264.7 cells through Fas activation and this was in agreement with previous observations made with THP-1 cells. Our studies indicated activation of TNFR1 through interaction with HP0986 and this elicited the aforementioned responses independent of TLR2, TLR4 or TNFR2. We found that mouse TNFR1 activation by HP0986 facilitates formation of a complex comprising of TNFR1, TRADD and TRAF2, and this occurs upstream of NF-κB activation. Furthermore, FADD also forms a second complex, at a later stage, together with TNFR1 and TRADD, resulting in caspase-8 activation and thereby the apoptosis of RAW 264.7 cells. In summary, our observations reveal finer details of the functional activity of HP0986 protein in relation to its behavior in a murine macrophage cell environment. These findings reconfirm the proinflammatory and apoptotic role of HP0986 signifying it to be an important trigger of innate responses. These observations form much needed baseline data entailing future *in vivo* studies of the functions of HP0986 in a murine model.

1. Introduction

Helicobacter pylori infection is the known cause of infectious gastritis, peptic ulcer disease and gastric cancer. Although more than 50% of world's population is infected with *H. pylori*, only 10–20% of total infected cases progress to form clinically significant morbidity [1]. The infection initiates with the adherence of *H. pylori* to the gastric epithelium with concomitant immune responses leading to infiltration of neutrophils and lymphocytes. The inflammation developed during this process is often not sufficient enough to clear off the infection. This leads to persistence of *H. pylori* infection for decades [2]. However, variations have been observed in the course and severity of infection in different individuals which can be attributed to bacterial strain heterogeneity, host genetic susceptibility and environmental factors [3–5].

Genetic heterogeneity of *H. pylori* is reflected in its varied functional effects occurring due to variations in the allelic structures and functional repertoires of certain virulence factors. Among the several known virulence factors, cytotoxin-associated gene (cag) Pathogenicity Island (*cag*PAI) and vacuolating cytotoxin (VacA) are most extensively studied [6–9]. In addition, factors contributing towards the adherence of *H. pylori* to gastric epithelial cells (SabA, BabA, AlpA/B, HopZ and OipA) also play crucial roles during pathogenesis [10–13]. However, the diversity in expression of these virulence factors in clinical strains is reflective of the existence of selection pressure on the colonizing strains infecting the same or different individuals. Such variability in strain specific virulence factors is also responsible for differences in virulence potentials of *H. pylori* isolates [10,14,15]. Many *H. pylori* strain-specific genes that encode putative virulence factors of unknown functional mechanisms are encoded within highly variable chromosomal plasticity zones (PZs) [3]. Most PZ genes are predicted to be of unknown function but the encoded products of some are considered to influence the virulence potential of infecting strains.

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The HP0986 protein encoded within the PZ of strain 26695 is one such example. Our previous studies have shown it to be naturally expressed and presented to the immune system and addition of purified recombinant HP0986 to cultured macrophages and gastric epithelial cells were shown to activate NF-κB leading to up regulation of proinflammatory cytokines such as TNFα and IL-8 [16,17]. Also, HP0986 actively induced apoptosis *via* TNFR1 and Fas mediated pathways in THP-1 cells [16], although specific steps and details of these pathways have remained elusive. These pathways urgently required to be dissected. Further, there was a need to confirm HP0986 induced signaling in a murine setup in order to widen the study towards reproduction of the effects *in vivo*.

2. Materials and methods

2.1. Murine macrophage (RAW 264.7) cell culture

RAW 264.7 cells were procured from NCCS, Pune, India. The cells were grown in RPMI 1640 medium (HyClone™, Thermo Fisher Scientific, USA) supplemented with 10% fetal bovine serum, 2 mM glutamine and 1% anti-bacterial and anti-mycotic agent (Gibco, Life Technologies™). Approximately 0.5×10^6 cells were grown until 60–70% became confluent in 12 well culture plates. Cells were induced with increasing concentration of recombinant (His-tagged) HP0986 (0.5 μg, 1.0 μg, 2.5 μg and 5.0 μg/ml). Cells without any stimulus and cells with proteinase-K treated HP0986 were used as negative controls. Cells treated with LPS (5.0 μg/ml) from *Escherichia coli* 055:B5 (Sigma Aldrich, USA) served as positive control. Culture supernatants from each treated set were collected after 10 h of incubation and stored at −80 °C until used for assays.

2.2. Cytokines assay

Cytokines/chemokines in the culture supernatant were analyzed by multiplexed bead based immunoassays using commercially available Cytometry Bead Array (CBA) Kit (BD Biosciences, USA). The assay was performed according to manufacturer's instructions. The samples' flow data were collected using a flow cytometer (BD FACS CantoII, BD Biosciences, USA) and analyzed using FCAP Array multiplex analysis software (BD Biosciences, USA). Cytokine/chemokine concentrations were determined with a standard curve plotted using the intuitive FCAP array software. The values were represented as mean ± SE.

2.3. Real-time PCR

Approximately 2×10^6 RAW 264.7 cells per well and per 2 ml of RPMI media were grown until 70–80% confluent in a 6 well culture plate. Cell were treated with recombinant (His-tagged) isocitrate dehydrogenase (ICD) (2.5 μg/ml), LPS (5.0 μg/ml) and HP0986 (2.5 μg/ml). An aliquot of cells was also treated with neutralizing anti-mouse TNFR1 antibody (5.0 μg/ml) before HP0986 (2.5 μg/ml) induction. The cells were incubated for 6 h. After incubation, cells from each well were harvested in 1 ml TRI reagent (Sigma Aldrich, USA). Total RNA was extracted as per the manufacturer's instruction. One microgram of total RNA was taken for generation of cDNA using Verso cDNA Synthesis Kit (Thermo Fisher Scientific, USA) according to the protocol described in their manual. The mRNA levels of MCP-1 and TNF-α were measured with the help of SYBR green (Applied Biosystems, Life Technologies™) in a Mastercycler® ep *realplex* thermal cycler (Eppendorf, Germany). Following primers were used for the amplifications: Mouse TNF-α Forward primer: TCCCAGGTTCTCTTCAAGGGA, Reverse primer: GGTGAGGAGCACGTAGTCGG; Mouse MCP-1 Forward primer: GAAGGAATGGGTCCAGACAT, Reverse primer: ACGGGTCAACTTCACATTCA and Mouse GAPDH Forward primer: TGTGTCCGTCGTGGATCTGA and Reverse primer: CCTGCTTCACCACCTTCTTGA. The relative expression levels of MCP-1 and TNF-α were normalized to expression values of GAPDH taken as a housekeeping gene reference.

2.4. Western blot analysis

RAW 264.7 cells were harvested and centrifuged at 500*g* for 5 min. The whole cell extract was prepared using non-denaturing lysis buffer and according to the protocol described earlier [18]. Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. The extracts were stored at −80 °C until use. Western blotting was preformed as per the previous description [19]. Primary antibodies used were against p65 (NF-κB), procaspase-8, procaspase-3, β-actin (Santa Cruz Biotechnology Inc., USA), lamin B and IκB-alpha (Sigma Aldrich, USA). Blots were developed using Super Signal West Pico Kit (Thermo Fisher Scientific, USA). β-actin was used as whole cell/cytoplasmic extract marker and for nuclear extract, lamin B was used as loading control.

2.5. Immunoprecipitation assays

For interaction analysis, cell extracts were prepared with RIPA buffer as described before [18]. An aliquot of 0.5 mg of the cell lysate was incubated overnight with HP0986 (10.0 μg) and polyclonal anti-HP0986 antibody (10.0 μg) at 4 °C. The immune complex was pulled down with protein-G agarose beads (Santa Cruz Biotechnology Inc., USA). The complexes were run on 12% SDS–PAGE and transferred over to PVDF membrane for western blot analysis using the primary antibodies directed against TNFR1 and TNFR2 (Santacruz Biotechnology Inc, USA), TLR2 and TLR4 (Imgenex, India). The immunoblots were developed with Super Signal West Pico Kit (Thermo Fisher Scientific, USA). Primary antibodies against TRAF2, TRADD and FADD (Santa Cruz Biotechnology Inc., USA) were used to analyze the respective co-precipitated molecules along with the HP0986-receptor complex by Immunoblotting.

2.6. Flow cytometric analysis

Cells were grown in 12 well plates till they became approximately 50–60% confluent. Cells were then treated with increasing doses of HP0986 (1.0, 2.5, 5.0 and 10.0 μg/ml) and incubated in a CO_2 incubator at 37 °C for 24 h for TNFR1 expression study. Similarly, for the Fas expression analysis, cells were treated with increasing doses of HP0986 (1.0, 2.5 and 10.0 μg/ml) and for different time periods (12 h, 24 h and 48 h). Unstimulated cells were taken as negative control for both Fas and TNFR1 expressions whereas TNF-α (0.2 μg/ml) treated cells acted as positive control. After incubation, cells were harvested in 0.2% EDTA solution (in PBS) and counted. Cells were centrifuged at 300*g* for 5 min and supernatant was completely removed. Cells were then resuspended in appropriate volume of blocking buffer (5% horse serum in PBS) to get 1×10^6 cells per ml of buffer. The resuspended cells were kept on ice for 30–45 min to ensure the blocking of the Fc receptor sites present on macrophage cells and therefore to enhance the specific binding of the primary antibodies to their respective antigenic sites on cells surface. Approximately 0.1×10^6 cells (100 μl) were taken in a polystyrene tube and the subsequent staining steps were performed in the same buffer. For TNFR1 expression analysis, cells were incubated with rabbit anti-TNFR1 antibody (Santacruz Biotechnology Inc. USA) for 45 min on ice, followed by incubation with secondary FITC-conjugated anti-rabbit antibody (Sigma Aldrich, USA) for another 45 min. For the Fas expression assay, approximately

0.1×10^6 (100 μl) cells were incubated directly with FITC-conjugated anti-CD95 antibody (BD Biosciences, USA) for 30 min on ice. After the incubation with labeled antibodies, the cells were washed twice and resuspended in 500 μl of the same buffer for immediate data acquisition on flow cytometer. Unstained cells were taken as internal negative control in both the assays to rule out any interference due to background fluorescence. All acquired data were analyzed with Flow Jo software (Tree Star Inc., USA).

2.7. MTT-assay for cell viability measurement

The RAW 264.7 cells' viability after HP0986 treatment was analyzed by MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (Amresco, USA) assay. The HP0986 induced cells were incubated for 48 h in a 96 well plate. An aliquot of 20 μl of MTT (5 mg/ml) was added to each well and kept for 3 h at 37 °C. The resultant insoluble Formazan complex was dissolved in dimethyl sulfoxide (Sigma Aldrich, USA). The colored solution thus formed was measured at 570 nm [20].

2.8. HP0986 mediated apoptosis analysis

HP0986 induced apoptosis in RAW 264.7 cells was analyzed by AnnexinV-FITC/PI assay. Cells were processed and staining was performed according to manufacturer's instructions (BD Biosciences, USA) and the sample data were acquired on flow cytometer (BD FACS Canto II, BD Biosciences, USA). The percentage of cells undergoing apoptosis were expressed as total of % $AnnexinV^+$ and PI^+ cells after subtracting the background fluorescence. Furthermore, apoptosis in HP0986 treated RAW 264.7 cells could be visualized through DNA fragmentation assay. The analysis was performed as per the method described previously [21]. In brief, 2×10^6 cells were washed with PBS and pelleted by centrifugation at 400*g* for 5 min. Cell pellets were then treated with a lysis buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl, pH, 7.5) by repeated pipetting up and down. Homogenates were then centrifuged at 13,000*g* for 5 min. Supernatants were then treated with RNase A at 37 °C for 2 h followed by proteinase-K at 37 °C for 2 h. Then a ½ volume of 10 M ammonium acetate was added to DNA solutions. To further facilitate the DNA precipitation, 2.5 volume of ethanol was added and centrifuged. DNA pellets were dissolved in gel loading buffer, run and analyzed on 1% agarose gel.

2.9. Statistical analysis

Results were expressed as means ± standard error (SE). Comparisons of cytokine inductions, TNFR1 and Fas expressions were performed by two-tailed *t*-test using GraphPad Prism version 5.01 for windows (Graph Pad software, San Diego, California USA: www.graphpad.com). The differences between two values were considered significant if $p < 0.05$.

