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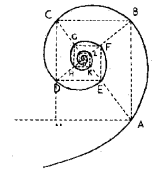

DOTTORATO IN MEDICINA MOLECOLARE E TRASLAZIONALE

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Development of a webtool for interactome-sequencing data analysis and identification of *H. pylori* epitopes responsible for host immuno-response modulation.

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ABSTRACT

To elucidate the molecular mechanisms involved in persistency/latency of the *H. pylori* infection or in its progression towards serious diseases, it is necessary to analyse the host pathogen interaction *in vivo*. The circulating antibody repertoire represents an important source of diagnostic information, serving as biomarker to provide a "disease signature".

The aim of this work is the identification of *H. pylori* epitopes responsible for host immuno-response modulation through: a discovery-driven approach that couples "phage display" and deep sequencing (interactome-sequencing) and the development of a specific webtool for interactome-sequencing data analysis.

We used this approach to identify novel antigens by screening gDNA libraries created from the pathogen's genome, directly with sera from infected patients. Two genomic phage display libraries from 26695 and B128 *H. pylori* strains have been constructed by using ß-lactamase ORF selection vector. Genomic DNA was sonicated, fragments cloned into the filtering vector, after transformation libraries of $1x10^6$ clones were obtained and sequenced by Illumina technology. More than 93% of Hp CDSs were represented in the phage genomic libraries therefore being representative of the whole *H. pylori* antigenic ORFeome.

A webtool for interactome-sequencing data analysis was developed and used to identify the *H. pylori* antigens/epitopes which could be considered specific for infection progression towards three different pathological outcomes.

Putative antigens were selected from libraries using sera from patients affected by: i) gastric adenocarcinoma; ii) autoimmune gastritis; iii) MALT lymphoma. The results, obtained thanks to the new interactome sequencing pipeline developed, show that the diversity of the libraries after selection is significantly reduced. Furthermore, individual ranks, for each infection condition, have been compared highlighting the pattern of putative antigens, shared by all the conditions, and some that can distinguish the different stages of infection.

One of this new antigens, that seems to be specific for infection progression towards more serious diseases, has been successfully validated through ELISA assay on a wide number of sera from patients. Other more specific antigens identified by our approach and by the application of the new data analysis pipeline here described are in validation.

SOMMARIO

Per comprendere al meglio i meccanismi molecolari coinvolti nella persistenza/latenza dell'infezione da *H. pylori* o nella sua progressione verso patologie più gravi, è necessario analizzare l'interazione ospite patogeno *in vivo*. Il repertorio di anticorpi circolanti rappresenta un importante risorsa di informazioni diagnostiche, utili per la definizione di una "disease signature".

Lo scopo di questo lavoro è l'identificazione degli epitopi di *H. pylori* responsabili per la modulazione della risposta immunitare attraverso un approccio guidato che accoppia la tecnologia del “phage display” e del sequenziamento ultra-massivo (interactome-sequencing) e lo svilippo di una specifica applicazione Web per l'analisi di questa tipologie di dati.

Questo approccio è stato applicato per identificare nuovi antigeni attravero lo screening di librerie fagiche costruite a partire dal DNA genomico di *H. pylori* direttamente con i sieri di pazienti affetti e con diversi outcome patologici. Sono state costruite due librerie fagiche genomiche dai ceppi *H. pylori* 26695 e B128 utilizzando un vettore di selezione delle ORF che sfrutta come folding reporter la ß-lattamasi. Il DNA genomico è stato sonicato e i frammenti sono stati clonati nel un vettore di filtraggio utilizzato poi per la trasformazione di *E. coli;* sono state così ottenute librerie di $1x10^6$ cloni e sequenziate con la tecnologia Illumina. Più del 93% delle sequenze codificanti (CDS) di *H. pylori* sono rappresentate nelle librerie genomiche che posso quindi essere considerate rappresentative dell'intero ORFe-oma/Domain-oma antigenico del patogeno.

Successivamente ho sviluppato un webtool per l'analisi dei dati provenienti da esperiemnti di interctome-sequencing e lo strumento di analisis sviluppato in questo lavoro di tesi è stato utilizzato nell'identificazione di antigeni di *H. pylori* specifici per le tre patologie prese in considerazione: i) adenocarcinoma gastrico ii) gastrite autoimmune iii) linfoma MALT. . I risultati prodotti grazie al nuovo strumento di analisis sviluppato, dimostrano che la diversità delle librerie dopo la selezione è significativamente ridotta. Inoltre le liste ottenute per ognuna delle tre patologie sono state confrontate evidenziando un

pannello di antigeni comuni e di antigeni specifici per i diversi outcome patologici.

Uno degli antigeni identificati, che sembra essere specifico per la progressione dell'infezione verso patologie più gravi, è stato già validato con successo tramite saggi ELISA su un numero ampio di sieri di pazienti. Altri antigeni identificati con questo approccio e grazie alla nuova strategia di analisi da me sviluppata e qui descritta sono attualmente in fase di validazione.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ABBREVIATIONS

AIG: Autoimmune gastritis

AP-MS: gel electrophoresis and mass spectrometry

ChIP-seq : Chromatin immunoprecipitation sequencing CDS: Coding sequence

DEG : Differentially Expressed Gene

FDR : False discovery rate

GA: Gastric Adenocarcinoma

HP : Helicobacter pylori

Interactome-Seq : Interactome-Sequencing

MALT: Mucosa-associated lymphoid tissue

NGS : Next-generation sequencing

ORF: Open reading frame

RUT : rapid urease test

Y2H: yeast two-hybrid

WHO: World Health Organization

1. INTRODUCTION

1.1. Helicobacter pylori

The pathogen, *Helicobacter pylori* is a gram-negative, neutralophilic organism, that colonizes half of the world's human population. *H. pylori* was discovered in 1984 by Barry Marshall and Robin Warren and for demonstrating its role in inducing gastritis, peptide ulcer formation and gastric cancer [1], they were awarded the Nobel Prize for medicine in 2005. This finding broke the dogma of the stomach as a sterile organ. In 1994 the International Agency for Research on Cancer classified *H. pylori* as a class I carcinogen [2]. *H. pylori* has evolved specific mechanisms to colonize and persist within the stomach in spite of the harsh acidic conditions encountered in this environment. Different genomic analysis has revealed symbiotic relationship between human host and *H. pylori*. Perturbation of this relationship leads to the dysregulation of this host-pathogen interaction, resulting in the development of severe gastroduodenal disease, including adenocarcinoma, autoimmune gastritis and gastric MALT lymphoma [3]. *H. pylori* is an epsilonproteobacterium and a member of the Helicobacteraceae family [4]. Helicobacters are classified into two types according to their customary niche: gastric and enteric. The Helicobacter species adapted to humans is *H. pylori* and is a gastric Helicobacter.

1.2. Epidemiology

The World Health Organization (WHO) has estimated that close to half of the world's population is positively infected with *H. pylori*. As human populations transmigrated across the world their endemic *H. pylori* strains diverged aboard them leading to phylogeographic differentiation of this infective agent that can be grouped into Amerindian, European,Asian and Africans subgroups. In various nonindustrial states, more than 81% of the population is positive affected by *H. pylori*, and infection occurs even among young people [5]. The *H. pylori* prevalence in developed countries is under 41% and is substantially higher in adults or in elderly individuals than young people [6]. In certain

geographical areas, the proportion of individuals affected bt *H. pylori* infection is reciprocally correlates with social and economic status, in particular there is a specific correlation to life conditions during prepuberty [7]. Meanwhile the prevalence of *H. pylori* infection in underdeveloped countries is relatively constant, in the industrialized countries is rapidly declining [8]. The causes of this decrease is due to the improvement of hygiene and sanitation via antimicrobial treatment [9].

1.3. Transmission

The mechanisms of transmission whereby *H. pylori* is acquired are not well known. *H. pylori* infection is believed to occur as a effect of direct person-to-person transmission, via either an oral-oral or fecal-oral route or both. *H. pylori* has been found in different biological specimens like saliva, feces, vomitus and also in gastric refluxate, but there is no clear evidence for prevalent transmission through any of these specimens [10,11]. Various reports have shown the contamination of *H. pylori* DNA in environmental water sources, but this is due to the contamination with dead *H. pylori* organisms [12]. An increased risk of infection was observed among hospitalized adolescent people during outbreaks of gastroenteritis [13]. Conjugated with the extreme sensitivity of the organism to atmospheric oxygen pressure, lack of nutrients, and temperatures outside the range of 34°C-40°C, direct human-to-human transmission is the most probable transmission route [14].

1.4. Genomic diversity and flexibility

The *H. pylori* genome consists of 1.65 million bp and codes for about 1,500 proteins and it has an average GC content of 38.9% [12]. *H. pylori* expresses an uncommon genetic variability that could potentially account for the bacterium's ability to adapt within the host gastric stomach [13]. These adaptations comprehend both temporary and permanent changes to the genomic conformation such as regulatory mechanisms that modulate gene expression [14]. Analyses of numerous whole genome sequences indicate that

H. pylori has evolved clusters of genes within genomic islands that create distinct areas of variability, called “plasticity zones”. These plasticity zones are are prevalent and widely distributed among almost all *H. pylori* strains and are also implicated in lateral gene transfer, they allow fitness advantages to the organism by promoting genetic recombination events, which are involved in immune evasion and host colonization mechanisms [15]. Research recent published supports this hypothesis and indicates that genetic modifications occur ten times faster during host infection, when *H. pylori* is initially encountering the host’s immune response. A study published by Linz B. et al have shown that *H. pylori* mutation rates of genes encoding for outer membrane proteins (OMPs) are higher than any other bacteria tested [16]. Brodsky IE et al. have shown that OMPs are involeved in host-pathogen interaction and are targets for the adaptive immune response [17]. *H. pylori* mutating these exposed antigens promotes the chronicization of infection and the genes encoding OMPs under high selective pressure [18].

2. *HELICOBACTER PYLORI* ASSOCIATED DISEASES

H. pylori typically is localized in the mucus away from the epithelial surface and does not directly adhere with gastric epithelial cells. Interaction of *H. pylori* with host cells activates many signaling pathways, the release of toxins or other signaling molecules [19]. In all subjects persistently colonized by *H. pylori* occurs a humoural immune response together with tissue infiltration by mononuclear and polymorphonuclear leukocytes [20]. The host inflammatory response is comparatively weak when compared with the response that occurs against others transient bacterial pathogens. As mentioned above, *H. pylori* persistently colonizes the human stomach for many years, causing alterations in the gastric niche and induces the host immune response, but its own peculiarity is to be a symbiotic organism usually and in many cases does not cause adverse effects [21]. What factors contribute to the stability of the *H. pylori*-host equilibrium? One important element is the presence of the bacterium within the gastric mucus layer, without any substantial adhesion of

host tissue. Another important element is the synthesis of *H. pylori* factors that are highly adapted to decrease the intensity of the host defense system [22].
H. pylori lipopolysaccharide (LPS) is distinguished by modifications of the lipid A component that make it less proinflammatory than lipoglycans found in the outer membrane of other Gram-negative bacteria [23]. Although *H. pylori* typically colonizes the stomach for many years without specific symptoms, its presence is correlated with an increased risk of several pathological outcomes [24]. The risk of these outcomes is related to different factors like host genetic and environmental factors host responses and interactions between host and microbe. The progression steps from normal stomach to inflammation and precancerous conditions were described by Correa in 1992 [25]. In Correa's cascade, host responses and interactions between host and microbe modulate the transition from one step to next. The main gastric pathological outcomes related to *H. pylori* infection are (Figure 2):

- Gastric adenocarcinoma (GA), which develops in gastric cancer in 1-3% of infected individuals;
- Autoimmune gastritis (AIG), developed by the great majority of patients but that mostly remains asymptomatic;
- Gastric mucosa-associated lymphoid tissue lymphoma (MALT lymphoma), which develops in 0.1% of infected subjects;

2.1. Gastric adenocarcinoma

Gastric adenocarcinoma (GA) is the second principal cause of cancer-related death worldwide and between the common cancer is the fourth, every year causes about 700,000 deaths. The incidence rates of GA is not uniform and varies depending on the country, with the highest rates found in Asia (mainly in the east), Central America, some regions of South America, and Eastern Europe [26] (Figure 1).