3. Results

3.1. HP0986 induces expression and secretion of MCP-1 and TNF-α from murine macrophages

The N-terminal His-tagged protein was over expressed in BL-21(DE3) cells and purified under native conditions using Co^{+2}-NTA affinity column. RAW 264.7 cells treated with HP0986 and incubated for 10 h released MCP-1 and TNF-α into the culture supernatant in a dose dependent manner. A significant secretion of MCP-1 and TNF-α was observed when 1 ml aliquot of media with about 1 million cells was treated with increasing doses of HP0986 (0.5 μg, 1.0 μg, 2.5 μg and 5.0 μg/ml). Highest secretion of both MCP-1 ($p = 0.0002$) and TNF-α ($p < 0.0001$) were observed at the protein concentration of 2.5 μg/ml (Fig. 1A, B). Cells treated with even higher dose (5.0 μg/ml) of HP0986 did not show any further increase in MCP-1and TNF-α levels. Proteinase-K-treated HP0986 failed to induce the production of both MCP-1 and TNF-α suggesting that the response was indeed triggered by HP0986. Cells treated with LPS (5.0 μg/ml) were taken as a positive control for the induction of both MCP-1 and TNF-α (Fig. 1A, B). In order to further confirm the effect specific to HP0986, His-tagged isocitrate dehydrogenase (ICD) of *H. pylori* purified in a similar manner was used as an internal negative control. A relative mRNA quantification analysis using real-time PCR showed that cells treated with His-tagged ICD protein (2.5 μg/ml) failed to trigger MCP-1 and TNF-α mRNA expression above the control level and therefore ruling out the possible involvement of 6x His tag of recombinant, purified HP0986 in the above responses (Fig. 1C, D). Thus, our data reveal HP0986 specific proinflammatory cytokine responses in RAW 264.7 cells.

3.2. MCP-1 and TNF-α secretion is mediated by activation of NF-κB

In order to determine whether the MCP-1 and TNF-α secretion is regulated by NF-κB activation, we analyzed the NF-κB levels in nuclear and cytoplasmic fractions of HP0986 stimulated RAW 264.7 cells. We observed an increase in nuclear translocation of p65 in HP0986 treated RAW 264.7 cells in a time dependent manner. A clear and enhanced translocation of p65 in the nuclear extract of HP0986 treated (2.5 μg/ml) RAW 264.7 cells was observed with increase in time of incubation (0.5, 1.0, 2.0 and 3.0 h). The time course followed for this study indicated that maximum level of nuclear localization of p65 was observed at 3 h post treatment when compared to the control (Fig. 2B). The corresponding observed decrease in levels of p65 from cytoplasmic extracts during the same time period was also consistent with increased translocation to the nucleus. TNF-α (50 ng/ml) was taken as positive control for NF-κB translocation analysis. In a further study, western blot analysis showed noticeable degradation of IκB-alpha in the cytosol after stimulation with HP0986 (2.5 μg/ml). A visibly clear degradation of IκB-alpha was observed at 30 min, 1 h and 2 h post treatment with HP0986 (Fig. 2A). Taken together, we found that time course for IκB-alpha degradation preceded NF-κB activation.

3.3. Interaction of HP0986 with murine TNFR1

Host receptor involved in eliciting the cytokine response was analyzed by pull down assay which showed mouse TNFR1 captured together with HP0986 when probed with anti-TNFR1 antibody. However, HP0986 did not involve in any complex formation with TNFR2/TLR2 or TLR4 as revealed by pull-down assay using anti-HP0986 antibody followed by western blot analysis with anti-TNFR2, anti-TLR2 and anti-TLR4 antibodies (to rule out any possible HP0986 binding to receptors other than TNFR1) (Fig. 3A). Furthermore, an increase in cell surface TNFR1 expression was observed by flow cytometry in HP0986 treated RAW 264.7 cells in a dose (1.0, 2.5, 5.0 and 10.0 μg/ml) dependent manner (Fig. 3B). These observations confirmed the interaction between HP0986 and murine-TNFR1, an event upstream of inflammatory response, and also ruled out any interaction between HP0986 and TNFR2/TLR2 or TLR4. Moreover, RAW 264.7 cells pretreated with neutralizing anti-mouse TNFR1 antibody (5 μg/ml) prior to induction by HP0986 (2.5 μg/ml) revealed reduced translocation of p65 to the nucleus (Fig. 3C) and also decreased induction of MCP-1 and TNF-α (Fig. 1C and D). These observations clearly suggest a link between TNFR1 stimulation through HP0986 binding and NF-κB activation leading to the MCP-1 and TNF-α secretion in murine macrophages.

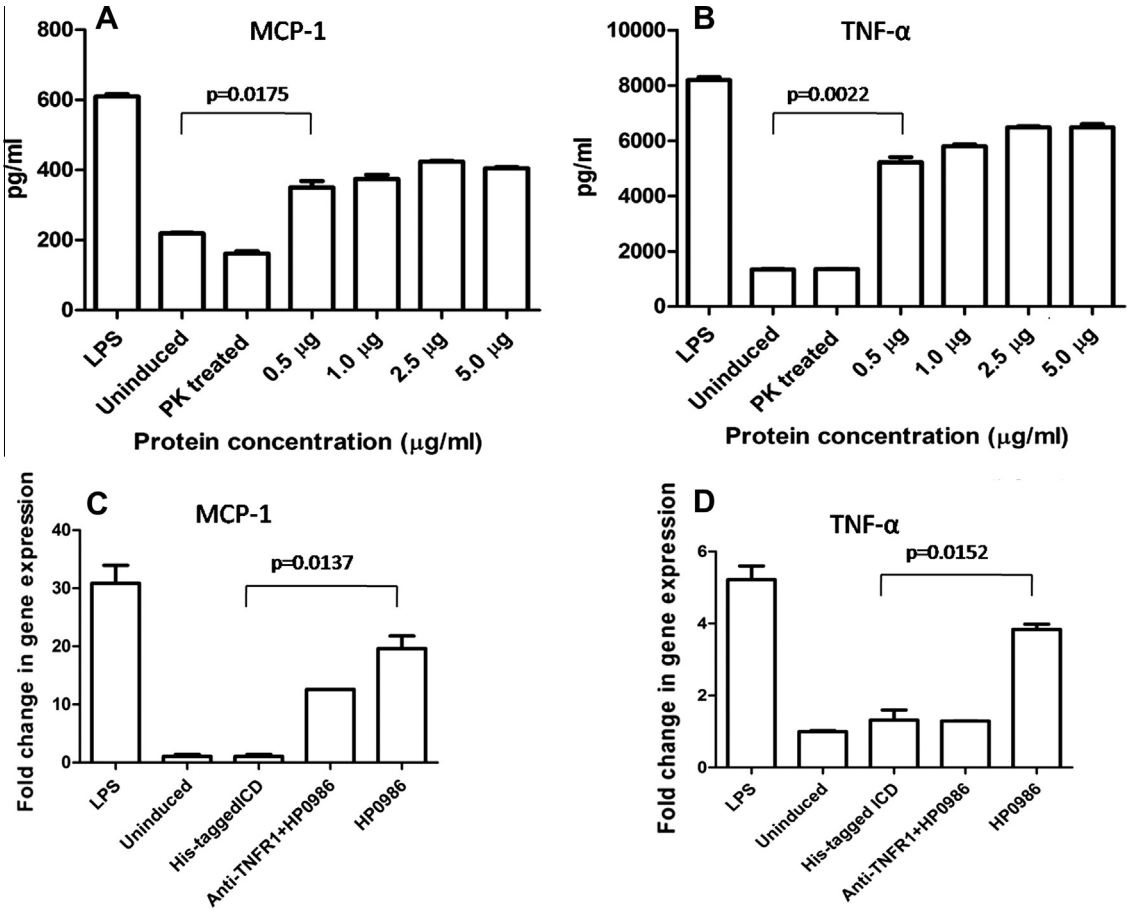

Fig. 1. (A) Secretion of MCP-1 and (B) TNF-α by murine macrophage (RAW 264.7) cells after treatment with HP0986. Uninduced cells, and cells stimulated with proteinase-K treated HP0986 were taken as negative control. LPS (5 μg/ml) treated cells were taken as positive control. Results are shown as mean ± SE. (C) Estimation of relative mRNA levels for MCP-1 and (D) TNF-α by real time PCR in HP0986 treated RAW 264.7 cells. Unstimulated cells, His-tagged ICD treated cells and the cells pretreated with neutralizing anti-mouse TNFR1 before HP0986 induction also served as negative controls for this study. Results are shown as mean ± SE.

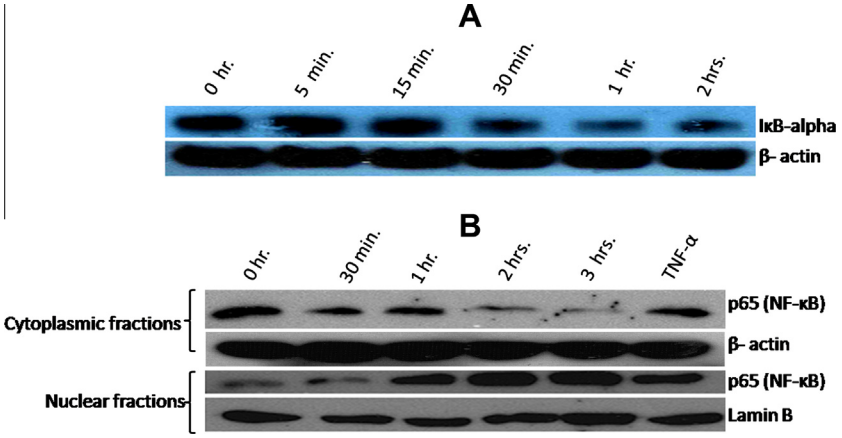

Fig. 2. (A) IκB-alpha degradation analysis in HP0986 treated murine macrophage (RAW 264.7) cells at different time intervals by western blotting. Reprobing of the blot with β-actin antibody was done to ensure equal loading of the protein. (B) Time dependent translocation analysis of p65 (NF-κB) in cytoplasmic and nuclear fractions from RAW 264.7 cells treated with HP0986. TNF-α treated cells were taken as positive control for this study. β-actin was used as a marker for equal loading of cytoplasmic fractions. Lamin B was used as a marker entailing nuclear fraction.

3.4. HP0986 induces Fas mediated apoptosis in murine macrophages (RAW 264.7)

Fas receptor on the cell surface induces apoptosis upon interaction with the ligand [22]. According to our previous observation with human macrophages (THP-1) that suggested a link between TNFR1 activation by HP0986 and Fas expression [16], we performed a similar analysis with murine macrophages. HP0986 were shown to induce increased expression of Fas on murine macrophages (RAW 264.7) in a dose (1.0, 2.5 and 10 μg/ml) and time (12, 24 and 48 h) dependent manner. However, at highest time point of 48 h incubation, the increment in protein doses did not reflect increase in Fas expression possibly due to apoptosis of the cells (Fig. 4). However, expression of Fas on murine macrophages was significantly higher than unstimulated cells. AnnexinV-FITC/PI assay also indicated an increase in apoptosis of

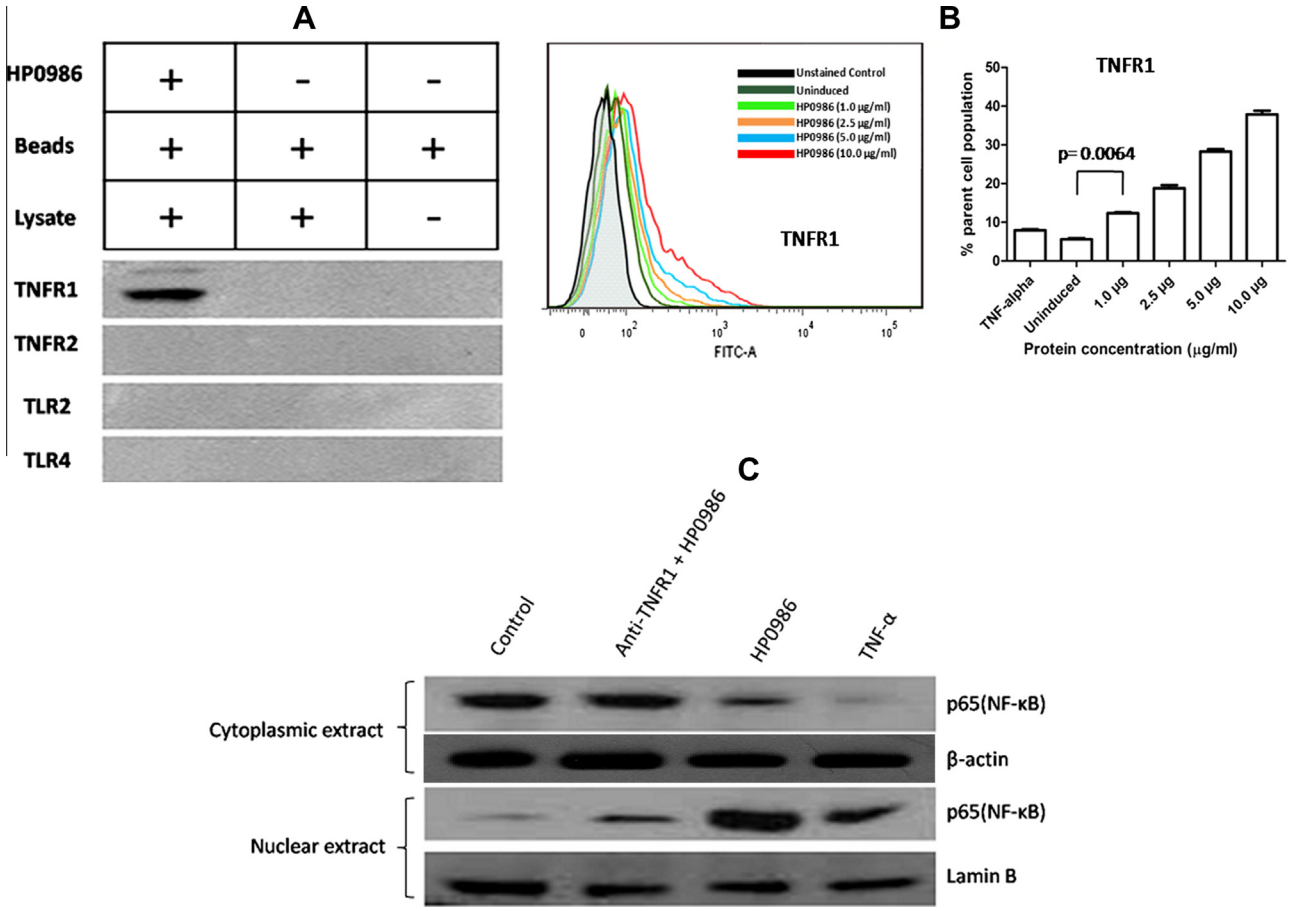

Fig. 3. (A) Pull down assays for binding analysis of HP0986 with receptors (TNFR1, TNFR2, TLR2 or TLR4). HP0986 bound receptor from the mouse macrophage cell lysate was pulled down with protein G-agarose beads and immunoblots were probed with anti-TNFR1, anti-TLR2, anti-TLR4 and anti-TNFR2 antibodies. Cell lysates incubated with protein-G beads and protein-G beads alone were used as negative controls for this assay. (B) Flow cytometry analysis showing increased effect of HP0986 on TNFR1 expression on murine macrophage (RAW 264.7) cells. Uninduced cells were taken as negative control while TNF-α treated cells were analyzed as positive control for TNFR1 expression. (C) Western blot analysis showing reduced translocation of p65 (NF-κB) in RAW 264.7 cells pretreated with neutralizing anti-TNFR1 antibody before induction with HP0986 when compared to cells treated with HP0986 alone. TNF-α treated cells served as positive control and unstimulated cells as negative control for this analysis.