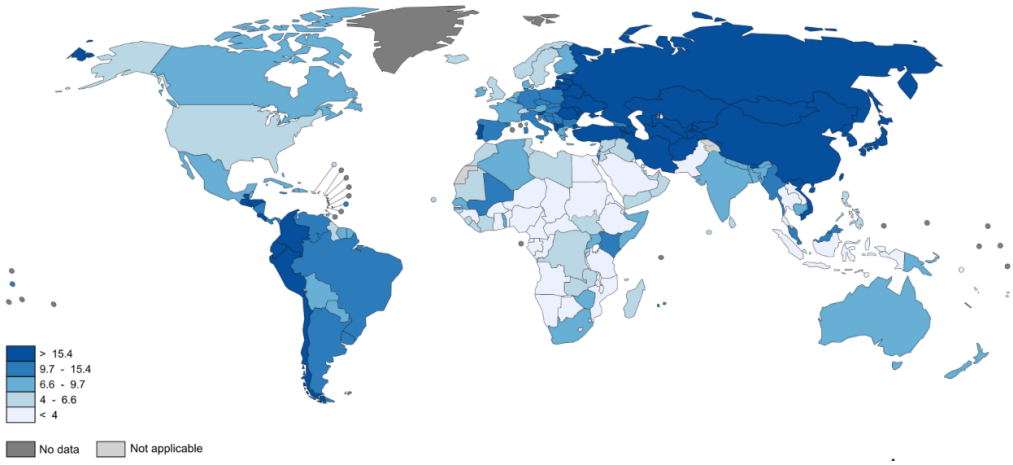


Figure 1 - Map of global distribution of Incidence from gastric cancer. Image reprinted by [26].

As shown in Figure 2 GA is initiated by the transition from normal mucosa to chronic superficial gastritis; this is followed by the development of atrophic gastritis and metaplasia, finally leading to dysplasia and adenocarcinoma [27]. GA affects male twice as frequently as female; it is thought that estrogen may confer some protection in women. Over the past century, the incidence rate of GA in developed countries is remarkably decreased [2]. This decline can primarily be attributed to a decline in intestinal-type adenocarcinomas in the distal stomach [26]. Currently, in the USA, distal GA is diagnosed most commonly in elderly people and occurs more often in African-Americans, Hispanic-Americans, and Native Americans than among other ethnicities. While the incidence of GA of the distal stomach has been declining, the incidence rates of proximal GA as well as those originating in the gastroesophageal junction have been increasing in both the United States and Europe [28].

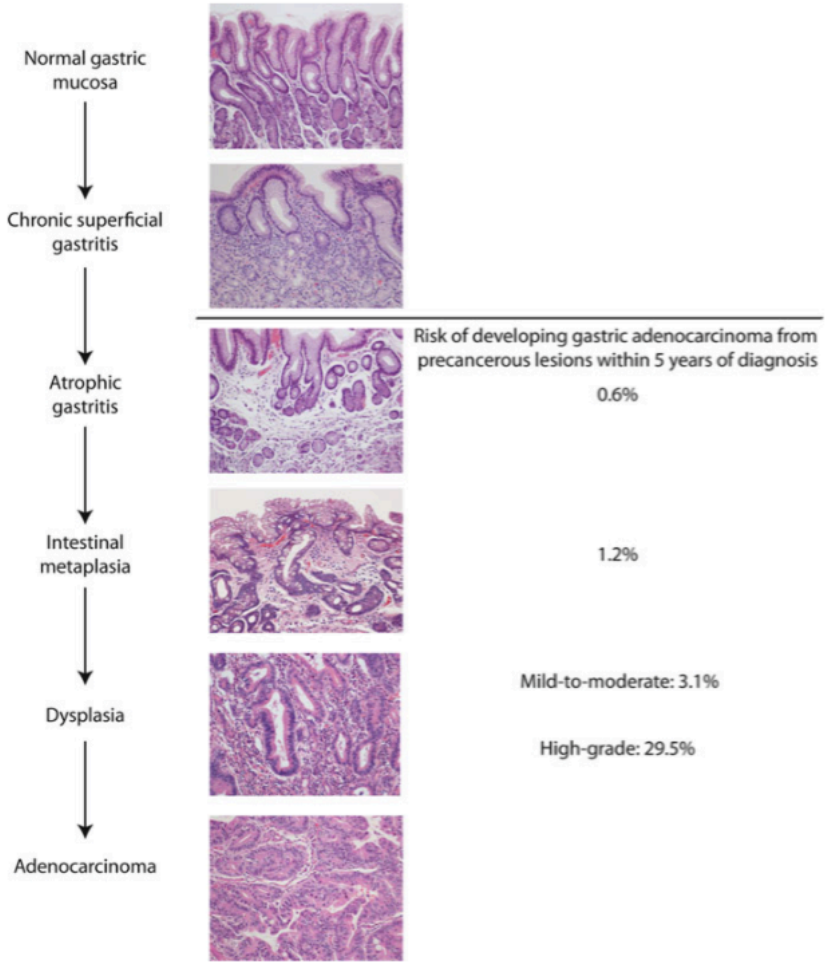


Figure 2 - Correa's cascade proposed in 1992. Image reprinted from [25]

2.1.1. Signs and symptoms

It is usually difficult to diagnose GA at an early onset stage of because it often does not cause particular symptoms. When symptoms do occur, they may be not specific and these symptoms can also be caused by many other illnesses, such as a stomach virus or an ulcer. The signs and symptoms associated with GA are similar to other gastric disease and they include indigestion, discomfort in the abdomen, nausea and vomiting, diarrhea or constipation, loss of appetite, sensation of food getting stuck in the throat while eating, unexplained weight loss [29].

2.1.2. Cause

Besides *H. pylori*, the pathogenesis of GA is correlated with diet and cigarette smoking. High dietary salt intake was found to be associated with an increased risk of GA [30]. Two studies, from South Korea and Japan stated that *H. pylori*-infected subjects taking high-salt diet had a greater risk of GA than subject who underwent a low-salt diet [31], even if the mechanisms that link a high-salt diet with the increase of GA development remains unclear. The risk of developing GA in *H. pylori* infected subjects is also associated with cigarette smoking. By several studies, it emerges that exists a relationship between *H. pylori* infection and sigarette smoking with enhanced risk of developing GA [32,33].

2.1.3. Pathophysiology

The pathogenesis of *H. pylori* associated to GA is a multi-factorial and multi-step process, and as mentioned in the previous chapter its development depends on a combination of host, bacterial and environmental factor as shown in Figure 3. Many studies focused on the effects of *H. pylori* infection on the host epithelium. The gastric epithelium has the function to protect the underlying tissue and has also the responsibility for digestive processes. *H. pylori* has developed sophisticated mechanisms to avoid host defence and to adapt to the immune system response in order to colonize the gastric niche, such, for example, disruption of epithelial junctions, stimulation of cytokine production, overproliferation, DNA damage, apoptosis and DNA transformation [34,35]. *H. pylori* disrupts intercellular apical junctions, these are critical in keeping the integrity of mucus layer, furthermore these intercellular junctions are indispensable for cellular functions because through the binding to specific cellular receptors they stimulate the signaling pathways [36]. The *H. pylori* virulence factor CagA is translocated into epithelial cells through the Type IV secretion system (T4SS) and interacts with membrane proteins like E-cadherin and ZO-1 and disrupts the tight of adherence junctions. It has been confirmed that E-cadherin interacts with β-catenin, a component of a cell adhesion

complex [37]. The complex E-cadherin/β-catenin play pivotal role in maintaining epithelial integrity and in the stabilization of cellular architecture [34,38]. During *H. pylori* infection, the structure of this complex is destabilized by the virulence factor CagA in a phosphorylation independent manner [39]. The evidences support that also the virulence factor VacA interacts with epithelial cells inducing autophagy [40]. Other studies demonstrated that to facilitate the intracellular survival and for promoting the carcinogenesis *H. pylori* suppress autophagy [41]. Saberi S. et al have shown that the fast turnover of epithelial cells is an important element to protect the gastric epithelium from infection, but *H. pylori* blocks this turnover inducing apoptosis in order to favour its survival [37]. A deleterious consequence of apoptosis iduction is an imbalance in normal gastric mucosal homeostasis leading to dysregulated tissue growth [42]. The mechanism behind this process is not clear, enzymes such as urease and virulence factors such as VacA lead to cellular apoptosis by damaging the gastric epithelium and activating the immune response [43]. The high level of free radicals generated by neutrophils and cytokines like IFN-γ in inflammatory response can damage DNA and stimulate apoptosis of gastric epithelial cells. Proinflammatory cytokines including IL-8, IL-6, MCP-1, TNF-α, MIF, IL-1α, TGF-β and IL-1β are secreted by epithelial cells and these play a fundamental role in pathogenesis of GA [44]. Another important aspect involved in pathogenesis of GA is the polymorphisms of cytokine genes. As reported by Amieva et al. the risk of GA in many populations was affected by the polymorphism of the genes encoding IL-1β, TNFα, IL-8, IL-17 and IL-10 [45]. These polymorphisms are important in gastric carcinogenesis because they affect the oxidative stress process and DNA mutagenesis, this phenomenon could also explain the occurrence of GA only in a small proportion of *H. pylori*-infected individuals [46].

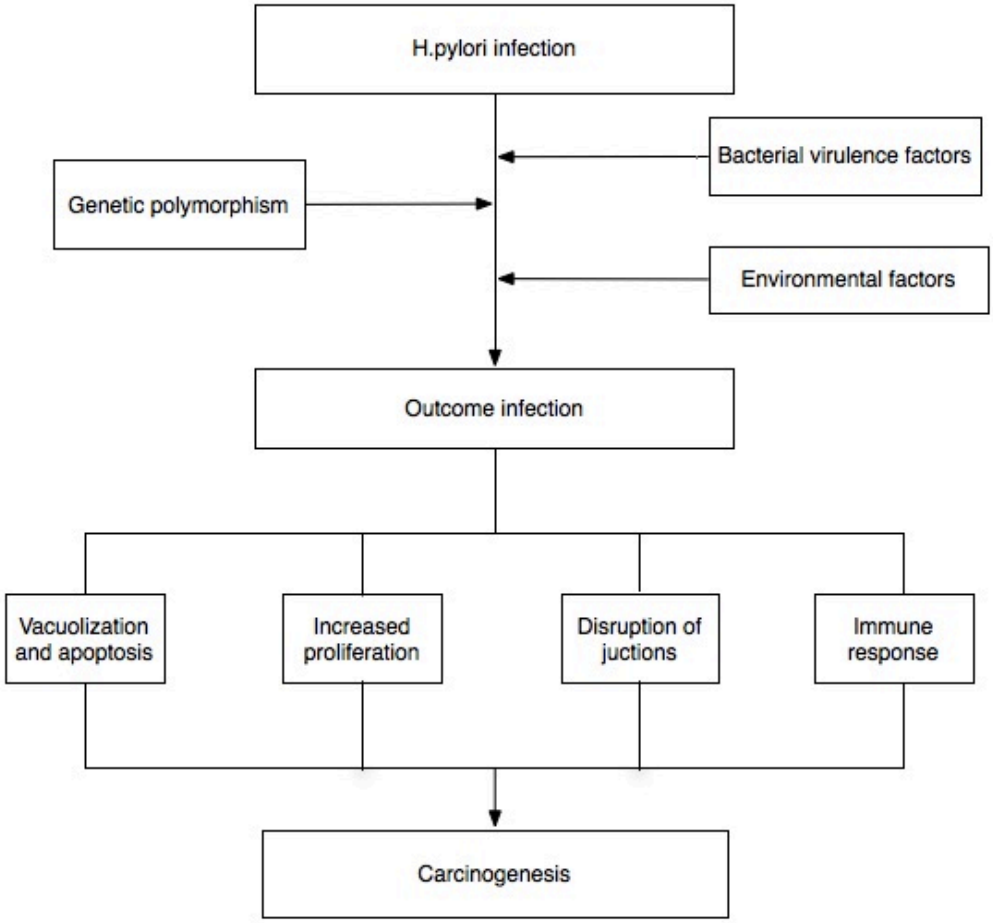


Figure 3 - Role of *H. pylori* in the gastric carcinogenesis. Host genetics, environmental factors, and bacterial strain differences in virulence properties contribute to disease progression and increased risk of negative outcomes. Image reprinted from [36]

2.1.4. Diagnosis

The diagnosis of GA is based on endoscopy and biopsy. Endoscopic ultrasonography and computed tomography (CT) of the chest and abdomen are currently the primary means of staging for the diagnostic of gastric cancer. Endoscopic ultrasonography and computed tomography are uncomfortable, painful and expensive, emphasizing the need for rapid and non-invasive tests for screening and monitoring the patients with dyspeptic symptoms. Recently has been developed a panel which combines a serum pepsinogen-I (PGI) and -II (PGII), gastrin-17 (G-17) and HP IgG antibodies (IgG-HP) [47,48]. These

biomarkers were tested in a cohort of 91 patients revealing a low sensitivity and specificity, for these reasons gastroscopic exams and CT remain the most commonly prescribed diagnostic test.

2.2. Autoimmune Gastritis

Autoimmune gastritis (AIG) is a chronic inflammation of the stomach caused by an indirect action of the *H. pylori* on the immune system. The term AIG includes a variety of definitions such as atrophic body gastritis and pernicious anemia [49].

2.2.1. Signs and symptoms

Patients with AIG are asymptomatic for many years, until parietal cell mass reduced capacity to secrete HCL acid causing hypochlorhydria or achlorhydria. Abnormal gastrin production (hypergastrinemia) occurs in achlorhydric patients, the clinical features of AIG are largely due to the pernicious anemia, weight loss occurs in about 50% of patients and AIG are occasionally associated also with sore tongue and neurological problems including numbness, weakness, and ataxia. Patients with AIG may have other autoimmune diseases such as Graves' disease, a condition in which the thyroid gland produces excessive hormones and idiopathic adrenocortical insufficiency [49].

2.2.2. Cause

H. pylori is the main aetiologic factor for AIG, the degree of mucosal inflammation results from the interplay of *H. pylori* virulence factor, genetic host susceptibility and environmental factors. AIG associated with *H. pylori* infection is limited to the antrum of the stomach (Type B gastritis). Other causes are the excessive and long term assumption of Nonsteroidal anti-inflammatory drug (NSAID); less common causes include alcohol, cocaine and Crohn disease [50].

2.2.3. Pathophysiology

AIG is an immune-based, non-infectious inflammation of the stomach, as a result of molecular mimicry between *H. pylori* antigens and H+/K+-ATPase. The hypothesis is based on the observation that *H. pylori*-infected patients with AIG harbour gastric CD4+ T-cells that recognize cross-reactive epitopes in common between gastric H+/K+-ATPase and *H. pylori* proteins. Cross-reaction antigens shared by H. pylori induce T-cell proliferation, production of Th1 cytokine and H+/K+-ATPase antibodies were identified in about 22-30% of *H. pylori* infected patients [51]. The clones deriving from T-cell proliferation reacted to epitopes different to the proton pump alone. Consequently, this autoimmune response induces the destruction of parietal cells and dysfunction of the oxyntic gastric mucosa [50].

2.2.4. Diagnosis

Despite the significant progress in the understanding of pathogenesis of chronic inflammation in AIG, diagnosis is based on histology and detection of the presence of Parietal Cell Antibody (PCA). Literature data on association of AIG with PCA is weak; in a clinical study of 2012 on a cohort of 108 patients with AIG 82% were positive for PCA while in a similara study a cohort of 140 patients with AIG 75% had PCA, which correlated positively with the grade of atrophy [50]. Histology coupled with endoscopy can provide a unequivocal diagnosis of AIG. Through endoscopy multiple gastric biopsy sampling is performed, diagnosis of AIG requires a minimum of 4 biopsies from antrum and corpus of stomach [48].

2.3. Gastric mucosa-associated lymphoid tissue lymphoma (MALT lymphoma)

Extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALT lymphoma) is the third most common subtype of non-Hodgkin's lymphoma [52]. The microbial pathogens that underlie such chronic inflammatory diseases, also play a pivotal role in both malignant transformation and successive clonal expansion of the transformed clone. This is well exemplified by the causative role of *H. pylori* infection in the development of gastric MALT lymphoma, the definition of this causative relationship led to successful innovative treatment of lymphoma with antibiotics [53].

2.3.1. Signs and symptoms

Gastric MALT lymphoma is an indolent disease and the clinical presentation is poorly specific. Symptoms ranging from fatigue, low-grade fever and nausea to alarm symptoms such as gastrointestinal bleeding or persistent vomiting [54]. Others symptoms like weight loss or night sweats are extremely rare in stomach MALT lymphoma. A systematic review published in 2010 including data of 2000 patients found that gastric MALT lymphoma occurs in a wide age range of patients and sex ratio incidence is essentially equal, and only in 42% of patients alarm symptoms were present [55].

2.3.2. Cause

Different infections have been involved in the progression of gastric MALT lymphoma. There is a clear correspondence between gastric H. pylori infection and MALT lymphoma diagnosed in the stomach (90% of cases) [56]. In fact, eradication of *H. pylori* leads to the disappearance of intratumoral *H. pylori* specific T-cells and thus to the removal of the growth support of neoplastic B-cells inducing the regression of lymphoma [57]. More or less, 5-10% of gastric MALT lymphomas are negative for *H. pylori* and can be associated to *H.heilmannii* infection [58].

2.3.3. Pathophysiology

The importance of *H. pylori* in the pathogenesis of gastric MALT lymphoma are confirmed by direct evidence obtained from in vitro studies. These studies show that lymphoma growth is stimulated in culture when exposed to the bacterium, through tumour-infiltrating lymphocytes (TILs) involving two costimulatory molecules: CD40 and CD40L. The infiltrated B cells are activated by T-cells specifically recognising *H. pylori* and undergo towards malignant transformation due to the acquisition of genetic abnormalities [59]. Epithelial cells are activated by chronic infectious stimuli, expressing on their surface high levels of HLA-DR (Human Leukocyte Antigen - antigen D Related) and costimulatory molecules, including CD80. Epithelial cells present antigens along with HLA molecules to T cells, CD40 ligand molecules expressed on the activated T cells can react with the CD40 molecule on B cells, consequently the expression of B cell CD80 is upregulated. CD80 is a protein found on activated B cells that provides a costimulatory signal necessary for T cell activation by binding CD28 [60]. Activated CD4 T-cells together with the action of various cytokines and chemokines can stimulate B-cells through CD40L-CD40 interaction. Activated epithelial cells, T cells and B cells allow to survive cooperatively in lymphoepithelial lesions (LELs), that are thought to be at the origin of lymphomas [61].

2.3.4. Diagnosis

Gastric MALT lymphoma associated with *H. pylori* infection presenting nonspecific symptoms, for this reason diagnosis is based on invasive approach and requires endoscopic biopsy [62]. Recent advancement in endoscopic techniques allow an accurate diagnosis in 91% of case [63]. Endoscopic biopsy appearances of MALT lymphoma are an atypical lymphoid infiltrate, lymphoepithelial lesions and moderate cytological atypia of lymphoid cells. Many biopsy specimens lack some of these characteristics [64]. In 1993, Dr A.C Wotherspoon proposed a useful scoring system, shown in the Table 1, currently in use, to better defining and differentiating the gastric lymphoid

infiltrates [65]. The diagnosis should also consider *H. pylori* infection, for this reason final diagnosis should include histology, rapid urease testing, urea breath testing, stool antigen testing, or serologic studies [66].

Score	Diagnosis	Histological features
0	Normal	Scattered plasma cells in lamina propria. No lymphoid follicles
1	Chronic active gastritis	Small clusters lymphocites in lamina propria. No lymphoid follicles. No lymphoepithelial lesions.
2	Chronic active gastritis with florid lymphoid follicle formation	Prominent lymphoid follicles with a surrounding manntle zone and plasma cells. No lymphoepithelial lesions.
3	Suspicious lymphoid infiltrate, probably reactive	Lymphoid follicles surrounded by small lymphocytes that infiltrate diffusely in lamina propria and occasionally into the epithelium
4	Suspicious lymphoid infiltrate, probably lymphoma	Lymphoid follicles surrounded by marginal-zone cells that infiltrate diffusely in lamina propria and into the epithelium in small groups
5	MALT lymphoma	Presence of dense diffuse infiltrate of marginal-zone cells in lamina propria with prominent lymphoepithelial lesions.

Table 1 - Histological criteria established by Wotherspoon. Table reprinted by [65].

3. MOLECULAR MECHANISM OF H. PYLORI INFECTION AND PATHOGENESIS

To promote chronic infection *H. pylori* has evolved a variety of mechanisms to survive in the acidic environment of gastric mucosa. To neutralize the hostile acid condition at beginning of infection *H. pylori* has developed an “acid acclimation mechanism” that promotes adjustment of periplasmic pH in the stomach by regulating activity of urease and carbonic anhydrase [67]. Once the bacterium has created a favourable environment in term of pH, the next step is the mobility through the protective layer of gastric mucus in the host stomach. Motility of *H. pylori* depends on the presence of up to 6 functional unipolar flagella [68]. *H. pylori* regulates cell motility by responding chemotactic cues: it has been demonstrated that the operon Tlp A B C D, and in particular TlpD is necessary for bacterial persistence in the murine stomach and growing in the infected and inflamed antrum [69]. Flagella-mediated motility is followed by specific interactions between bacterial adhesins with host cell receptors,

which thus leads to successful colonization and persistent infection [70]. Finally, *H. pylori* releases several effector proteins/toxins causing host tissue damage.