HP0986 (5.0 μg/ml) treated RAW 264.7 cells. In the same analysis, upon HP0986 (5.0 μg/ml) stimulation, we observed decreased apoptosis of cells pretreated with neutralizing anti-mouse TNFR1 antibody (5.0 μg/ml). His-tagged ICD (5.0 μg/ml) failed to induce apoptosis and therefore ruling out the possible interference by 6 × His-tag attached to recombinant HP0986. Staurosporin (50 nM) treated cells in all cases were taken as positive control for apoptosis (Fig. 5A). Further, we observed an increase in procaspase-8 and procaspase-3 degradation in RAW 264.7 cells induced with HP0986 for 48 h in a dose (2.5, 5.0 and 10.0 μg/ml) dependent manner when compared with uninduced cells (Fig. 5B). As a positive control, Staurosporin (50 nM) also showed an enhanced degradation of pro-caspase-8 and procaspase-3. β-actin was taken as loading control for the above analysis. This observation is also in agreement with earlier reports suggesting that Fas engagement with its ligand results in accumulation, cleavage and therefore activation of caspase-8 which further activates the effector caspases [23,24]. Furthermore, apoptosis process in RAW 264.7 cells was validated through DNA fragmentation in HP0986 (5.0 μg/ml) treated cells (DNA ladder assay) as compared to unstimulated cells (Fig. 5C).

3.5. TNFR1 activation by HP0986 and recruitment of TRADD, TRAF2 and FADD

Both TRADD and TRAF2 were co-precipitated together with TNFR1-HP0986 complex as shown in immunoblots using lysate from the RAW 264.7 cells treated with HP0986 and incubated for 30 min (Fig. 6). This suggests that TRADD forms a complex with TNFR1 after interacting with HP0986 which in turn provides a platform for the recruitment of TRAF2 [25] leading to the NF-κB activation. Further, we also observed in a similar study, the co-precipitation of FADD along with TNFR1-HP0986 complex in the lysate from cells treated with HP0986 for 24 h (Fig. 6). However since FADD interacts with both TRADD and Fas, this indicates two possible pathways for HP0986 induced apoptosis: one mediated through TNFR1 and the other involving Fas activation [25,26].

4. Discussion

HP0986 or TieA is an importantly proinflammatory protein and putative type II endonuclease encoded by the plasticity region of *H. pylori* [16,17]. The protein has been profiled either (genotypically) using patient biopsies and clinical isolates corresponding to different populations or through (serological) testing for humoral immune responses and in (cell signaling) studies involving cultured human cells, and was also shown to induce proinflammatory cytokines through TNFR1 and NF-κB mediated signaling pathways [16,17]. However, its function has not been verified in an animal model such as mouse. The present study therefore assumes an important extension of the works of Alvi et al. [16] and Devi et al. [17] to reproduce the interaction of this protein with mouse immune apparatus (including TNFR1 and other receptors) and

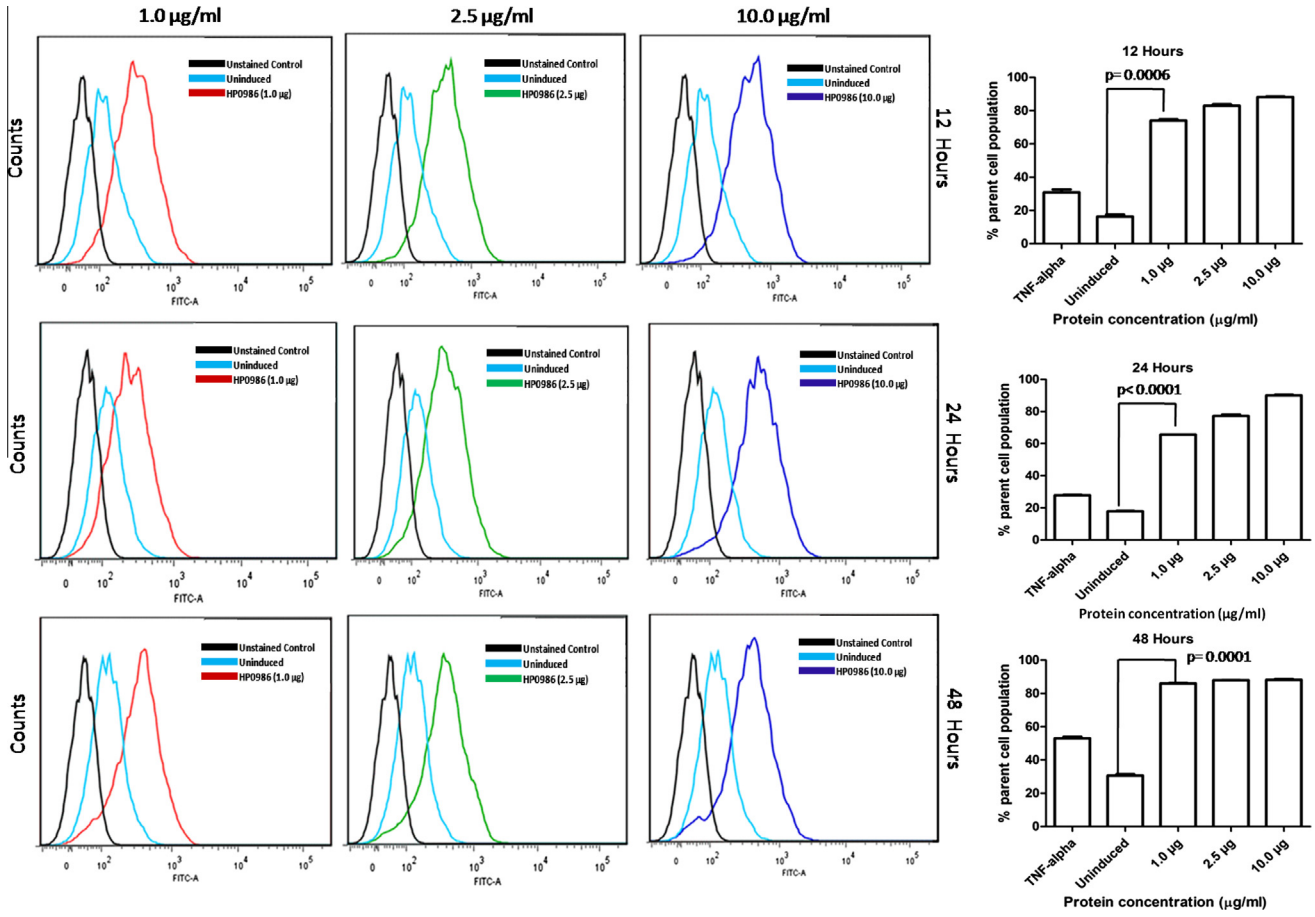

Fig. 4. Fas expression on the surface of murine macrophage cells (RAW 264.7) induced by HP0986 in a dose and time dependent fashion and analyzed using FITC-conjugated anti-Fas antibody by flow cytometry. Cells without protein stimulus and/or unstained uninduced cells served as negative controls and TNF-α treated cells as positive control. Results are also shown as mean ± S.E in bar diagrams.

characterize the downstream signaling events to unravel finer details of the apoptotic pathways in order to understand the complete scenario of the proposed 'survival advantage' to the bacterium [16,17] in a mammalian system.

In that pursuit, the strongest observation made in this study entails consistent proinflammatory and apoptotic behavior of HP0986 in a murine macrophage cell (RAW 264.7), similar to what has been observed earlier for the human cell lines and PBMCs. Induction of proinflammatory cytokines by the mouse cells confirms HP0986 to be an important antigen that excites mammalian cells. As a potent chemoattractant for monocytes/macrophages and T-lymphocytes, MCP-1 is reported to be actively involved in the inflammation process [27–30]. TNF-α secreted by macrophages could have several implications during bacterial infection. TNF-α binding to TNFR1 triggers Fas mediated apoptosis of macrophages [31–33]. Reports also indicate that TNF-α down-regulates phagocytosis of apoptotic cells by macrophages [34]. Recently, it has been shown in case of *H. pylori* infection that TNF-α accumulation during gastric injury has negative effect on the clearance of apoptotic gastric epithelial cells [35]. The apoptotic 'cell loads' further enhance inflammatory state of gastric mucosa through release of proinflammatory mediators from the dying cells following their secondary necrosis [35].

The present results reconfirmed involvement of NF-κB in induction of MCP-1 and TNF-α which are consistent with the previous reports [36,37]. The activation of NF-κB as observed through translocation of p65 to the nucleus after HP0986 induction was also supported by the degradation of the IκB-alpha in RAW 264.7 cells (Fig. 2). NF-κB activation is a key determinant of the immune cell response to the bacterial infection which in turn regulates expression of many inflammation promoting genes encoding several chemokines, cytokines and members of the IgG superfamily [38–42].

Fas expression by RAW 264.7 cells after HP0986 induction (Fig. 4) was in accordance with our previous observations in THP-1 cells [16]. This also suggests that the virulence potential and activity of HP0986 is similarly placed in the mouse system. In corroboration with previous reports, we also showed by immunoprecipitation method the interaction between HP0986 and murine TNFR1 (Fig. 3A). Furthermore, RAW 264.7 cells pretreated with neutralizing antibody against TNFR1 revealed reduced translocation of NF-κB to the nucleus upon induction with HP0986; this confirmed the dependency of the expression of inflammatory response genes on TNFR1 (Fig. 3C). This has thus indicated that HP0986 interaction with TNFR1 leads to a downstream inflammatory response as well as Fas mediated apoptosis.