The molecular mechanism by which *H. pylori* causes disease in humans can be described as a multi-step process. In summary, four steps are critical for H. pylori colonization and pathogenesis (Figure 4):

- Survival under acid stomach conditions;
- Movement toward epithelium cells through flagella-mediated motility;
- Attaching to host receptors by adhesins;
- Causing tissue damage by toxin release;

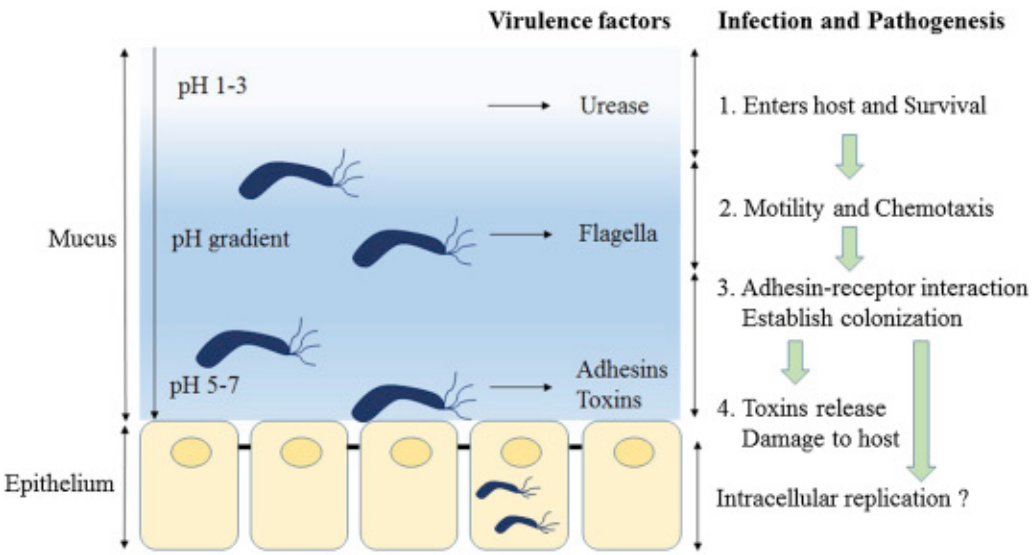


Figure 4 - Schematic representation of *H. pylori* infection and pathogenesis mechanism. Image reprinted from [71].

3.1. Urease and survival under acidic stomach conditions

H. pylori has adapted an original mechanisms to colonize and persist within the gastric environment, for acid resistance the organism uses urea to produce ammonia through the metalloenzyme urease, that catalyzes the conversion of urea into ammonia and CO_2 .The NH_3 produced by this reaction increases the local pH surrounding the bacterium thus allowing its survival in the hazardous acidic environment of the human stomach [72]

The constitutive production of the urease accounts up to 10% of the proteins synthesized by the organism at pH 7.0. In several bacterial species ureases are entirely cytoplasmic, instead *H. pylori* urease is found both inside the cytoplasmic and outside adhering to the surface of the organism. For many years it has been believed that external urease provides the acid resistance of *H. pylori* but there is no evidence supporting this hypothesis, furthermore the extreme acidity encountered in the stomach irreversibly inactivates this enzyme [22].

Instead urease localized in cytoplasmic protects the organism by producing uncharged ammonia inside this compartment, after synthesis ammonia is carried out to periplasmic space where it can bind protons raising the periplasmic pH and preventing a high concentration of protons from migrating into the cytoplasm and at the same time avoiding the pH decreasing to toxic levels [66]. The massive ammonia synthesis by urease activity constitutes the principal protection against proton overload, acid acclimation depends both on urea degradation and ammonia synthesis but also on urea uptake across the bacterial inner membrane [73]. Urea is transported from the periplasmic space into the cytoplasm where it is hydrolysed by urease for the production of ammonia through the channel transporter UreI [74]. The control of the periplasmic pH on acid exposure is unique to *H. pylori*, and make *H. pylori* colonization durable and persistent [75].

3.2 Bacterial motility toward epithelium cells

Flagella-mediated motility is essential for the *H. pylori* colonization of gastric mucosa, flagella can be considered as an early stage colonization/virulence factor [70]. *H. pylori* flagellum is mainly composed of the basal body hook and flagellar filament, that consists of two flagellins (FlaA and FlaB) [76]. The hook is composed of FlgE, and it links the basal body and flagellar filament. The basal body is composed of several structures, and it plays a role in providing the energy source for motility [77]. Several study have indicated *flaA* and *flaB* as necessary genes for the complete motility of *H. pylori* [76]. Recently, the

role of flagellins in bacterial adherence to mammalian hosts has been demonstrated by constructing flagellins (*flaA* and *flaB*) and flagellar regulator (*fibA*) mutants and it was shown that all mutants are still able to adher to gastric cells, supporting the hypothesis that flagella do not play a direct role in adhesion of *H. pylori*. Although a lower adhesion rate in a *flbA* mutant suggested that in addition to regulating flagella, FlaA may regulate some *H. pylori* adhesins [76] (Figure 5).

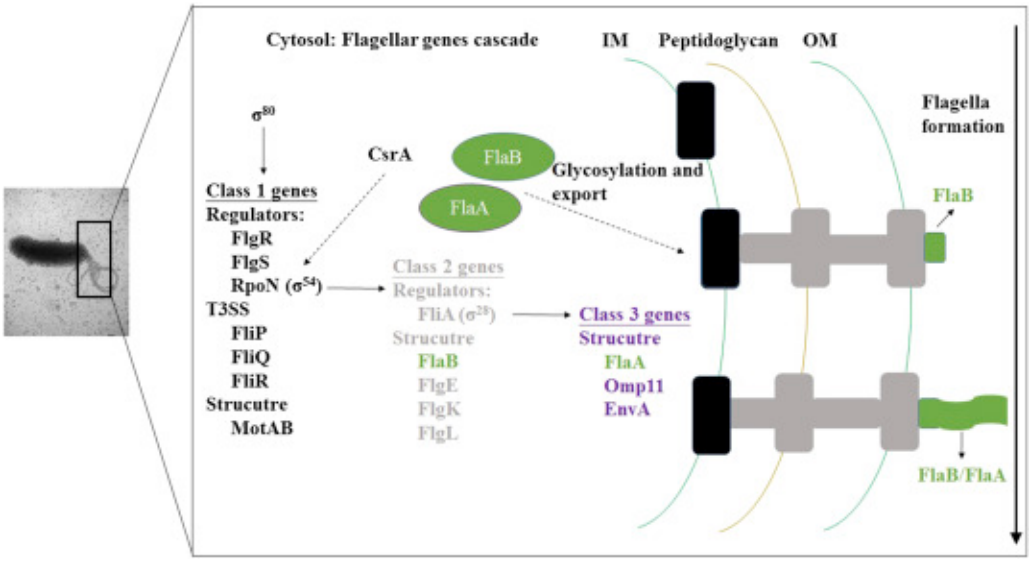


Figure 5- Schematic model of the flagellar transcriptional regulatory cascade for *H. pylori* flagellar biosynthesis. Image reprinted by [71].

3.3. Adhesins

Adhesins are a large group of bacterial cell-surface proteins that enable a strong interaction with epithelial cells. The adherence of pathogens to mucosal epithelial cells is considered to be the first important step in pathogenesis onset in the stomach. Bacterial adherence to the gastric mucosa is crucially important for protection from acidic pH, mucus, and exfoliation [78].

H. pylori adhesins are involved in numerous processes during early and chronic phases of infection. They also contribute to the differential outcome in infected patients by triggering disease development [79]. *H. pylori* adhesive factors belong to the largest outer membrane protein (OMP) family of the

bacterium, namely, the Hop family [80]. The Hop family contains the most well-known adhesins of *H. pylori* like BabA, BabB, SabA. Additionally, there are other proteins, such as NAP and Hsp, which have been implicated in cell adhesion and mediate the tropism of H. pylori to the gastric tissue [81].

3.3.1. Blood group binding adhesin (BabA and BabB)

Three *H. pylori* bab allelic types have been identified, including babA1, babA2 and babB. The molecular mass of the BabA protein is nearly 78 kDa, encoded by babA2. The babA1 and babA2 coding sequences are highly similar, but the translational starting codon is lacking in babA1 [71]. *H. pylori* employees BabA to bind to fucosylated Lewis B blood-group antigen (Leb) expressed on host gastric epithelium cells, when *H. pylori* initially infects the human stomach [82]. BabA and BabB are nearly identical in their 5′ and 3′ regions, with most of their sequence divergence being in their mid-regions. Importantly, the middle region of the BabA sequence determines the adhesion ability of BabA. The function of BabB is still unclear, but the expression of BabB was associated with increased gastric histologic lesions in patients [72]. To study the dynamics of Leb adherence during infection paired H. pylori isolates obtained sequentially from chronically infected patients were analysed [73]. The results showed that a complete loss or significant reduction of Leb binding was observed in strains from 5 out of 23 individuals, indicating that the BabA-Leb binding phenotype is quite stable during chronic human infection. Sequence comparisons revealed that most amino acid changes were found in the putative N-terminal extracellular adhesion domain [73]. This recombination mediates dynamic changes in adherence properties, which suggests that it contributes to the persistence and adaption of H. pylori in ever-changing gastric environments.

3.3.2. Sialic acid-binding adhesin (SabA)

SabA binds to sialylated structures such as the sialyl-Lewis X/A (sLex/a) antigens also found on mucins and epithelial cells [74]. This suggests that SabA adhesin plays a critical role to assist H. pylori to adhere to and colonize

the gastric epithelium cells of patients with gastritis [75]. Especially when lacking gastric Leb expression, Lex and Lea were closely related to H. pylori colonization. The sabB gene is homologous to sabA, but appears not to be involved in sLex binding. Therefore, the function of sabB in bacterial adhesion and pathogenesis is worth investigating. SabA is transcriptionally repressed by the acid sensitive ArsRS regulonand is therefore down regulated during acidic conditions [76]. In addition to this direct regulation by transcription factors, expression of the sabA gene is regulated through slipped strand mispairing through length variation in the poly(T) repeat tract that lies upstream of the promoter element of sabA (Berg et al., 2014). Additionally, the gene contains cysteine-thymidine (CT) repeats in the 5' part of the coding sequence, causing on/off phase variation. These dynamic adaptations may allow H. pylori to specialise for individual host variation in mucosal glycoprotein sialylation during persistent infection. Recently, the crystal structure of the extracellular parts of the SabA protein was resolved. The structure is dominated by alpha helices and resembles a club with a handle and a head. The head part contains the ligand-binding cavity, which is constrained by two highly conserved disulphide bridges. One of these pairs of conserved cysteines is also present in BabA, but the major part of the ligand-binding region is not homologous between the two proteins [78].

3.3.3. Neutrophil activating protein (NAP)

Neutrophil activating protein (NAP or HP-NAP) was first identified to stimulate high production of oxygen radicals from neutrophils, leading to damage of local tissues, and promote neutrophil adhesion to endothelial cells during *H. pylori* infection [83]. NAP upregulates the expression of β2 integrins in neutrophils and monocytes [80], and also is highly associated with the hallmark of chronic gastritis, and infiltration of neutrophils and mononuclear cells into the gastric mucosal barrier, caused by *H. pylori* infection. The glycosphingolipids expressed on the neutrophil surface serve as a major receptor to interact with the NAP expressed on bacterial surface [81]. Moreover, NAP is supposed to

facilitate SabA-mediated binding of sialyl-Lewis antigens on the membrane of the host cell [82]. To study *H. pylori* colonization in vivo Wang et al. [83] infected mice with both *H. pylori* napA mutant strain and wild-type strain, compare to the mice infected with wild-type strain the degree of survival was lower than the mice infected with napA mutant. However, for pathological outcomes related to *H. pylori* infection only one report showed that in sera from patients with GA the level of NAP-specific Ab was remarkably higher than that from infected patients with AIG [84]. No other studies have shown the direct involvement of NAP with gastric inflammation among patients *H. pylori* positive.

3.3.4. Heat shock protein 60 (Hsp)

Heat shock proteins (Hsp) are a highly conserved protein family found in prokaryotes and eukaryotes. Hsp are induced by several factors like a variety temperature, pH change, ischemia, and microbial infection [84]. *H. pylori* produces mainly two heat shock proteins, GroES-like HspA (Hsp10), and GroEL-like HspB (Hsp60). The high expression of Hsp60 at low pH, which interacts with the receptor-like sulfatide (sulfoglycolipid), indicates that the acid stress may change the specificity of *H. pylori* to receptors [85]. Hsp60 induces activation of NF-κB via TLR2 and the mitogen-activated protein kinase pathway, and thereby induces human monocytes to secrete IL-8 [86]. Moreover, anti-Hsp60 antibodies are consistently detected in *H. pylori*-infected patients, and these antibodies are associated with the progression of gastritis or gastric cancer [85].

3.3.5. Other adhesins

There are several others know adhesins used by *H. pylori* to adapt to different hosts/tissues, including AlpA, AlpB, HopZ, OipA and HopQ, all of them belong to the major Hop family of OMPs. The adherence-associated lipoproteins AlpA (HopC) and AlpB (HopB) are highly related and can be found in basically all *H. pylori* isolates. They are transcribed from the same operon, and their loss

impairs bacterial binding to the apical side of human gastric tissue sections, and in animal models. Unlike other OMPs, they are not subjected to phase variation but are expressed in virtually all clinical isolates [87]. The ligand of AlpA and AlpB has not yet been determined, although extracellular matrix laminins have been proposed as possible candidates, and the understanding of their role in human infection is still incomplete. The outer inflammatory protein A OipA (HopH) has been proposed to amplify IL-8 secretion and activate β-catenin, in parallel to cagPAI. OipA is phase-variable and can be switched "on" and "off" by slipped strand mispairing during chromosomal replication [88]. OipA expression status is associated with the presence of cagPAI and VacA s1m1 alleles in western isolates, and it has therefore been difficult to assess the separate influence of OipA on clinical manifestations [19]. Like AlpA/AlpB, the host surface receptor/interaction partner of OipA has not yet been identified. Other Hop proteins that have been implicated in adhesion are HopZ and HopQ. HopZ has been shown to be involved in the early phase of colonisation and is regulated by phase variation of CT repeats in the region encoding for the signal sequence. HopQ has also been implicated in binding to epithelial cells, but the binding partner has not yet been identified [89].

3.4. Virulence factors

Several *Helicobacter pylori* genes have been shown to modulate virulence of bacterial isolates. *H. pylori* yields various virulence factors that may dysregulate host intracellular signaling pathways and decrease the threshold for neoplastic transformation. Among all the virulence factors, *cagA* (cytotoxin-associated gene A) and its pathogenicity island (*cag* PAI) *vacA* (vacuolating cytotoxin A) are the most relevant.

3.4.1. cag Pathogenicity Island (*Cag*PAI) and CagA

Cag pathogenicity island (*cag*PAI) is a genetic element of exogenous origin that has been inserted by horizontal transfer into the glutamate racemase gene and is made up of 27-31 genes, with a total size of approximately 37 kb [90].

These genes encode structural components of a type four secretion system (T4SS) and an effector protein, the cytotoxicity associated virulence factor CagA. The T4SS pilus is used to inject CagA into the host cells, where it interferes with a series of host proteins and signalling pathways. The presence of this pathogenicity island highly varies between different strains: almost all East Asian isolates but only approximately 60-70% of Western isolates are cagPAI positive [91]. The actions of CagA can be divided into phosphorylation dependent inside the host cell, and phosphorylation-independent. The crucial region for CagA phosphorylation is represented by EPIYA (glutamic acid-proline-isoleucine-tyrosine-alanine) motifs in the C-terminal region. The tyrosines of these motifs can be phosphorylated by host Src-family kinases, and show a variability that has been linked to strain virulence and geographical origin [90]. On the basis of sequences flanking the EPIYA motifs, 4 different segments, termed EPIYA-A, -B, -C and -D, have been described (Figure 6).

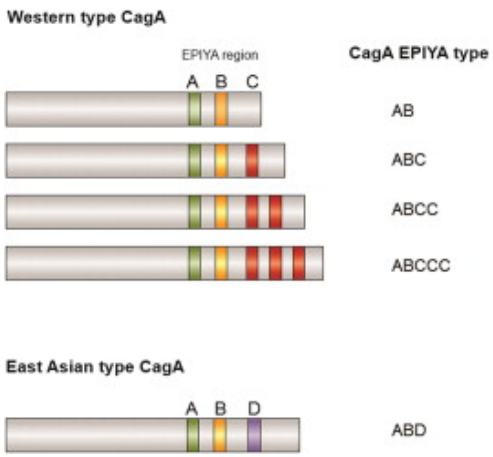


Figure 6 - Schematic Representation of the Western type CagA and the East Asian type CagA. Image reprinted by [92].

Most CagA positive strains have the A and B segments, while EPIYA-C is characteristic only of European strains and it is thus termed Western CagA. EPIYA-D is specific of East Asian strains CagA and consequently it is known as East Asian CagA. The East Asian CagA has been shown to have more

oncogenic potential than Western CagA, but Western CagA can also vary in the number of C regions, where more repeats are correlated to greater oncogenic potential. The effects of CagA on host cells include dysregulation of cell-cell adhesion and loss of polarisation in the epithelial cell, cellular elongation that resembles epithelial to mesenchymal transition (EMT), and the activation of the Ras-Erk cascade. The latter increases interleukin 8 (IL-8) release and the activation of NFκB, which leads to an increase in tumour necrosis factor alpha (TNF-α) and cyclooxygenase 2 (COX-2) pathway, among other effects [27]. Within the cell, phosphorylated CagA activates the SHP-2 phosphatase and the MAPK-ERK signalling cascade, causing effects resembling those induced by growth factor signalling. Reported phosphorylation-independent effects include the interaction with E-cadherin, the c-met receptor, and phospholipase C, which disrupts cell-cell junctions and induces loss of cell polarity, and pro-inflammatory and mitogenic effects [19].

3.4.2. The vacuolating cytotoxin A (VacA)

Along with CagA, the vacuolating cytotoxin (VacA) is the best-studied virulence factor. VacA is a toxin that forms hexameric, anion-selective pores through lipid bilayers such as the cytoplasmic and organelle membranes and, as the name implies, induces vacuolisation in the host epithelial cells [92]. There is significant variation among strains in their capacity to induce cell vacuolization [93]. This variation is attributed to the genetic structural diversity of the *vacA* gene that can assume different polymorphic rearrangements (Figure 7) [94].

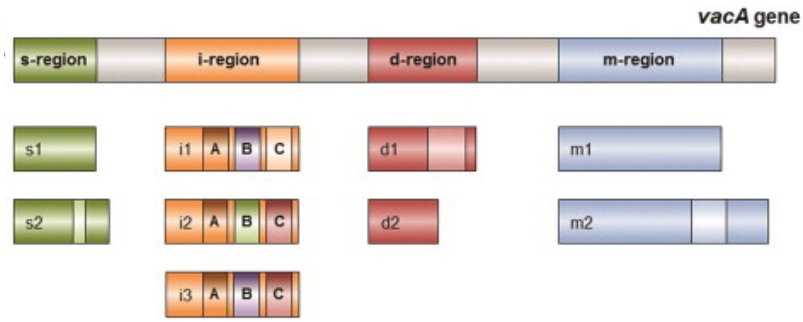


Figure 7 - Schematic representation of *H. pylori* vacA gene showing the s-, i-, d- and m-polymorphic regions. Image reprinted from [92].

The initial studies on *vacA* detected two main polymorphic regions, the signal (s)- and the middle (m)- regions. The s-region encodes a different signal peptide and assumes two forms, s1 or s2, s1 form is active while s2 form encodes a different signal peptide cleavage site which results in a short N-terminal part that display attenuated vacuolating activity. The m-region encodes the VacA binding site to host cells, and like s-region it can have two isoforms: the m1 and the m2 forms; m1 is the more effective of the two [92]. More recently, new regions of variations were found in *vacA*, the intermediate (i)- and deletion (d)-regions [92]. Like signal (s)- and middle (m)- regions they have two main polymorphic forms (i1,i2 and d1,d2) [92]. the mosaic combination of these forms discriminate among *H.pylori* strains vacuolating and strains non-vacuolating, for example the s2 type of VacA has been shown to be virtually nontoxic [95], while strains with the s1/m1/i1 genotype have been shown to be associated with a higher risk of advanced disease and are found in patients with higher gastric cancer risk. However, this genotype commonly coincides with cagA positivity, making the distinct contribution of the two virulence factors in these strains hard to be evaluated [92]. VacA can be transferred to host epithelial cells either by secretion or by contact-dependent transfer and is cleaved during its transport through the bacterial outer membrane [92]. The precise mechanism of VacA secretion and entry to the host cell is still controversial but both epithelial derived growth factor receptor (EGFR), the receptor tyrosine phosphatase alpha and beta (RPTPa/RPTBb), and sphingomyelin in lipid rafts have been shown to act as interaction partners [87]. Apart from its capacity to induce vacuolisation by forming pores in endosomes, VacA can also induce apoptosis in epithelial cells and lymphocytes by interfering with membrane trafficking, leading to loss of mitochondrial membrane potential, mitochondrial instability and the subsequent release of cytochrome C [96]. VacA interacts with epithelial cells as well as with immune cells, including B- cells, T-cells and phagocytes. In phagocytes, VacA can inhibit processing and presentation of antigen peptides

to T-cells; however, VacA can also interfere with T-cell function directly by blocking antigen-dependent proliferation or by inhibiting the activation of nuclear factor of activated T-cells (NFAT) [69].

3.4.3. Other virulence factors

Aside from VacA and the Cag pathogenicity island, several other factors have been proven to play a role in the virulence and severity of *H. pylori* infection. High temperature requirement A (HtrA) is a serine protease that is well conserved in gram-negative bacteria and has been described to increase the viability of the organism under stressful conditions. H. pylori HtrA is secreted into the extracellular space in its active form where it can cleave E-cadherin, a tumour suppressor commonly lost in several cancer settings, that is involved in cell-cell adhesion [94]. The extent to which the cleavage of E- cadherin affects E-cadherin signalling and function is not yet known, but this cleavage has also been shown to allow for bacterial entry into the intercellular space by disrupting the adherence junctions [72]. Gamma-glutamyl transpeptidase (GGT) is a virulence factor that has gained increased attention in recent years. GGT is an enzyme that converts glutamine into glutamate and ammonia, and glutathione into glutamate and cysteinylglycine. This has been shown to lead to glutamine and glutathione consumption in the host cells, which interferes with the oxidative capacity of the cell, resulting in the production of ammonia and ROS. These products affect many central cell functions, inducing cell-cycle arrest, apoptosis, and necrosis in gastric epithelial cells. GGT can also induce immune tolerance by influencing dendritic cell differentiation and inhibition of T cell-mediated immunity, affecting the efficiency of the immune response towards *H. pylori* [97]. Duodenal ulcer promoting gene A (DupA) is another virulence factor that was identified based on its positive relationship to duodenal ulcer and inverse relationship to gastric cancer [98]. DupA has been suggested to form a Type IV secretion systems (T4SS) with *vir* genes that make up the *dupA* gene cluster. The presence of a complete *dupA* cluster was

found to significantly increase the risk of duodenal ulcer compared to *H. pylori* infection with an incomplete *dupA* cluster or without the *dupA* gene [99].