To gain further insight into the signaling events, we have shown through immunoprecipitation analysis the interaction of HP0986 to TNFR1 receptor and activation of downstream signaling molecules including TRADD, TRAF2 and FADD (Fig. 6). Given these results, and on the lines of previous observations [43–46,26,16], we propose a signaling cascade similar to that mediated by TNF-α. In brief, stimulation of TNFR1 by HP0986 triggers the recruitment of the adaptor molecule TRADD to the cytoplasmic domain of TNFR1. Further, TRAF2 binds to the N-terminal domain of TRADD leading to the activation of NF-κB. TNFR1 activation also contributes to another pathway through the recruitment of FADD to the

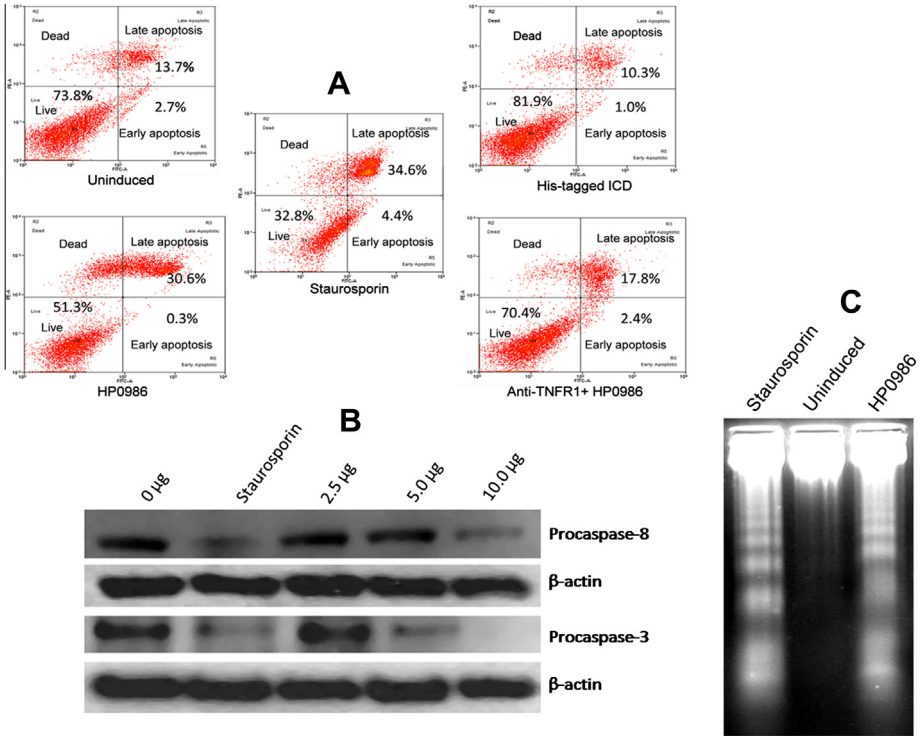

Fig. 5. (A) AnnexinV-FITC/PI staining to analyze apoptosis in murine macrophages cells by flow cytometry using (i) HP0986 induced cells, and (ii) cells treated with neutralizing antiTNFR1 antibody and HP0986; controls comprised of (iii) cells without protein stimulus (negative control), (iv) cells treated with recombinant His-tagged ICD protein (unrelated protein control) and (v) cells treated with Staurosporin (positive control). (B) Immunoblotting showing procaspase-8 and procaspase-3 cleavage analysis as a marker for apoptosis in murine macrophage cells in a dose dependent manner. Cells without any stimulus were taken as negative control. Staurosporin treated cells were used as positive control. (C) DNA ladder assay to assess the proapoptotic nature of HP0986 in RAW 264.7 cells. Cells without protein stimulation were taken as negative control. DNA isolated from Staurosporin induced cells was taken as positive control for apoptosis.

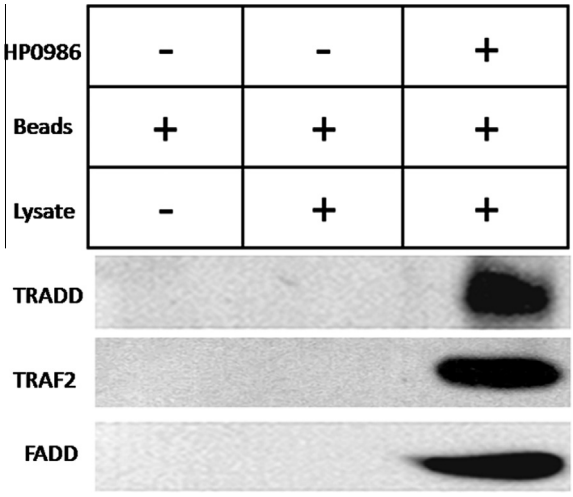

Fig. 6. Co-precipitation of TRADD, TRAF2 and FADD recruited to activated TNFR1 bound with HP0986. These adaptor molecules were pulled down together with HP0986-TNFR1 complex and detected by western blotting. Protein-G beads incubated with cell lysate, and protein-G beads alone were used as negative controls for this analysis.

C-terminal domain of TRADD and subsequent activation of caspase-8 leading to apoptosis. However, since FADD also connects Fas to caspase-8 activation [26], there could be a possibility of involvement of an additional death pathway (other than that mediated through TNFR1) in HP0986 treated mouse macrophages.

In view of our observations, it is now possible to clearly portray the role of HP0986 in inflammation and apoptosis at the interface of various possible cellular pathways that conjoin TNFR1, Fas and NF-κB. These new data unleashing in a mouse macrophage background would definitely underpin future efforts directed at understanding the complexity and nature of *H. pylori* induced cancer signaling. The immunological co-ordinates so established would also facilitate *in vivo* validation of HP0986 function in a mouse system in order to study the secretion, localization and regulation of this important virulence factor.

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Helicobacter pylori Antigen HP0986 (TieA) Interacts with Cultured Gastric Epithelial Cells and Induces IL8 Secretion *via* NF-κB Mediated Pathway

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Keywords
Gene expression, *Helicobacter pylori*, *Helicobacter pylori* seropositivity, IL-8, virulence factor.

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Abstract

Background: The envisaged roles and partly understood functional properties of *Helicobacter pylori* protein HP0986 are significant in the context of pro-inflammatory and or proapoptotic activities, the two important facilitators of pathogen survival and persistence. In addition, sequence analysis of this gene predicts a restriction endonuclease function which remained unknown thus far. To evaluate the role of HP0986 in gastric inflammation, we studied its expression profile using a large number of clinical isolates but a limited number of biopsies and patient sera. Also, we studied antigenic role of HP0986 in altering cytokine responses of human gastric epithelial (AGS) cells including its interaction with and localization within the AGS cells.

Materials and Methods: For *in vitro* expression study of HP0986, 110 *H. pylori* clinical isolates were cultured from patients with functional dyspepsia. For expression analysis by qRT PCR of HP0986, 10 gastric biopsy specimens were studied. HP0986 was also used to detect antibodies in patient sera. AGS cells were incubated with recombinant HP0986 to determine cytokine response and NF-κB activation. Transient transfection with HP0986 cloned in pEGFPN1 was used to study its subcellular localization or homing in AGS cells.

Results: Out of 110 cultured *H. pylori* strains, 34 (31%) were positive for HP0986 and this observation was correlated with *in vitro* expression profiles. HP0986 mRNA was detected in 7 of the 10 biopsy specimens. Further, HP0986 induced IL-8 secretion in gastric epithelial cells in a dose and time-dependent manner *via* NF-κB pathway. Serum antibodies against HP0986 were positively associated with *H. pylori* positive patients. Transient transfection of AGS cells revealed both cytoplasmic and nuclear localization of HP0986.

Conclusion: HP0986 was moderately prevalent in clinical isolates and its expression profile in cultures and gastric biopsies points to its being naturally expressed. Collective observations including the induction of IL-8 *via* TNFR1 and NF-κB, subcellular localization, and seropositivity data point to a significant role of HP0986 in gastroduodenal inflammation. We propose to name the HP0986 gene/protein as 'TNFR1 interacting endonuclease A (TieA or *tie*A)'.

Helicobacter pylori infection is characterized by the infiltration of mononuclear and polymorphonuclear cells into the gastric mucosa in addition to the accumulation of various cytokines, including IL1β, IL6, IL-8, and TNFα secreted by gastric epithelial and immune cells [1, 2]. Among these cytokines, IL-8 is a potent pro-inflammatory mediator secreted mainly by the gastric epithelial cells and plays an important role in the recruitment and activation of neutrophils [3]. The clinical phenotype of *H. pylori* infection depends on several determinants that

include virulence factors, the host susceptibility to infection and other environmental co-factors [4–7]. It was previously believed that CagPAI was the chief virulence factor that strongly associates with severe gastric inflammation while encoding proteins that induce IL-8 secretion by epithelial cells [8]. However, subsequent studies could not identify a strong association between IL-8 induction and the presence or the functionality of the cagPAI; they found that several other genes unrelated to CagA status, such as *oip*A, flagellar proteins, heat shock proteins, and several other *H. pylori* products could also induce IL-8 secretion [9,10]. Chronic active inflammatory response is the hallmark of *H. pylori* infection and the main underlying molecule seems to be IL-8 [11]. Many putative virulence genes have been described to determine the clinical outcome; among these are the genes present in the plasticity region cluster (that is located outside the CagPAI) that correspond to nearly half of the strain-specific genes [12]. Plasticity region is recently being considered to be a novel transposable element and the genes present in this cluster possibly affect bacterial fitness and phenotypes [13]. Similar to CagPAI, plasticity region displays some characteristics of a genomic island such as its large size and a different percentage of G+C content than in the rest of the bacterial genome [14].

Most of the genes in the plasticity region of *H. pylori* are functionally unknown although they may epidemiologically associate with the strains from different disease conditions in certain human populations [15,16]. Previous studies have shown that there are regional and ethnic differences in the distribution of *H. pylori* subtypes with respect to strain variable genes; this in turn suggests that, in a given geographic area, the *H. pylori* genotypes may play a significant role in infection or progression of infection [17,18]. Moreover, plasticity region encoded proteins such as JHP0940 and HP0986 have already been reported to stimulate pro-inflammatory cytokines and activate NFκB mediated pathway in cultured mammalian cells [19–21]. In this regard, it is prudent to functionally characterize these genes/proteins with respect to their putative roles in persistence and virulence of *H. pylori*.

Our group previously reported that HP0986 was a pro-inflammatory protein that upregulates tumor necrosis factor alpha (TNF-α) and triggered IL-8 secretion and at the same time induced apoptosis through Fas-mediated pathway [21]. Although this pioneering study focused on the function of HP0986 outside the bacterial environment (as it was based on the effects of recombinant HP0986 on cultured human macrophages and peripheral blood mononuclear cells), the interaction of HP0986 with human gastric epithelial cells was not analyzed. Further, the study [21] did not explore intrinsic role of this protein (such as the predicted restriction endonuclease activity). Given this, there is a clear need to dissect the functional capacity of HP0986 in different cellular environments. We therefore, sought to extend this study to another cell type to ascertain the role of HP0986 in altering the cytokine responses by human epithelial cells (AGS cell line) and to understand the underlying mechanism. We also explored if HP0986 is presented to humoral immune system. This study also analyzed the prevalence as well as expression of HP0986 in clinical isolates and gastric biopsies obtained from an ethnically complex setting such as Malaysia. We also describe the localization of HP0986 in human gastric epithelial cells and discuss its potential to undergo a possible cytoplasmic-nuclear shuttling.

Materials and Methods

Ethics Statement

The present study was approved by the Ethics committee of the University of Malaya Hospital, Kuala Lumpur, Malaysia. Written informed consents were obtained from the patients as per the University protocol.

Patient Selection: (For *in vitro* Expression Study)

We screened more than 500 patients in the present study who underwent gastric endoscopy at the University of Malaya Hospital, Kaula Lumpur, Malaysia, during 2012–2013. In total, 110 adult patients were selected in this study, and these were the patients of functional dyspepsia ($n = 102$) (93%) and peptic ulcer disease ($n = 8$) (7%), determined on the basis of 2 inclusion criteria: those who had no history of *H. pylori* eradication therapy and those positive for rapid urease test**.** Functional dyspepsia was endoscopically and pathologically defined as *H. pylori* associated functional dyspepsia. Sixty out of 110 patients were from Indian ethnic group (mean age 48.5), 38 were of Chinese ancestry (mean age 59.7), and 12 were Malay (mean age 51.6). In all, 51% ($n = 56$) were males and 49% ($n = 54$) were females**.**

Patients and Gastric Biopsies (for *in vivo* Expression Study)

In total, 10 patients were selected in this study module; these patients underwent gastric endoscopy at the University of Malaya Hospital, Kaula Lumpur, Malaysia during 2013. All the 10 patients had functional

dyspepsia. Among these, 6 were from Chinese ethnic group (mean age 51.7) and 4 were of Indian ethnic group (mean age 59.7). Individual gastric biopsy specimens were placed in sterile vials after a positive diagnosis of *H. pylori* infection and were stored at −80°C. Out of these patients, four gastric biopsy specimens each were collected from antrum/body. One biopsy was immediately processed for bacterial culture, one for histologic examination, and two for total RNA extraction. Biopsy material was stored in formalin for histopathology and frozen in liquid nitrogen and stored at −80°C for total RNA extraction.

Helicobacter pylori Culture from Gastric Biopsies

Gastric biopsies (n = 110) were processed for *H. pylori* culture by homogenization of the tissue. Homogenates were inoculated on blood agar (Oxoid, Thermo Scientific) containing 7% horse blood and incubated at 37°C under 10% CO_2 for 5–7 days [22]. *H. pylori* growth was confirmed by microscopy and rapid urease test. Single translucent colonies that were positive for urease test were further subcultured for DNA and RNA isolation. PCR-based detection of HP0986 was performed on all *H. pylori* strains using gene-specific primers as described elsewhere [14].