3.6. Diagnosis of *H. pylori* infection

A number of tests with varying accuracy and sensitivity have been developed to diagnose *H.pylori* infection. These tests could be separated into invasive and non-invasive approaches depending on whether an endoscopy is needed. The choice of the test depends on factors such as age, clinical situations, availability, cost and prevalence of infection in the population. The numerous tests used to diagnose *H. pylori* infection in adults may also be used in children but some non-invasive tests like urea breath test (UBT) and serology have limitations because at age below 6 years, the UBT can lead to nonspecific results, and serology may not be enough sensitive [100]. Non-invasive tests can identify *H. pylori* in a sample, but do not reveal the amount of tissue damage, on the contrary invasive approaches not only diagnoses the amount of damage in the stomach but also allows the clinician to directly sample the tissue to test it for the presence of *H. pylori* infection [101].

3.6.1. Invasive tests

Invasive procedures available for detection of *H. pylori* are endoscopy, histology, culture, rapid urease test (RUT) and polymerase chain reaction (PCR) [102]. The procedures are rather expensive and laborious and scarcely applicable to wide samplesets. Histopathology staining remains one of the best methods for detecting *H. pylori* infection and together with endoscopy, it provides important information on the pathological condition of the stomach. Endoscopy is expensive, unpleasant for the patients and requires highly specialized operator to be used as a routine diagnostic test. Bacterial culture is the gold standard for diagnosis of *H. pylori* infection, because at the same time it is possible to test the antibiotic susceptibility [101]. The clinical application of bacterial culture is limited because *H. pylori* is difficult to cultivate on culture media, costly, and compared to other methods time consuming, for this reason

the use of this procedure is limited to research and epidemiologic purpose [103]. Measuring urease production from biopsy specimen can be accomplished by rapid urease test (RUT). A small biopsy sample is placed in a vial containing the urease test solution and incubated overnight, the sample is observed for a change in a color, pink indicates positive result. This test has the advantages of being more rapid than histology or culture, and has a high sensitivity, but false negative urease tests may occur if patient has taken antibiotics or compounds containing bismuth [102]. Molecular methods like PCR have been used successfully to detect *H. pylori* DNA in gastric tissues by amplifying genes such us urease gene, adhesion genes and 16S rRNA gene. The 16S rRNA is one of the specific targets for PCR diagnosis of *H. pylori* infection [104]. Urease is another specific gene for detecting *H. pylori* infection and positive amplification can be considered as a direct evidence of the presence of the pathogen [105]. The disadvantages of PCR as a routine test are that it is a technically demanding and expensive test compared to culture and the rapid urease tests, and it requires special laboratory conditions. The technique is highly sensitive, but it is subject to false-positive results due to possible environmental contamination. A positive result obtained by PCR does not indicate active infection but could also detect the presence of DNA of dead bacteria.

3.6.2. Non-invasive tests

Non-invasive procedures available for detection of *H. pylori* are urea breath test, fecal antigen test and serological tests. The first two are considered active tests because can detect the presence of *H. pylori* and provide an evidence of an active infection, while serological tests are considered passive tests because provide the evidence of exposure to *H. pylori* and are usually not indicated in cases of eradication [106]. These tests are based on the detection of antibodies to *H. pylori*. The urea breath test is the preferred non invasive choice for *H. pylori* diagnosis before and after treatment. This test exploits the fact that *H. pylori* metabolizes urease (see above), so for this test patients

ingest a small amount of urea that is radioactively labeled (^{13}C), bacterial urease split off radio-labeled carbon dioxide, which is detect in the breath. In patients not infected by *H. pylori* radio-labeled carbon dioxide will not be detected in their breath and the urea ingested will be completely eliminated within urine. This test is considered the "gold standard" for in vivo detection of *H. pylori* infection and is also a good measure for the follow-up of the eradication therapy [107]. The disadvantage of this technique is that the detection of ^{13}C requires a mass spectrometer, which may not be easily available in clinics. Fecal antigen test is a relatively new diagnostic procedure that uses an enzyme linked immunosorbent assay to detect the presence of *H. pylori* stool antigen (HpSA) in the stool with the use of polyclonal anti-*H. pylori* antibody. This test has a high sensitivity and specificity but its disadvantage is the increasing of false-negative results for the stool antigen test during proton pump inhibitor (PPI) treatment [108]. Serological tests measure circulating IgG, IgM and IgA antibodies in a patient's serum and have sensitivity and specificity of 94% and compared to invasive techniques are extremely cheap. The heterogeneity of *H. pylori* strains has been well documented and is characterized by a considerable variation in the prevalence of specific strains, especially from different geographical areas [109], thus the success of a serology test depends on the use of antigens that are present in *H. pylori* strains from a given population. Serological tests have several advantages, namely they are non-invasive and they do not produce false negative results in patients receiving treatment (proton pump inhibitors and antibiotics) or presenting acute bleeding [110], for this reason several *H. pylori* immunogenic proteins have been presented as candidates to detect infection, such as the FlidD protein, multiple recombinant (CagA, VacA, GroEL, gGT, HcpC and UreA) proteins, CagA or Omp18 [111]. All current diagnostic procedures have its own advantages, disadvantages and limitations, between non-invasive methods and especially between serology tests there's the need to improve the diagnostic yield of *H. pylori* infection detection in specific clinical conditions [100]. At the same time, for wider applications, it would be

fundamental to identify novel biomarkers that could allow the diagnosis and/or prognosis of the progression of infection towards specific different pathological outcomes such ad Gastric Cancer, Autoimmune Gastritis and MALT lymphoma [19].

4. PHAGE-DISPLAY TECHNOLOGY

Biomarkers are indispensable tools for diagnostics and play roles in molecular medicine in the identification, prevention, and diagnosis being also very important for practical clinical disease management. Nowdays there is an increasing awareness of the need for new biomarkers that could contribute to diagnosis and prognosis, various protein detection techniques have been applied in biomarker discovery. SDS-PAGE and Western blotting are the most basic and traditional techniques, these techniques have been combined with high-throughput techniques, indeed different experimental methods, such as two-dimensional electrophoresis (2-DE), protein or peptide microarray, high-performance liquid chromatography (HPLC) and mass spectrometry (MS) have been employed for the discovery of immunogenic proteins [112]. However, the identification of the antigens that trigger specific systemic antibody responses is still difficult and limits the applications of these technologies. To serve this purpose phage display technology was proposed in 1985 by George P. Smith to identify polypeptides with specific binding activity and subsequently evolved with many versatile applications [113]. The phage-display technology was recently used to identify novel potential biomarkers from phage genomic libraries produced from different Mycoplasma species [114], Salmonella Typhimurium [115] and Neisseria gonorrhoeae [116]. However, the ability of phage display libraries to be enriched for epitopes of monoclonal antibodies has long been demonstrated [117]. Phages contain the genetic information of the proteins displayed on their capsids, thus genotype and phenotype are linked together, in this way this technology allows the direct determination of the genetic information (ORF/ORF fragment) from which the protein/antigen derives [118].

4.1 ORF PHAGE-DISPLAY

A link between the genotype and phenotype of the phage is provided by inserting DNA inside of the phage that encode for peptides which are displayed on the phage surface [105]. Each phage expresses on his capsid a unique peptide but the whole library includes a large number of different displayed peptides that afterwards will be used by affinity selection in order to identify specific ligands for different target [106]. The affinity selection technique, called biopanning, is the procedure of selecting specific binding partners from phage display libraries [107]. One interesting advantage of ORF Phage-Display is that this technique does not need prior knowledge of the identity and attributes of the target, furthermore once generated, libraries can reuse for an unlimited number of screenings [108]. This technology has applications in drug discovery [109], antibody engineering and epitope mapping [110], gene/drug delivery [111], enzyme technology, organ targeting [112], bioimaging and biosensing [113], study of interactions protein–DNA and protein–protein [114], antiviral research [115].

4.1.1 Phage display and ORF selection

Phage display technology application was expanded to include the display of antibodies and many other proteins [116]. As mentioned in the previous paragraph cDNA phage display has been successfully and widely applied both to identify antibody epitopes or binding partners [108,117] but the large number of non-functional clones present made the cDNA libraries inefficient and difficult to use. Although this high rate of non-functional clones may be tolerable when starting to work with DNA from a single gene or even a small genome but become unfunctional if employing more complex DNA sources.

To solve this problem the first strategy applied was to use λ-based vectors for cDNA display, despite this vector C-terminal intracellular vectors increase the likelihood that ORFs will be displayed, they do not provide any selective pressure for ORFs suggesting then the need for a further selective step to filter DNA fragments encoding ORFs [118]. Different attempts have been made to

filter DNA fragments, the first strategy was proposed in the 1992 by Seehaus [119], the selection was made with an antibiotic resistant gene to remove deletion mutants from antibody library with a plasmid in which antibody library was cloned upstream of a β–lactamase gene, thus only those antibody genes in frame were capable to allow ampicillin resistance by the creation of an antibody–lactamase fusion protein, whereas those that contained deletions or frameshifts were not conferring ampicillin resistance. Zacchi et al in 2003 [120] applied a similar strategy with a phagemid, they inserted the cDNA fragment followed by the gene of β–lactamase delimited by two homologous lox sites. To obtain efficient display of foreign polypeptides at N-terminus, the selection step with ampicillin , β–lactamase selection was removed by Cre recombinase-mediated recombination [120]. Faix et al [104] further improved this approach, they first selected fragments with a β–lactamase gene in a plasmid, after this fragments were extracted from ampicillin-resistant plasmids and re-cloned into a phagemid and afterwards rescued by hyperphage system. D'Angelo e al. [121] in order to demonstrate the feasibility of filtering method with antibiotic resistant gene have applied β–lactamase filtering vector at whole genome level in Clostridium thermocellum for domain-based functional annotation purposes. They demonstrated that domainome libraries are easy to generated by applying β–lactamase filtering vector to randomly fragmented bacterial gDNA libraries and that once a library is generated, it can be used for an unlimited number of screenings.

4.1.2 Comparison of Phage display with other techniques for interactome analysis

To elucidate protein physical association and elucidate protein functions different technologies have been widely used such as protein affinity purification coupled with 1D or 2D gel electrophoresis and mass spectrometry (AP-MS) or yeast two-hybrid (Y2H to identify protein-protein interactions [119]. Both AP-MS and Y2H have been applied for the recognition of binding peptides for specific protein bait as well as for domain-scale interactome

mapping. AP-MS has been well demonstrated for efficient mapping of interactome for yeast, *E.coli* [120] and human [121] .

AP-MS technology is more flexible than Y2H but has several limitations, it has a low sensitivity, consequently, less abundant proteins my not be detected (Table 2). Although Y2H and AP-MS have been widely used to identify protein-protein interactions, they are limited to high cost, technical complexity, instruments requirements, Li and Caberoy. [122] estimated the error rate that is more or less 50% for AP-MS (15% with tandem affinity purification), more or less 48% for Y2H while for ORF phage display is 29% (Table 2). One of the advantages of ORF phage display is that can significantly reduce the time required to identify a peptide with binding characteristics. Phage has a robust growth rate and another advantage is the versatility, Y2H is applicable only to protein-protein interactions studies. Several studies have shown that phage display is applicable to proteins, antibodies, peptides, multi-molecular complexes and affinity selection may be performed in vivo and in vitro [123]. One disadvantage of bacterium-based ORF phage display is that proteins displayed on phage surface lack appropriate post-translational modifications but this approach facilitates the identification of binding compounds with high affinity in a large scale with an efficiency and sensitivity comparable to Y2H and AP-MS [119]. Other limiting factors for these experiments are the availability of expression constructs, data analysis and the downstream validations.