Quantitative Real Time PCR assay

Helicobacter pylori cultures (n = 110) were pelleted and washed twice with 1X phosphate buffer saline and further pelleted by centrifugation at 4000 rpm for 5 minutes RNA was extracted from each pellet using Qiagen RNeasy Mini Kit as per the manufacturer's instructions and treated with DNase I (Qiagen, Hilden, Germany) on columns and further purified by RNA clean up. In a similar way, total RNA from each of the 10 frozen biopsies were also extracted. RNA samples were quantitated by Nanodrop spectrophotometer and stored at −80°C until further use. Expression analysis was carried out by IQ5 real-time PCR (BioRad Laboratories, Hercules, CA, USA). Briefly, the reaction was performed in 25 μL volume containing 12.5 μL of SYBR green (iScript^{T-M}One-Step RT-PCR kit, Qiagen), 1.25 μL of forward primer GAAAAGAGTTTA GAAAAGATACA, 1.25 μL of reverse primer CTTGAT GGTCTTTGTAAAACA, 0.25 μL of reverse transcriptase (Qiagen), 1 μL of RNA template, and 8.75 μL of Dnase/RNase free water. PCR conditions for both HP0986 and 16S RNA (control) were denaturation at 94°C for 15 minutes; 40 cycles of 94°C for 15 seconds; annealing at 45°C for 30 seconds, extension at 72°C for 30 seconds followed by 61 cycles of melting curve analysis at 65°C for 10 seconds. Reaction without RNA template was included as negative control for each primer tested and a control without reverse transcriptase was also included for each test. The analysis was performed in triplicates and IQ5 real-time PCR software (Biorad) was used to generate the quality control of the replicates, data extraction and initial analysis. Data were analyzed by ΔΔCT method as described earlier [23]. Relative expression level of HP0986 normalized to the 16SrRNA was checked in clinical isolates and in gastric biopsies when compared with the levels of HP0986 mRNA in strain P12. Also, HP0986 specific amplification was confirmed by a single amplicon on 1% agarose gel.

Cloning, Expression and Purification of HP0986 Using Prokaryotic and Eukaryotic Expression Vectors

The ORF/gene hp0986 was cloned in prokaryotic expression vector pRSETA and was purified to homogeneity as described earlier [21]. To clone hp0986 into eukaryotic expression vector, the gene was amplified from the genomic DNA of *H. pylori* strain 26,695 using the following primers: 5′CCCCTCGAGATGGTGGAACT TTTTTCTCTTTGCATGTC 3′ (xho I), 5′AATAAGCTTACG CCTAGAGTTATTAATATAATTCTCAATATTTT 3′ (Hind III). The amplified 714 bp product and TA cloning vector (Fermantas, Lafayette, CO, USA) were incubated together for an overnight ligation at 16°C. Insert was confirmed by double restriction digestion (Hind III, xho I) and was further subcloned into pEGFPN-1 (Clonetech) vector. The clone was again confirmed for the insert by double restriction digestion and sequencing.

Stimulation of AGS Cells with HP0986 and Estimation of IL-8

AGS cells were obtained from the National Center for Cell Sciences, (Pune, India). For all the experiments described below, the cells were grown in Ham's-F12 media (Sigma-Aldrich, St. Louis, USA) with 10% heat inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA) and 1X Antibiotic –Antimycotic (Invitrogen) and maintained at 37°C with 5% CO_2 in a humidified incubator.

AGS cells were seeded into a 6 -well plate at a density of 2×10^5 cells per well. They were maintained for 2 days until 70% confluent. To rule out the possibility of LPS contamination, HP0986 was incubated with Polymixin B-Agarose (Sigma-Aldrich, St. Louis, MO, USA) for 4 hours at 4°C. The cells were then treated with the following concentrations of HP0986 protein: 5 μg/mL, 10 μg/mL at different time intervals; LPS-treated AGS cells (5 μg/mL) were included as positive control. The

culture supernatants were then collected at 6, 12, 24, and 36 hours post-treatment so as to measure the levels of various cytokines such as IL6, IL-8, and TNF-α by BD CBA flex kit; acquisition of the data was carried out in the BD FACS Canto II flow cytometer (BD Biosciences, USA) using BD FACS diva software (BD Biosciences) and the analysis was performed by plotting the standard graphs for each cytokine using FCAP (BD Biosciences) array software.

Western Blot Analysis

The AGS cells were seeded and treated with HP0986 as described above. The cells were washed twice with 1X PBS for the preparation of nuclear and cytoplasmic extracts three hours after treatment with HP0986 and an equal aliquot of all the samples was loaded on 12% SDS-PAGE from respective extracts that were quantified by the MicroBCA protein assay kit (Thermo Scientific, Lafayette, CO, USA). The separated proteins were then transferred onto PVDF membrane, blocked in 5% BSA, and probed with rabbit anti phospho–p65 antibody (Santa Cruz, Dallas, TX, USA) overnight at 4°C followed by 1 hour incubation with peroxidase-conjugated goat anti-rabbit IgG (1 : 80000) (Sigma-Aldrich) to detect NF-κB. Further to detect IκBα, the blots were probed using rabbit polyclonal IκBα (Sigma-Aldrich) overnight at 4°C at a dilution of 1 : 1000 and probed with secondary antibody, anti-rabbit IgG (1 : 80,000, Sigma-Aldrich). Finally, membranes were washed thrice with 1X TBST and then developed using ECL plus chemiluminescence kit (Thermo Scientific). The membrane was blocked with 5% BSA in 1X PBST for 2 hours and then incubated in 1X TBST. It was then washed thrice with 1X TBST at room temperature and subjected to ECL plus chemiluminescence detection (Thermo Scientific).

Detection of Antibody Responses by ELISA

Antibody response against HP0986 was examined in a total of 40 human serum samples comprising of 20 *H. pylori* positive and 20 *H. pylori* negative sera which were obtained from different patients by indirect ELISA as described previously [21].

Transient Transfection of AGS Cells

AGS cells in Ham's F12 medium supplemented with fetal bovine serum were grown on 13 mm cover slips in 24-well plates until they reached 50–60% confluency. Spent medium was aspirated next day and cells were supplemented with Ham's F12 medium without fetal bovine serum and transfected with pEGFPN-1HP986 construct using Lipofectamine 2000 (Invitrogen) transfection reagent as per manufacturer's instructions. Briefly, AGS cells were treated with a mixture of lipofectamine and plasmid DNA in a ratio of 1 : 3 for 4 hours. Thereafter, the serum-free medium was aspirated and cells were grown in Ham's F12 medium with 10% fetal bovine serum for 24 hours. The coverslips were then washed thrice with 1X phosphate buffer saline and fixed in 4% paraformaldehyde and probed with anti-rabbit HP0986 antibody. This was followed by 1 hour incubation with peroxidase-conjugated goat anti-rabbit IgG. Slides were washed and mounted with Vectashield mounting medium containing DAPI (Invitrogen). Expression vector pEGFPN-1 without any insert was used as negative control.

Statistical Analysis

Two tailed student *t*-test was used to demonstrate the level of secretion of IL-8 in treated cells when compared with untreated cells. Further, Mann–Whitney's *U* test was carried out to compare antibody responses. All the data were expressed as mean ± SEM. *p* values of less than .05 were read as statistically significant.

Results

Genotyping and *in vitro* Expression of HP0986 in *H. pylori* Clinical Isolates

To investigate the *in vitro* expression of HP0986 in *H. pylori* clinical isolates and to confirm it as a virulence marker linked to disease outcome, we checked the epidemiologic consistency of HP0986 across the Malaysian patient population selected by us (see in methods). Firstly, we performed PCR-based detection of HP0986 using the genomic DNA isolated from all ($n = 110$) *H. pylori* isolates and with the help of gene specific primers as described earlier [14]. Among the 110 clinical isolates, HP0986 gene was found in 31% ($n = 34$) of the samples. To investigate if PCR-based detection of HP0986 also entails expression of HP0986, a quantitative real time PCR was performed on the above 34 isolates so as to analyze the *in vitro* expression of the gene. A single primer set, complementary to a highly conserved region, which specifically amplifies HP0986 was used to perform the *in vitro* expression analysis. This precluded the possibility of any primer mismatches. The mRNA expression of HP0986 was recorded as cycle threshold relative to the strain P12 of *H. pylori*. (Fig. 1). Our results revealed that the presence of HP0986 genotypes corroborated with the *in vitro* expression of

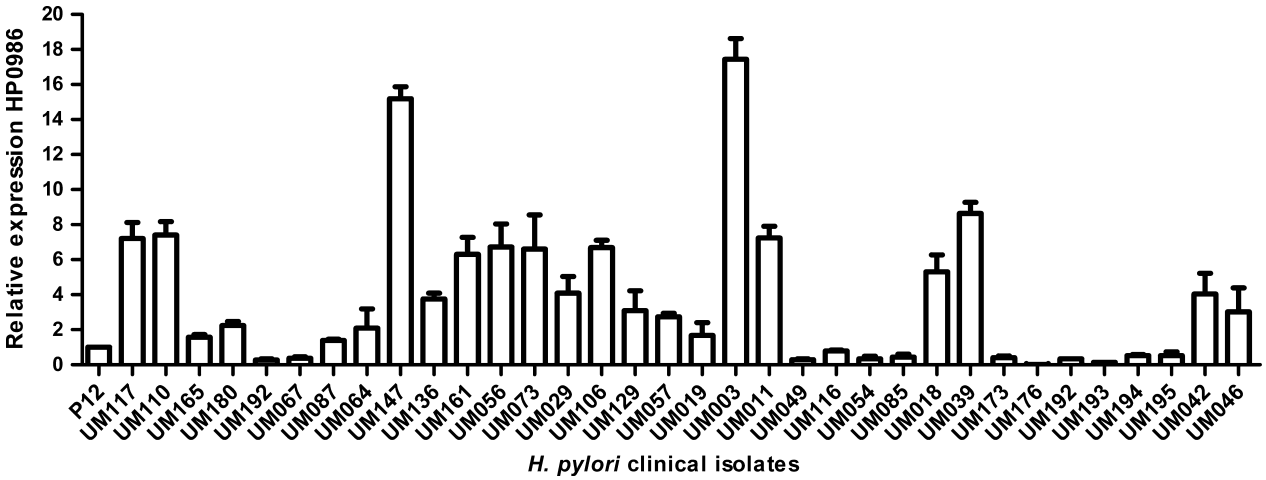

Figure 1 Relative quantitative real time PCR analysis of HP0986 gene expression in *Helicobacter pylori* clinical isolates cultured from gastric biopsies. Values represent Mean ± SEM of three replicates.

HP0986. The prevalence of HP0986 in the clinical samples varied among three ethnic groups and it was highest among the Indian origin patients (88%) followed by Chinese (10%) and Malay subjects (2%). This demonstrated that there was a differential prevalence of HP0986 in *H. pylori* clinical isolates corresponding to different ethnic groups in Malaysia.

Expression of HP0986 in Biopsy Samples

We analyzed the expression of HP0986 mRNA in gastric biopsy specimens and the analysis of relative HP0986 transcript levels was performed by quantitative RT PCR. On a pilot scale, relative mRNA was measured only in 10 gastric biopsy specimens. These 10 biopsy specimens were different from the 110 clinical isolates used for *in vitro* expression analysis. Our results demonstrated that 7 out of the 10 (70%) gastric biopsies had detectable HP0986 mRNA. To further confirm that the positive *in vivo* expression of HP0986 was reflective of presence of the gene, a PCR-based confirmation of HP0986 from the same sample was obtained. We found all the seven biopsy specimens positive for HP0986 mRNA expression and these were also positive by PCR. This indicates the specificity of our qRT-PCR in detection of *in vivo* expression. We also checked the profile of constitutively expressed 16S rRNA as an internal control. The presence of HP0986 in the biopsies correlated with the expression of HP0986 *in vivo,* wherein, all the 7 biopsies showed significant expression as determined by their cycle threshold (Fig. 2). Similar to the *in vitro* expression, among the 7 biopsies positive for HP0986, 4 were from ethnic Indians and 3 from Chinese. This observation corroborated with our *in vitro* expression results that

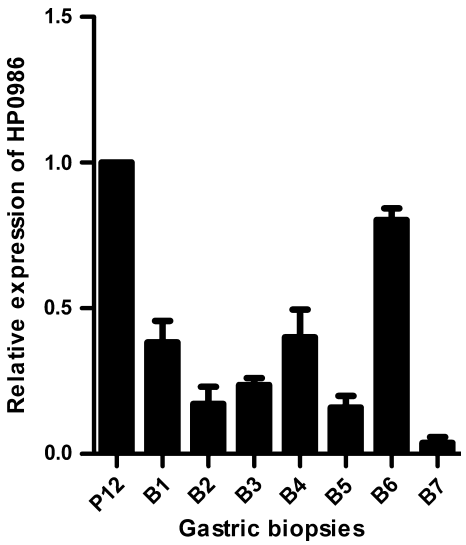

Figure 2 HP0986 expression profiles in gastric biopsies: Bar diagram represents relative expression of HP0986 mRNA transcripts in gastric biopsies. Values represent Mean ± SEM of three replicates.

HP0986 was perhaps more prevalent among Indian ethnic group followed by Chinese. In sum, expression of HP0986 under *in vivo* conditions conveys its possible role during infection and that the protein was naturally produced and presented to the immune system (see later).

HP0986 Elicits Antibody Response

Twenty *H. pylori* positive and an equal number of *H. pylori* negative patients' sera were used for an indirect ELISA to evaluate antibody response of *H. pylori* infected patients to HP0986 when compared with

healthy controls (Fig. 3). All the 20 *H. pylori* positive patients' sera showed seropositivity of HP0986. The high titers of serum IgG observed in *H. pylori* positive patients when compared with *H. pylori* negative patients ($p = .0025$) confirmed that HP0986 was expressed *in vivo* and recognized during natural infection.

HP0986 Induces IL-8 Secretion by AGS Cells

To examine the ability of HP0986 to stimulate IL-8 secretion from AGS cells, a bead-based immunoassay was performed wherein we monitored the cytokine secretion profiles in a dose and time course manner in culture supernatants of AGS cells. We observed that the IL-8 induction by HP0986 had increased in a dose- and time-dependent manner (Fig. 4), which was strongly enhanced at 36 hours post treatment (1200 pg/mL), whereas, no detectable levels of other proinflammatory cytokines such as IL6 and TNF α were observed.

To ensure that the cytokine response by the cells is specific to HP0986, we simultaneously tested proteinase K digested HP0986 preparations; as expected, they did not show any significant IL-8 secretion. Further, our previous report ensured that inclusion of another histidine tagged (6X His) protein purified in the same manner (namely, HP0023 encoding *H. pylori* isocitrate dehydrogenase) [24] did not induce any pro-inflammatory response. Secretion of IL-8 by AGS cells following stimulation with HP0986 and the previous data related to IL-8 secretion by macrophages and PBMCs [21] hints

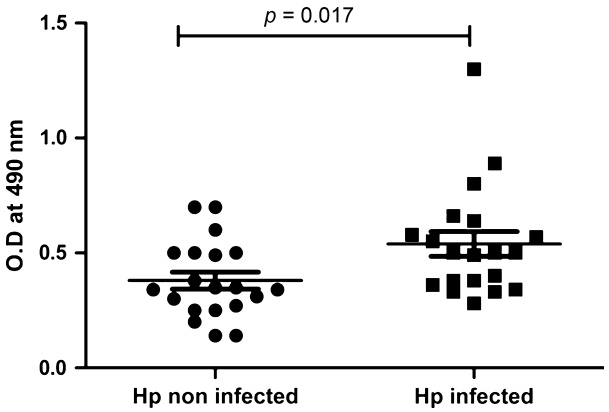

Figure 3 Detection of anti HP986 imunoglobulin G (IgG) in *Helicobacter pylori* positive patients: IgG levels in sera of 20 *H. pylori* infected patients versus 20 controls were quantitated using ELISA. We carried out a Mann–Whitney's *U* test to evaluate differences in antibody responses. Statistically significant difference was found among the levels of IgG antibody reactivity with respect to the two groups (p value: .017).

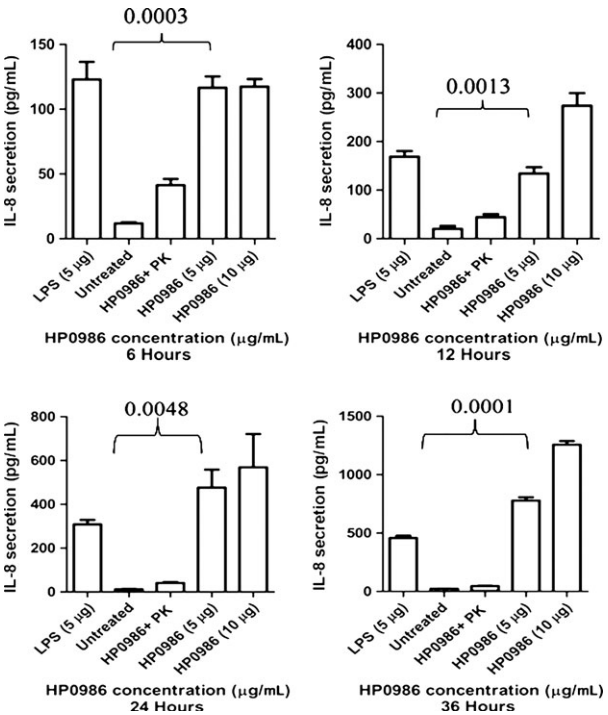

Figure 4 Time- and dose-dependent increase in IL-8 secretion by AGS cells upon treatment with HP0986: Supernatant was collected at 6, 12, 24, and 36 hours and IL-8 secretions were measured by flow cytometry. Maximum secretion was observed at 36 hours (p values are mentioned on the top of brackets in each bar chart and they indicate significant increase in induction of IL-8 when compared with untreated cells at four different time points). Each bar in the graphs shows the mean ± SEM of three replicates. PK: proteinase K.

that HP0986 is more likely to be associated with gastroduodenal inflammation.

HP0986 Triggers IL-8 Secretion by AGS Cells Through Activating NF-κB *via* Degradation of IκBα

To unravel the specific signaling pathway involved in HP0986 mediated IL-8 secretion, we treated the AGS cells with HP0986 and Western blotting was performed using antibodies specific to IκBα and phospho-p65. We were interested to determine the role of NF-κB pathway in HP0986 induced IL-8 production in gastric epithelial cells. In addition to this, we also determined the levels of IκBα in AGS cells upon treatment with HP0986. Treatment with HP0986 resulted in a decrease in the cytoplasmic levels of IκBα. The IκBα started degrading at 60 minutes post-treatment and proceeded up to 90 minutes after the treatment. We also observed the increase in the levels of NF-κB in the nucleus at 90 minutes post-treatment, followed by translocation of p65 into the nucleus of AGS cells (similar to what was observed upon LPS treatment) (Fig. 5).

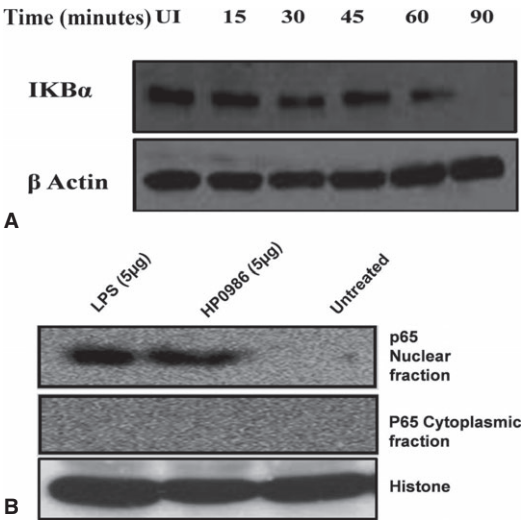

Figure 5 Western blot analysis of the effect of HP0986 (5 μg) on NF-κB activation and IκB degradation in AGS cells: (*A*) Western blot analysis for degrading levels of IκB from 0 to 90 minutes. UI indicates uninduced cell fraction used as negative control. (*B*) immunoblot representing activation of NF-κB in AGS cells upon treatment with HP0986 for 3 hours. β-actin and histone were used as equal loading controls respectively in (*A* and *B*). LPS was used as a positive control.

Cellular Localization of HP0986

Gastric epithelial surface is the main site of host pathogen interaction in *H. pylori* infection [25–28]. Several of the *H. pylori* virulence factors can enter epithelial cells either by direct injection *via* T4SS [29] or by endocytosis etc. Recent reports suggest that *H. pylori* virulence factors accumulate in the cytoplasm of several immune cells *in vitro* [30,31]. But, the cellular localization of *H. pylori* virulence factors in the gastric epithelium is sparsely studied. Therefore, to know the exact cellular localization of HP0986, we transiently transfected the AGS cells with pEGFPN1-HP0986 and the cellular location of HP0986 was visualized (Fig. 6). The fusion protein (pEGFPN-1-HP0986) was detected in the cytoplasm as well as in the nucleus. These results demonstrated that HP0986 localized both in cytoplasm and nucleus. Transfection of expression vector pEGFPN-1 alone did not produce similar localization pattern with respect to AGS cells.

Discussion

The role of strain-specific genes of the plasticity region in *H. pylori* has been of recent interest particularly concerning gastric mucosal inflammation and adaptation [32–34]. The plasticity region of *H. pylori* harbors different combination of genes and consequently, the gene content of different strains and isolates is significantly variable; this may be important in the context of different disease outcomes [35,36]. Several studies have reported the role of plasticity region genes in *H. pylori* induced gastroduodenal diseases. Some of these genes are proposed to be good candidate markers for clinical outcome, such as jhp0947and dup A etc. [37–39]. Moreover, several genes of the plasticity region have still not been characterized.

HP0986 is an important candidate antigen and a proinflammatory protein encoded by the plasticity region ORF hp0986 of *H. pylori* strain 26695. The protein has been characterized *in vitro* and was shown to be inducing proinflammatory cytokines through TNFR1- and NF-κB- mediated signaling [21]. However, the secretion, localization, and regulation of this seemingly important protein have not been worked out in an *in vivo* system. This study therefore, forms a logical extension of the work of Alvi *et al*. [21] with an emphasis on the interaction of this protein with AGS cells and its localization therein including its genotypic, serologic and expression profiling based on clinical isolates, patient sera, and biopsies. Our results broadly confirm the results of Alvi *et al*. obtained with cultured macrophages and PBMCs, by showing an upregulation of IL-8 secretion by cultured AGS cells upon stimulation with recombinant HP0986 protein. Our results also support the conclusion of Alvi *et al*. that the HP0986 protein is presented to the human immune system as demonstrated by serologic profiling of patient samples in both the studies using sera from patients with varied geographic descent.

Our results revealed presence of hp0986 locus in a significant number (31%) of clinical isolates (Fig. 1) and its prevalence was highest in strains obtained from the Indian ethnic group (88%) in Malaysia. Further, our study appears to be in agreement with the previous observation on the prevalence of *dupA*, in different ethnic groups in Malaysian and Singaporean populations [40]. Similarly, the prevalence rates of other plasticity region genes were also reported by others to be in a similar range, for example, jhp0940, jhp0945, jhp0947, and jhp0949 had a prevalence rate of 23.9, 39.1, 37.7, and 45.7%, respectively, in Colombia, United States, South Korea, and Japan [41]. As all these studies have been carried out on the basis of PCR-based genotyping, it will be necessary to stress that the absence of a PCR amplicon in *H. pylori* could be due to different allelic structures or sequences in different clinical isolates given that it is a highly recombining and geographically compartmentalized pathogen. However, our approach included consensus sequence-based primers nested within the hp0986 gene

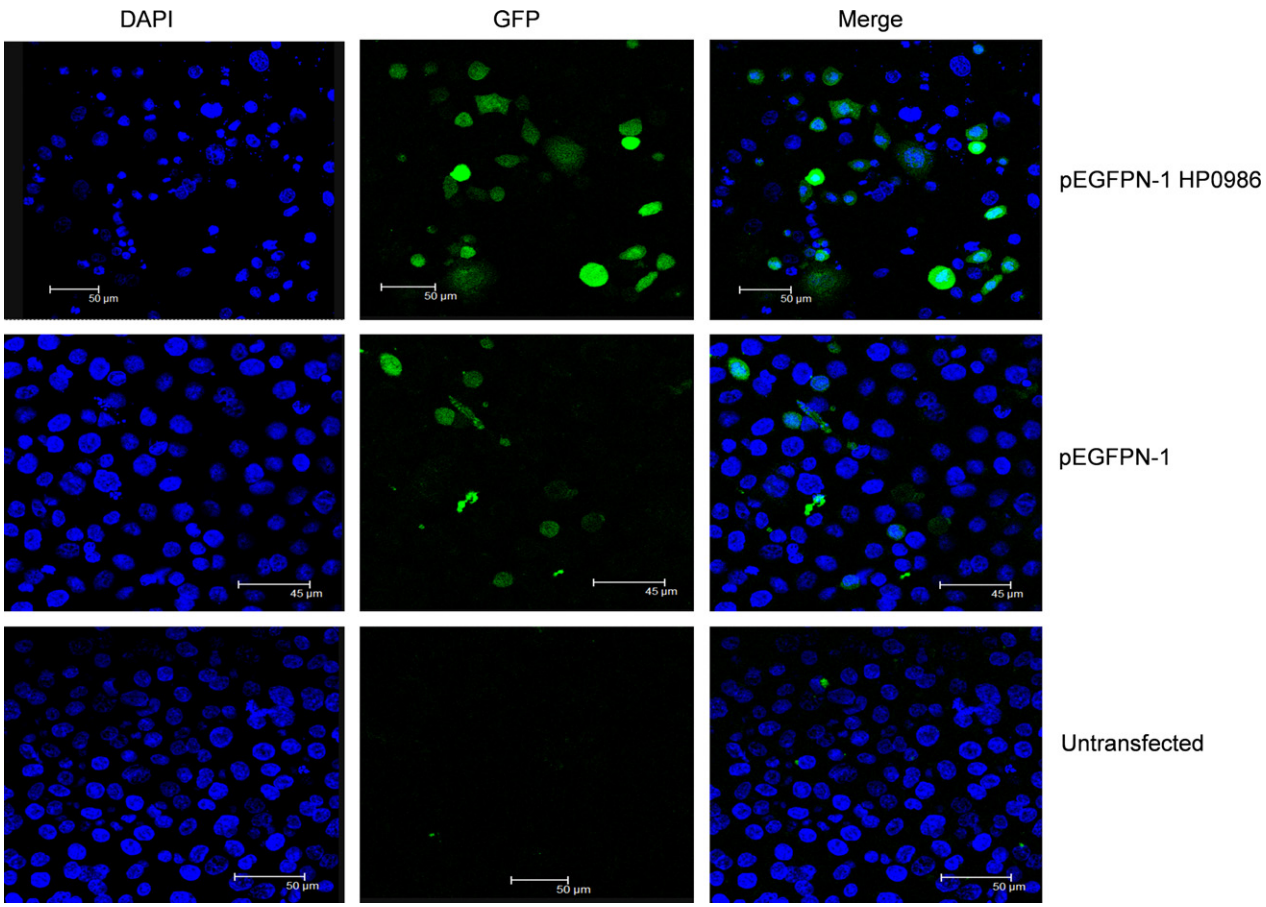

Figure 6 Expression and cellular localization of HP0986 in AGS cells: Subcellular localization of GFP+HP0986 was examined by transient transfection. Green fluorescent signal represented homing of HP0986 inside and on the surface of the AGS cells. Upper panel (pEGFPN1-HP0986) represents expression of hybrid protein GFP+HP0986 by the AGS cells. The middle panel indicates transfection of the AGS cells by the GFP vector alone whereas the lowest panel represents untreated AGS cells. DAPI indicates the position of the nuclei 24 hours after transfection. The cellular localization of HP0986 was visualized by confocal microscopy.

which appears to be highly conserved when tested in a single strain colonizing a single patient for about 10 years [42]. Although it is possible that the gene length in some strains could be larger than the gene annotated in strain 26695, but it may not affect the outcome of a PCR-based survey.