	ORF Phage Display	Y2H	MS-based approach
Capacity of the technologies			
Protein-protein interaction	Yes	Yes	Yes
Protein-polysaccharide interaction	Yes	No	Yes
Protein-lipid interaction	Yes	No	Yes
Protein-antibody interaction	Yes	No	Yes
Protein-DNA interaction	Yes	Yes	Yes
Protein-RNA interaction	Yes	Yes	Yes
Protein-virus interaction	Yes	No	No
Protein-cell interaction	Yes	No	No
Protein-tissue interaction	Yes	No	No
In vivo selection	Yes	No	No

Table 2 - Comparison of different technologies for functional proteomics. Table reprinted [124] with modifications.

4.2 Phage Display evolution: INTERACTOME-SEQUENCING

Nowadays the advent of high-throughput sequencing (HTS) techniques is transforming multiple research fields as well as the analysis of the intrinsic heterogeneity of phage libraries [125]. The complex high throughput data provided by deep sequencing enables the analysis of highly complex samples such as phage display libraries and holds the potential to circumvent the traditional laborious picking and testing of individual phage rescued clones [126]. In the recent times there have been significant attempts in coupling HTS with phage display, in 2009 Dias-Neto et al. [127] proposed the use of NGS coupled with RT-PCR to improve phage analysis of the library inserts encoding phage-displayed variants.

Pyrosequencing was employed for deep-sequencing amplicons obtained from phage ssDNA libraries, recovered directly from four human tissue biopsies, using primers flanking the library gragment within the fusion by Di Niro et al (2010) [129]. Thus combining phage display and NGS they increased of two orders the number of affinity-selected clones. Authors screened an ORF-

filtered cDNA phage library, obtained from several human cell lines mRNA , to identify proteins in contact with tissue transglutaminase 2 (tTG2), an enzyme involved in different clinical outcomes. This approach has driven the characterization of a “landscape” of binding variants from the phage display library. Another relevant application of NGS coupled with phage display was shown by Gourlay et al. [128] , these authors generated a ORF-filtering library using the *Burkholderia pseudomallei* genome in order to develop a high-throughput tool to identify soluble protein domains from the entire protein repertoire of the bacterium. With this approach they demonstrated that the extension of the filtering power of β–lactamase based filtering selection strategy to identify outer membrane protein domains is very useful in the context of host-pathogen interaction studies. Between the 1279 ORFs represented in the library the focus fell on a potential antigen, BPSL2063, which was recognized by positive antibodies from patients that tested positive for *B. pseudomallei*. To identify the domains of this antigen a second single-gene domain filtering library was constructed and after a specific bioinformatic analysis two domains were revealed at N- and C- termini, respectively; afterwards they were produced in soluble form and successfully crystallized.

In conclusion the ORF-filtering library approach allows the characterization of the whole ORF-eome and Domain-ome of any intronless genome, favoring the selection of ORFs encoding for proteins that are potentially exposed on the cell surface or involved in transport mechanisms. It allows the identification of outer membrane localized protein domains supporting the identification of antigens.

The Domain-filtering library approach applied to single gene/proteins (very long more than 1000 aa and transmembrane) allows the selection of protein domain boundaries, and can accelerate and improve the steps leading to soluble protein production for crystallogenesis.

4.3 ORF-filtering library analysis

The output generated by ORF-filtering libraries sequencing can't be analyzed with the existing bioinformatics resources, as a matter of facts the kind of data do not resemble other sequencing data like ChIP-seq or RNA-Seq for which there are many free tools and softwares that are constantly released or updated. Phage display technology coupled to sequencing was introduced only in 2010 by Di Niro et al [129] and from a bioinformatic point of view the analysis of this kind of data was always performed by adapting tools designed for other purposes. In two recent works from D'Angelo et al. [130] and Gourlay and et al. [128] the analysis for the identification of specific domains/antigens was performed through NGS-TreX developed by Boria et al. [131]. NGS-TreX is a free available web-tool designed for the analysis of RNA-Seq data and it is not specific for the analysis of Interactome-Seq data. In literature, up to date, there aren't dedicated pipelines able to reveal enriched domains from dataset generated by Interactome-Seq technology. Therefore, at the beginning of my thesis work, there was the urgent need to develop a robust workflow that should be able to manage Interactome-Seq data, both with fixed or user-defined parameters, and to perform specific testing for the identification of enriched domains/antigens. At the same time the visualization and the sharing of the features identified was not possible so I decided not only to develop a new pipeline of analysis but to implement it into a web-tool easy to use, user friendly and available to the whole scientific community interested in analysing and consulting Interactome-Seq data.

5. AIMS OF THESIS

The aim of this thesis was the identification of *H. pylori* epitopes responsible for host immuno-response modulation through: a discovery-driven approach that couples “phage display” and deep sequencing (interactome-sequencing) and the development of a specific webtool for interactome-sequencing data analysis.

To this purpose my thesis work was focused on the:

- Identification of novel biomarkers to provide a “disease signature” by screening genomic DNA libraries, created from *H. pylori* genome, directly with sera from infected patients with different pathological outcome.

- Development of a new pipeline for interactome-sequencing data analysis.

- Implementation of a Webtool for functional analysis and visualization of interactome-sequencing data.

6. MATERIALS AND METHODS

6.1. Bacterial strains

Bacterial strains HP-26695 and HP-B128 used in this study were isolated at the Carregi Hospital and gently provided by Dott. Mario Milco D'Elios.

6.2. H. pylori *genomic ORF-filtering library construction and sequencing*

To identify all the potential soluble domains on a genomic scale we applied our strategy, based on the construction of an ORFeome library from two *H. pylori* genomes. The procedure initially involves the fragmentation of a whole genome into DNA fragments of 200-800 bp (D'Angelo et al., 2011, Heger & Holm, 2003) with the purpose to create a library of fragments coding for potential domains (or parts of). DNA fragments encoding well-folded protein domains, fused upstream of ß-lactamase, allow the reporter enzyme to fold correctly and allow bacteria to survive the selective pressure posed by the antibiotic. Genomic DNA from *H. Pylori* strains HP-26695 and HP-B128 was fragmented by ultra-sonication (Covaris) (Duty Cycle 10%; Intensity 5.0; Cycles per burst 200; Duration 2X60 seconds, total 120 seconds; Mode frequency sweeping; temperature 6°C) to obtain fragments in a length range between 200 and 800 bp (about 65–250 aa). Fragments were collected, blunt-ended using the Quick Blunting Kit (New England Biosciences) and cloned into the pFilter vector using EcoRV cloning site between a pelB leader sequence and the mature β-lactamase gene [132]. After ligation the *H. pylori* genomic DNA library was electroporated into DH5a F' cells and plated on chloramphenicol plates (34 mg/ml), supplemented with ampicillin (25 µg/mL). A small dilution of the library was also plated and grown in parallel on both chloramphenicol and chloramphenicol plus ampicillin resistance in order to calculate the size of the library and the efficiency of the filtering.

10^7 bacterial cells were plated and after overnight growth approximately 1% cells survived at the highest ampicillin concentration, yielding one library with an estimated size of $1x10^6$ clones.

Bacteria were harvested, mixed, resuspended in 20% sterile glycerol and stored in small aliquots at –80°C. One aliquot was used to extract plasmid DNA used for the preparation of the phage-library and for sequencing analysis of the inserts. To this aim the HP genomic DNA inserts were recovered from the pFILTER vector, by amplification with specific primers. The primers used to rescue the inserts were linked at their 5' end to Illumina adaptors sequences, thus allowing the direct sequencing of the HP genomic DNA inserts by using the MiSeq Illumina sequencer (Figure 8). The libraries were indexed, pooled together and sequenced on a MiSeq Illumina sequencer; 250nt paired end reads were generated

Forward primer:

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG*GCAGCAAGCGGCGCG CATGCC*;

Reverse-primer:

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA*GGGGATTGGTTTGCC GCTAGC*;

In bold are indicated the Illumina adaptors,in italic are indicated the specific primers.

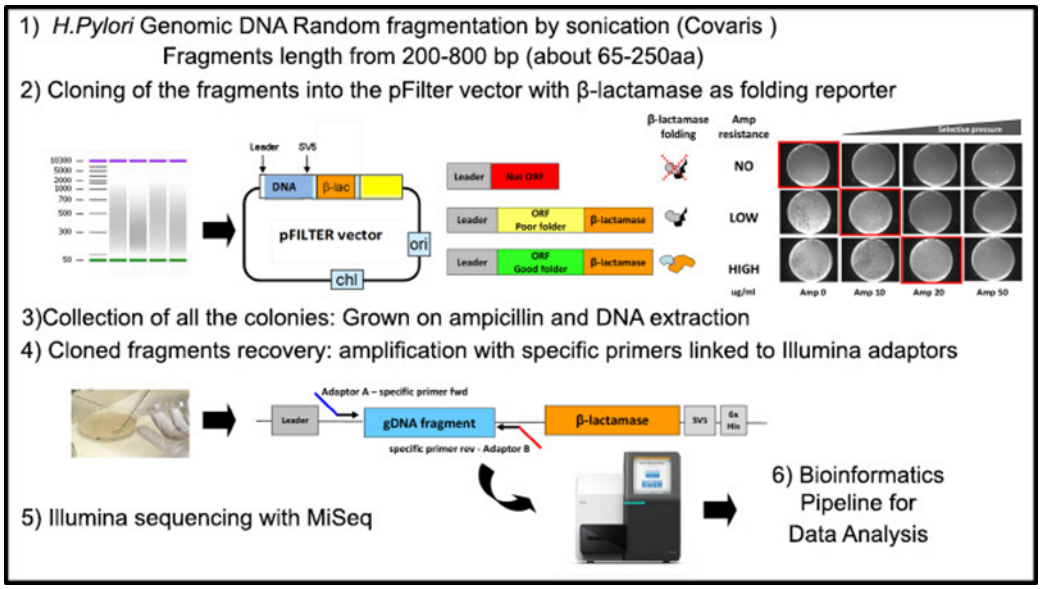


Figure 8- Schematic Overview of the main steps for the construction of the *H.pylori* genomic ORF-filtering library: 1) Random fragmentation of genomic DNA 2) gDNA fragment cloning into the pFILTER vector and filtering using β-lactamase as a folding reporter 3) Collection of all colonies and DNA extraction 4) gDNA fragment recovery by amplification using specific primers linked to adaptors for sequencing 5) deep sequencing 6) data analysis.

6.3. H. pylori Phage-library construction

In order to create a phage-display library, filtered DNA fragments were collect and cloned inside phagemid vector. Phagemid enables the expression of the foreign DNA, purposely introduced in the vector in such a way that it is expressed in conjunction with a phage protein, as a fusion protein, for display on the phage surface. This instrument is necessary to perform the selections with sera, it allows to couple genotype and phenotype in the same phage (Figure 9). For the *H. pylori* phage-library preparation the filtered HP ORF were recovered from the pFILTER3 vector and cloned in the pDAN5 [133] upstream to the cDNA codifying for the g3p protein. After ligation the library was electroporated in DH5aF' competent bacterial cells and grown for 18h at 37°C on 2XTY-Agar plates supplemented with 100 µg/ml of Ampicillin. Bacteria were harvested, mixed, resuspended in 20% sterile glycerol and stored in small aliquots at -80°C. One aliquot was used to extract plasmid DNA and subjected to sequencing analysis of the inserts, performed as described above.