We observed variation in transcript levels of HP0986 in some *H. pylori* clinical isolates which may be due to multiple factors such as difference in environmental conditions of the stomach (pH, stress, level of mucin secretion etc.) or it may be an intrinsic property of an individual clinical isolate [25]. However, *in vivo* expression of HP0986 was limited when compared with *in vitro* expression (Fig. 2); decreased *in vivo* expression may likely be due to several reasons such as less bacterial load as the bacterium maintains low profile during infection, or it may also be due to loss of RNA during sample processing etc. [43,44]. It was also shown that *in vivo* expression of CagA in gastric biopsies was lower than its *in vitro* expression [45] which was further consistent with a DNA microarray study wherein the *in vivo* CagA expression was downregulated by low pH levels [46].

The antibody response in the sera of *H. pylori* infected patients to HP0986 was evaluated by comparing them with the healthy controls. Our results (Fig. 3) clearly demonstrate the possibility that HP0986 is expressed and presented to the immune system as *H. pylori* possibly harnesses different means of releasing its antigens into the extracellular space (T4SS, autolysis, and formation of membrane vesicles etc.). HP0986 could therefore be secreted by one of these mechanisms [47]. We have earlier shown seropositivity of HP0986 [21] in a geographically distinct and mixed patient sera collection [24]. In this study, an ethnically diverse but geographically related patient population was used to

demonstrate that HP0986 induced antibody response was not population specific.

Our observation that HP0986 induces IL-8 in a cagA-independent manner supports the notion that CagA alone may not be the sole pro-inflammatory trigger during *H. pylori* infection and that many other players could be involved in the proinflammatory activity independent of CagA. These observations are in agreement with previous reports by Selbach *et al*. and Gorrell *et al.* where they concluded that IL-8 secretion in gastric epithelial cells was independent of CagA [48,49]. This then opens up the possibility that the strains lacking cagA gene could also produce clinical symptoms linked to inflammation. It is now certain that several other genes also encode proinflammatory proteins of the sorts of flagellar antigens, outer membrane proteins and Hsp60 etc. [50]. Other investigators also reported similar findings while working on strain-specific proteins that are found outside the cagPAI; particularly, the plasticity region proteins/genes such as *dupA* and JHP0940 were shown to be able to induce IL-8 secretion [19,51]. Another plasticity region locus, jhp947-jhp949 was found to be associated with duodenal ulcer disease and IL-12 production by monocyte cells [37].

Induction of pro-inflammatory cytokine responses involving NF-κB activation is mostly described to be associated with the type IV secretion system (T4SS) in *H. pylori* [52]. Apparently, it may be possible that HP0986 is also secreted through T4SS although there is no direct evidence at this stage to show the same. We have also shown the localization of HP0986 in gastric epithelial cells using a mammalian expression vector. Our results revealed that HP0986 localizes in cytoplasm as well as in the nucleus. However, further studies are required to understand detailed mechanisms involved in HP0986 entry and regulation of host cell machinery. Nonetheless, our results appear consistent with previous observations in which CagA was also shown to localize in the inner leaflet of host cells [53]. However, as HP0986 does not have a secretion signal and that it did not offer any structural or sequence homology to some of the known effector proteins or toxins that are secreted through T4SS, such as CagA or members of any other T4SS in *H. pylori*, it will be interesting to find out its secretory mechanism and regulation.

A somewhat intriguing property of HP0986 gene/protein is its size variability. The hp0986 locus consists of an ORF encoding from 231 amino acids to 558 amino acids in various sequenced genomes of *H. pylori*. There are over 200 different sequences, available in the public domain, that show that the average size of the ORF for this protein is longer than that of the reference strain 26695. However, multiple sequence alignment revealed that an ORF of 237 amino acids corresponding to HP0986 of the strain 26695 was highly conserved in most of the sequenced genomes. Nevertheless, it will be worthwhile to study the functions of larger variants in different strains as they may be relevant to our understanding of some of the critical aspects of HP0986 (such as its mode of secretion and regulation), although they may not encode functions dramatically different than the hitherto described roles of this protein. In case of latter possibility, it will indeed be appropriate to reconsider the choice of strain 26695 as a reference strain to study HP0986 and other putative/novel gene functions.

Detailed analysis of HP0986 sequence using Pfam webserver [54] revealed that HP0986 also possesses a domain similar to type II restriction endonucleases, but whether it corresponds to functional restriction endonuclease or methylase activity needs to be proved. Given this, HP0986 could perhaps be a 'moon lighting' antigen similar to isocitrate dehydrogenase of *H. pylori* and *Mycobacterium tuberculosis* and aconitase of *M. tuberculosis*; these proteins participate in core metabolic activities such as energy cycles and also have immunological and regulatory roles respectively [24,55–58]. Perhaps, HP0986 could be very similar to another important virulence factor, IceA, which is proinflammatory and has also been shown to be a restriction endonuclease [59]. Alvi *et al*. [21] could not shed light on possible new functions of HP0986 and remained focused on TNFR1-mediated proinflammatory and proapoptotic roles of HP0986. Given their demonstration of a TNFR1-mediated signaling and our present findings, it is tempting to name this important gene/protein as 'TNFR1 interacting endonuclease A (TieA or *tie*A)'. Further, it will be possible to direct future efforts at understanding the functional promiscuity of this protein and the regulation of proinflammatory as well as methylase activities.

In conclusion, our study demonstrated that HP0986 induced IL-8 secretion in gastric epithelial cells *via* NF-κB activation and localized both in the cytoplasm as well as in nucleus of the cells. mRNA expression profiling of bacterial cultures and gastric biopsy specimens clearly conveyed that HP0986 was expressed naturally. The antibody profiles of patient sera further confirm this and point to the role of HP0986 in *H. pylori* infection-induced pathogenesis. Future studies involving mechanistic confirmation of the cellular and extracellular roles of the protein are pertinent. Our next experiments will be directed at identification of upstream and downstream signaling events with respect to NF-κB activation in epithelial cells as well as establishing the cause and effect relationships by means of isogenic knockout strains.

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Competing interests: The authors have no conflicts of interest to declare.

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Review

Helicobacter pylori - a seasoned pathogen by any other name

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Abstract

Helicobacter pylori is a well known inhabitant of human stomach which is linked to peptic ulcer disease and gastric adenocarcinoma. It was recently shown in several studies that *H. pylori* can be harnessed as a surrogate marker of human migration and that its population structure and stratification patterns exactly juxtapose to those of *Homo sapiens*. This is enough a testimony to convey that *H. pylori* may have coevolved with their host. Several protective effects of *H. pylori* colonization have been considered as evidence of a presumed symbiotic relationship. Contrary to this assumption is the presence of a strong virulence apparatus within *H. pylori*; why a co-evolved parasite would try inflicting its host with serious infection and even causing cancer? The answer is perhaps embedded in the evolutionary history of both the bacterium and the host. We discuss a hypothetical scenario wherein *H. pylori* may have acquired virulence genes from donors within its environment that varied with change in human history and ecology. The *H. pylori* genomes sequenced to date portray fairly high abundance of such laterally acquired genes which have no assigned functions but could be linked to inflammatory responses or other pathogenic attributes. Therefore, the powerful virulence properties and survival strategies of Helicobacter make it a seasoned pathogen; thus the efforts to portray it as a commensal or a (harmless) 'bacterial parasite' need rethinking.

Introduction

The human gastric pathogen *Helicobacter pylori* is the most successful colonizer in the stomachs of almost half of the world's population. As a result of this colonization, a majority of infected individuals show histological signs of chronic gastritis; only a small fraction of infected individuals develop *H. pylori*-associated diseases, such as peptic ulcers and, more rarely, gastric adenocarcinomas [1]. Consequently, each year, about half a million patients die from gastric cancer worldwide, making it one of the three major causes of cancer related deaths, leading to considerable socioeconomic costs. As for many other cancers, chemotherapy of gastric adenocarcinoma hardly leads to any improved outcome; however, it is possible that the development of *H. pylori*-associated gastric cancer can be prevented by eradication or abrogation of the infection. The pathology of *Helicobacter* induced chronic gastric inflammation entails a highly coordinated interplay [2] of several virulence factors (encoded mainly by the accessory component of the bacterial genome), although CagA being the most important single virulence determinant that has been investigated extensively for mechanistic and functional evidence to its being cytotoxic and carcinogenic [3-7]. Host genetics and the environment play important roles in imparting susceptibility (or otherwise) towards more serious outcomes of the colonization.

On the other front, *H. pylori* has been projected to have co-evolved with its human host [8], and several protective roles played by the colonizer have been speculated [9,10]; accordingly, low incidences of gastroesophageal reflux disease and childhood diarrhea, and lately asthma, have been suggested to be linked to the presence of *H. pylori* [1,9-11]. Despite the deemed protective roles/associations of the *H. pylori* colonization, the pathogen needs to be carefully monitored especially in developing countries, where widespread drug resistance makes it difficult to be eradicated creating thus a persistent colonizer. In the future, global climate change is likely to impact transmission dynamics of *H. pylori* as there has been an active link of the same with the climate change due possibly to its being transmitted through contaminated water amidst poor community hygiene (at least in the developing countries) and it is shown to be a predisposing factor for the incidence of water-borne diseases such as cholera [12]. Given this, novel intervention strategies are needed at the level of prevention of transmission and therapy. Replicate genome sequences of the pathogen appear promising [13] to understand acquisition and maintenance of virulence in an evolutionary sense; consequently, novel information could emerge to contribute in terms of understanding virulence mechanisms leading to chronic adaptation and survival.

Co-evolution and acquisition of virulence

Polymorphisms in the genomes of pathogens potentially provide support for the reconstruction of ancestral human population migrations and settlements. This is particularly true for microorganisms that persist lifelong and cause overt chronic diseases. Population genetic structure of such pathogens (that are supposedly co-evolved with humans) juxtaposes to genetic distribution patterns of their host. Human DNA analysis in the recent past has revealed that the farther from Eastern Africa a population is, the more diverse genetically it is (as compared to other human populations) [14]. Comprehensive genetic analysis of *H. pylori* found almost exactly the same dispersal scenario for this pathogen [8]; genetic affinities estimated based on multilocus sequence typing (MLST) of many different sets of *H. pylori* isolates revealed a co-evolutionary pattern [15-17], meaning that population genetic structure of its human host could be similar to the population stratification patterns of the pathogen. Apart from this, genetic analyses incorporating human and bacterial data sets lend support to the idea that *H. pylori* may have migrated from Eastern Africa at almost exactly the same time as early humans, approximately 60,000 years ago [8]. This ultimately conveys that humans and this bacterium have been intimately linked at least for the last 60,000 years. However, the question that has not been answered clearly until now is whether this 60, 000 year old *H. pylori* was as virulent as today's *H. pylori*? In other words, it is not clear if *H. pylori* harbored its virulence genes since the beginning or it acquired them later (from early microorganisms that surrounded humans as a result of gradual change in history and ecology of the early societies through their transition from hunter gatherer's lifestyle to the agrarian type). Also, it is not clearly known which of the deleterious genes were lost from the genome to achieve a peaceful coexistence with the host.

The acquisition of the *cag* pathogenicity island (PAI) has therefore been the subject of debate on its origin and circumstances under which the PAI was imported from a foreign source [16]. Taking into account the comprehensive genetic analyses that have been performed, it is possible to predict a possible evolutionary scenario (Fig. 1) that supports the proposition that the *cag*PAI was acquired by ancestral *H. pylori* populations that arose on different continents before agriculture began in the civilized world. The acquisition of the PAIs might have occurred in *H. pylori* populations quite recently, possibly due to close contact of humans with domesticated animals, crops or rodent pests surrounding them. Such an inter species gene transfer could be explained partly based on the fact that many constituent genes of the *cag*PAI reveal well-established homologies to the type IV systems of *Agrobacterium tumifaciens* [18] and that *cag*A-like sequences have been reported from some *Aeromonas* isolates [19], obtained from environmental samples. Subsequent environmental changes and evolution of the food habits might have led to further continent specific adaptation of *H. pylori*. To date, the genetic structure of *H. pylori* is highly geographically oriented, both with respect to the core and the flexible genome components.

Also, the gain of pathogenicity islands might have augmented the fitness of the organism to infect and spread, thus giving rise to modern populations capable of out competing any residual, native strains. This has in fact been suggested in case of South American (Amerindian) strains of Helicobacter which are gradually disappearing as a result of colonization by more 'aggressive' European strains [16]. Many of the genetic elements supposedly of foreign origin in *H. pylori* have been described to be virulence-linked in a strain specific manner. That means, for some strains, enhanced pathogenic and proinflammatory potentials are imparted by novel elements which may not be universally conserved [20,21].

Survival tactics, chronicity and disease association - role of strain specific genes

H. pylori induced chronic gastritis is a definitive risk factor for the development of gastric cancer. However, it was found that the statuses of some of the chief virulence factors (CagA and VacA) do not always correlate with particular outcomes of infection, as also discussed previosuly

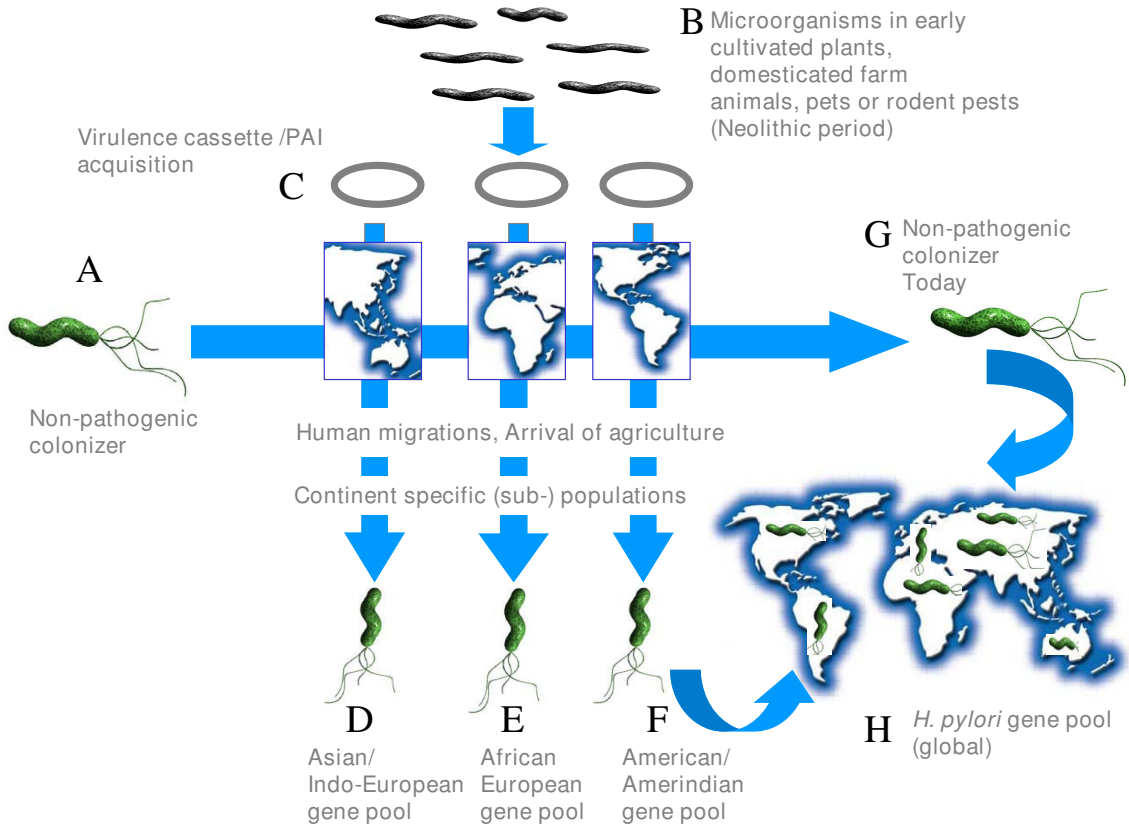

Figure 1

Genome evolution, global diversification and spread of *H. pylori* (sub-) populations. Horizontal gene transfer and genome plasticity likely contributed to the evolution of pathogenic variants from non-pathogenic colonizers. Modern *H. pylori* populations thus derived their gene pools from ancestral populations that arose on different continents and can be correlated with different migrations of human populations and other Neolithic events such as arrival of agriculture. The beginning of agriculture and the domestication of farm animals (which seem to have occurred hand in hand but across multiple domestication events in a continent specific manner) suggest a scenario, as depicted here, which can be linked to the acquisition of virulence by *H. pylori*. It can be hypothesized that early bacterial communities originating from crop plants, animals or rodent pests etc. rampant in the vicinity of early human societies may have served as donors of some of the virulence gene cassettes. Such genetic elements may have been acquired by *H. pylori* either bit by bit or en-bloc, at some point of time, through horizontal gene transfer events. There are indirect evidences to this effect in the form of sequence and structural similarities of some of the *H. pylori*'s virulence genes to their homologues in plant pathogens and environmental bacteria. Also, we believe that the extraneous virulence genes may have conferred some survival advantage upon *H. pylori* making them fitter in different human and animal hosts and, as a result, the pathogen may have spread selectively in a geographically compartmentalized manner.

[22]. In view of this, it appears that virulence of *H. pylori* is a complex phenotype that need to be seen as a function of bacterial strategies aimed at survival and adaptation. However, it is not clear how the bacterium maintains its niches for almost an entire life span of its host without being cleared. Perhaps, there operate highly orchestrated, biological interactions between the host and the pathogen; the nature of such interactions is not clearly understood. Of late, roles of new virulence determinants are becoming plausible. *H. pylori* harbors up to 45% strain specific genes [23], mostly gained through horizontal gene transfer events [24]. Recently, some of the members of the plasticity region cluster were proposed to be likely involved in promoting proinflammatory capacity of some of the strains [22,25] thus imparting a survival advantage. Our experiments with one such protein, from the plasticity region cluster suggest that some of the members of this cluster encode proinflammatory and/or proapoptotic roles (Alvi *et al.*, unpublished data). Most persistent microbes seemingly evolve strategies to avenge innate responses to gain niche and to maintain growth fitness. For example, *H. pylori* traditionally harness its chief virulence factors, CagA and VacA to cause pathology *via* a two pronged approach: 1) downregulate T-cell responses

(through the VacA mediated cell cycle arrest) and 2) upregulate mucosal proinflammatory pathways (by CagA). Surprisingly, in our studies, one of the plasticity region cluster protein appeared to be able to perform both the immune stimulatory (macrophage proliferation, secretion of IL8 and TNF-alpha) and immune evasion (apoptosis of activated macrophages) tasks single handedly (unpublished observations). Thus we believe that some of the bacterial proinflammatory proteins [such as JHP0940 [22] and others] are capable of taking up the functions of Vac A/Cag A, especially in the case of the deficiency of the latter and probably function as 'persistence factors' (Alvi *et al*., unpublished data); this however awaits validation using appropriate animal models.

In a recent study [25], 42 isolates of *H. pylori* were profiled to find that 1,319 genes were present in all isolates, while 341 (20.5%) genes were variably present among different isolates. Of the variable genes, 127 (37%) were interspersed within the plasticity region cluster. They observed disease association of such genes and found thirty genes to be significantly associated with nonatrophic gastritis, duodenal ulcer, or gastric cancer, 14 (46.6%) of such putative disease-linked genes were operational from within the plasticity region and the cag PAI (many of the constituent genes of the cag PAI form part of the plasticity region cluster of *H. pylori*). In the observation of Romo-Gonzalez [25], two genes (HP0674 and JHP0940) were absent in all gastric cancer isolates. In our own studies (Tenguria and Ahmed, unpublished), strains representing intestinal metaplasia cases failed to amplify JHP0940 gene (data not shown). It is therefore possible that some of the genes are deleted by the pathogen as the disease progresses through intestinal metaplasia to gastric cancer. However, such observations need functional validation and mechanistic explanation. Nevertheless, the disease-linked genes as discussed above, may be pursued as (putative) biomarkers of the risk for progression of *H. pylori* induced inflammation towards more serious gastroduodenal illnesses such as atrophic gastritis, intestinal metaplasia and gastric adenocarcinoma.

Association with other enteric infections

In the last two decades, several researchers have predicted mass migrations as a consequence of climate change. They have foreseen millions of people fleeing from rising sea levels, floods, disease outbreaks and drought, leading to serious consequences for both migrants and receiving societies. Many enteric infection outbreaks occur during shortage of drinking water wherein populations are forced to drink from un-conventional or unreliable sources which might be contaminated by sewage and human excreta. Enteric pathogens such as *H. pylori* also co-migrate with their human host. Population dynamics and disease potentials of these pathogens are likely to change with the change in history, geography and ecology of their hosts. *H. pylori* is thus one of the prominent candidates whose epidemiology and evolution within different stationary and migratory communities will be of interest as the impact of climate change as well as change of lifestyle [26] on enteric infection has emerged as a concern in recent years. Chronic *H. pylori* infection has already been described to be a predisposing factor for enteric infections such as cholera [12], which occurs mostly as a result of groundwater contamination - a potential sequel of local or global climate change.

Replicate *H. pylori* genomes - how many do we need?

Like many other pathogenic bacteria, *H. pylori* is being sequenced to generate replicate, whole genome sequences. Such replicate genomes [13,27], are likely to yield novel, 'back up' functions encoded from within a 'dockyard' of accessory genes called the 'plasticity region cluster' [20]. Previous studies point to such pool of strain specific genes in pathogens such as *H. pylori*, which could be useful in adaptation to a particular host population [21,23-25]. Another important reason to sequence replicate genomes of *H. pylori* entails the need to study chronological evolution within a single host. The nature and extent of genetic rearrangement that the chronically inhabiting pathogens such as *H. pylori* accumulate (across wide timescales) and during colonization of different host niches are not known; the advantages of polymorphisms that impart needed fitness to pathogens or commensals to colonize and inhabit their preferred host (niches) need additional in-depth studies [13]. While some experiments have been conducted to explore chronological strain diversity through multilocus genotyping [28] microarrays [29] and limited sequencing [22], whole genome profiling of such isolates has not been performed. This needs to be done at the earliest, especially, for those strains which are obtained at different intervals and sampled from different sites of individual patients to investigate the occurrence of possible insertions, deletions and substitutions (and mechanisms thereof) including their functional significance related to host adaptation and gain of niche. Apart from this, geographically distinct strains and their multiple representatives could be sequenced to explore local advantages that prevail in certain geographical regions in terms of host adaptation or disease outcome; for example, *H. pylori* infection in the Indian population (despite a very high colonization rate of up to 90%) rarely leads to serious consequences such as gastric cancer in a significant majority of patients who test positive for *H. pylori* infection [26]. Biological co-ordinates of such 'protection', if any, should be studied with the help of bacterial genome sequence data obtained from a number of strains. This appears not a distant possibility given that the next-generation sequencing methods are becoming

increasingly affordable. Also, the costs of whole genome sequencing should be low given that the genome sequence is approximately about 1.67 Mb.

Parasite, commensal or a mutualist?

If we discuss survival advantage to the pathogen, we should also see if there is any protective advantage for the host. Although there is no direct evidence in this direction, recent studies point to the possibilities that *H. pylori* infection protects against childhood diarrohea, gastro-oesophagial reflux disease, oesophagial cancers and asthma [9-11]. Eradication of *H. pylori* by antibiotic therapy has shown augmented incidence of some of these diseases in different populations. Also, due to eradication, *H. pylori* is at steep decline in the west and has been rightly dubbed as an 'endangered species' in the stomach [30,31]. Do we need to save and conserve *H. pylori* as an important beneficial organism and a marker of human history; or should we eradicate it completely? Is eradication that simple? In most developing countries, it is not achievable because of rampant drug resistance among local strains. Also, even if eradicated using future effective drugs, re-colonization will be difficult to avoid due to poor water hygiene and frequent contamination. Apart from need for future functional studies to link *H. pylori* to human disease or to project it as a commensal or a mutualist, region-centered epidemiological studies may be required to ascertain need for eradication or otherwise for different populations and societies. As far as a biological level definition of this pathogen goes, it is possible to term prehistoric versions of it as a 'bacterial parasite' [32], as it was so, prior to its acquiring the virulence genes (again it needs to be proved whether *H. pylori* has been always benign prior to the emergence of *Homo sapiens*). The present day *H. pylori* should not be dubbed as a beneficial organism or a commensal but only after its 'disarmament' - meaning that its powerful virulence machinery makes the organism a 'classical pathogen' and nothing less than that.

Competing interests

The authors declare that they have no specific competing interests related to this manuscript except that NA is the Co-Editor in Chief of the *Gut Pathogens* journal.

Authors' contributions

NA developed the hypothesis, wrote the text and sketched Figure 1. ST participated in molecular genotyping studies on *H. pylori* and NN worked on functional characterization of plasticity region proteins.

All authors have read and approved the final manuscript.

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