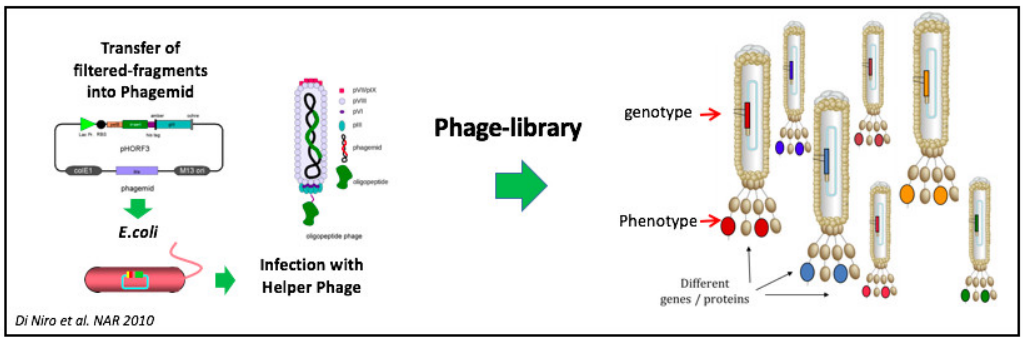


Figure 9 - Schematic Overview of the main steps for the construction of the phage library.

6.4. Sera selection

Once recloned into a phage display context, we directly used the ORF-filtering libraries to selected putative antigens by capturing the antibodies present in the sera from patients affected by *H. pylori* and presenting increasing degrees of infection: A) gastric adenocarcinoma (pool of 2 sera); B) autoimmune gastritis and pernicious anemia (pool of 3 sera); C) MALT lymphoma (pool of 2 sera); at the same time control sera from healthy patients both affected or not by *H. pylori* (D) were used to get a background control for successive normalization of the signals. The sera were grouped into different pool and each pool was independently used for three consecutive rounds of selection, in order to enrich the library for immunoreactive clones, with increasing washing and binding stringency Two cycles of selection and amplification were performed, and mini-libraries of selected phages were collected after each cycle (Table3). Test set antibodies were incubated with library phages, immune-complexes were recovered by protein A coated magnetics-beads and bound phages were eluted by standards procedures. Selected phages were used to infect *E. coli* and to produce mini-libraries from each cycle of selection. DNA inserts were recovered from each individual selected library, purified and sequenced by using the Illumina MiSeq sequencer (Figure 10)

Library description	Library name
H. pylori ORF-filtering library	Genomic
Healthy patients both affected or not by *H. pylori*	Control
Patients affected by gastric adenocarcinoma	SelectionA
Patients affected by autoimmune gastritis	SelectionB
Patients affected by MALT lymphoma	SelectionC

Table 3 - Library description and library name assigned**.**

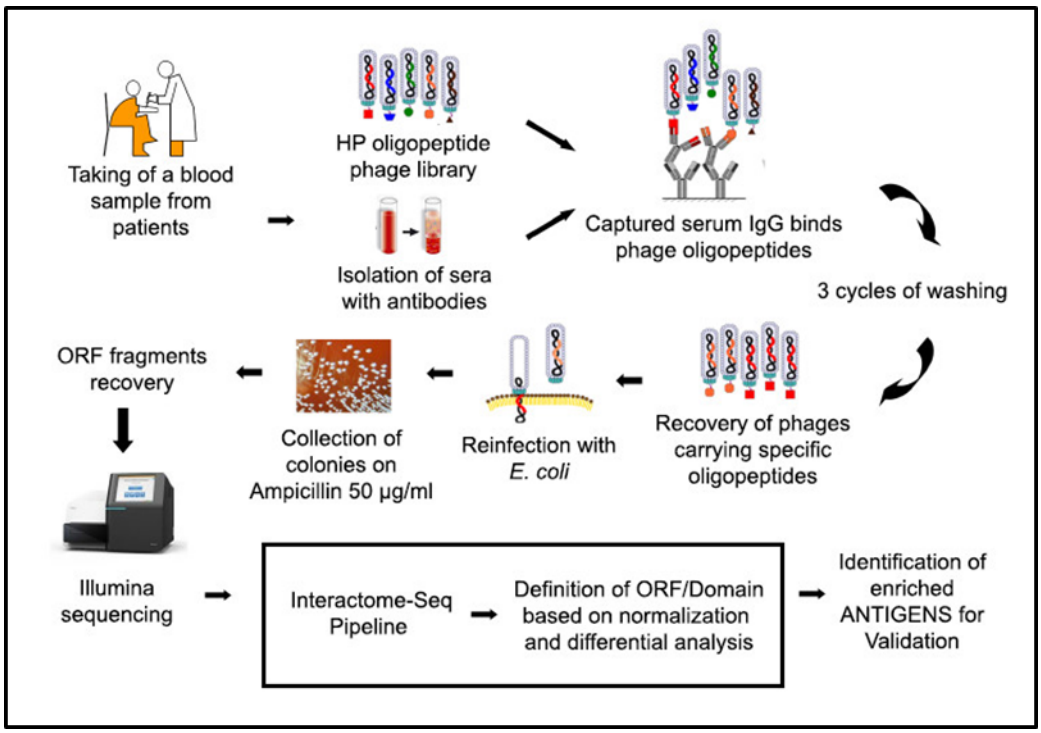


Figure 10 - Schematic overview of sera selection. Phage library were used for selection on patient serum antibodies immobilized on magnetic beads, three cycles of washes were followed by phage amplification on bacteria. *E.coli* cells plates supplemented with Amplicillin were deep-sequenced allowing the identification of enriched gene sequences. After data analysis, a list of putative antigens were produced and validated with an independent set of sera and assassed for their specificity.

6.6. Bioinformatics analysis and web-tools design

6.6.1. *De-Novo* Assembly of B128 strain

The assembly tool used in this study was Velvet 1.2.10 [134]. Large contigs (length >500nt) obtained were ordered and orientated with CONTIGuator version 2.7.4 [135]. Scaffolds were annotated with RAST [136].

6.6.2. Genomic comparison with other *H. pylori* strains

For comparative analysis, complete genome sequences of *H.pylori* were downloaded from NCBI database and compared using Gegenees tool (see Appendix 10.10) [137]. The tool utilizes a fragmented alignment algorithm to calculate average similarity among the compared genomes using BLASTn. The fragment size can be optimized according to the user. The tool was run with the fragment size set to 200 and a step size of 100 using BLASTn. The average similarity was calculated with a BLAST score threshold of 40% generating a heat plot matrix that was further used to deduce phylogenetic relationships exported in the form of a .nexus file. This nexus tree file was supplied as an input to SplitsTree [138] program for building an un-rooted phylogenetic tree employing Neighbor-Joining algorithm.

6.6.3. Implementation of Interactome-Seq pipeline and web-tool.

I developed a data analysis workflow called Interactome-Seq that is a combination of custom shell, AWK and python scripts. As part of the analysis several tools have been included in the pipeline, namely 1) FastQC (v. 0.11.4) for quality check and processing of fastq sequences, 2) Cutadapt (v. 1.10) [139] for trimming primers and discarding reads with low Phred quality score, 3) Bowtie2 (v. 2.2.9)[140] for alignment step, 4) Samtools (v. 0.1.19-44428cd) [141] for converting alignment in different formats, 5) Bedtools (v. v2.24.0) [142] for working with genomic intervals, 6) EdgeR (v. 3.14.0) [143] for differential signal analysis. The Interactome-Seq pipeline can be accessed

through an interactive web-based graphical user interface (http://cerbero.ba.itb.cnr.it/cricket/ITBHelicobacter).

Analysis steps are implemented and distributed inside server environment through a dispatching application and the whole execution can be launched and monitored using a common up-to-date web browser. Interactome-Seq is powered on a Ubuntu 14.04LTS Linux operating system. The Web Framework application used for development was Google AngularJS, combined with HTML5 and CSS3 standards, to enable a better user interaction The server was an Intel® Xeon® Processor, 64 GB of RAM, 9 TB (SATA) of hard disk.

6.7. ELISA validation

Helicobacter pylori ORFs obtained from the second round of selection, and resulting specific for progression towards pathological outcomes, were excised from the phagemid vector and cloned into a compatible pGEX-FLAG (D'angelo et al Clin Immunol, 2013) vector, creating for each output of selection a library for the expression of the selected ORFs as GST-fusion products. Plasmid DNA was isolated from an aliquot of these libraries and used as a template for an inverse PCR-based cloning strategy performed with two back-to-back outward specific primers designed centered on the epitope region identified by the overlapping reads. Colonies grown on Ampicillin plates were sequenced. Positive colonies were grown at 37°C until OD600nm=0,5 and induced by IPTG with a final concentration of 0,2mM for 16h at 28°C. Recombinant protein was purified with GST affinity resin (Sigma Aldrich) using standard procedures. An in-house ELISA assay was used to validate the antigenicity of the selected ORF. Briefly recombinant GST-protein was diluted in PBS to 2µg/ml and 100µl were coated in ELISA wells (Greiner), overnight at 4°C. Wells were washed with PBS and blocked with 200µl of blocking solution (PBS- 0.05% Tween20-2% milk) for 1h at 37°C. Wells were again washed for three times with PBS-0,05% Tween20 and incubated with sera samples diluted 1:500 in blocking solution, for 2h at 37°C. Extensive washes were performed with PBS-0,05% Tween20 and PBS. Secondary antibody was a goat anti-human-IgG HRP

conjugated (Sigma Aldrich) diluted 1:5000 in blocking solution, for 1h at 37°C. After extensive washing, immune-complexes were revealed with TMB and the plate read at 450nm. Samples with absorbance ≥ of the mean OD450 value obtained with control sera plus 2 standard deviations (SD) were considered positive.

7. RESULTS AND DISCUSSION

My thesis work is divided in four main parts:
1) construction and comparative analysis of two *H. pylori* genome ORF-filtering libraries; 2) bioinformatics analysis of sequencing data: development of a new data analysis pipeline and implementation of a web-tool, 3) phage-library production and analysis of the enriched domains after sera selection; 4) validation of the results.
I participated to all the parts of the work, but my personal involvement was mainly focused in the bioinformatics analysis of the sequencing data generated, on the pipeline development and on the web-tool implementation.

7.1 HP-26695 and HP-B128 ORF-filtering libraries comparison

H. pylori is known for its remarkably high level of genetic diversity creating a dynamic pool of genetic variants, this pool of genetic variants delivers a sufficient genetic diversity to allow *H. pylori* to occupy all the potential niches in the stomach (for example, antrum and corpus mucosa) [144].
Since one of the aims of my thesis work was to: identify novel biomarkers, to provide a "disease signature", by screening genomic DNA libraries created from *H. pylori* genome, directly with sera from infected patients with different pathological outcomes, first of all I wanted to be sure that the strategy here adopted and the instrument here proposed (i.e phage-library coupled with interactome-sequencing) could be considered universally valuable.
For this reason two ORF-filtering libraries were produced: one from the genome of the HP-26695 strain and another from the genome of the HP-B128 strain. Strain HP-26695 originates from patients suffering from a chronic gastritis and is considered the reference HP strain, having a complete and very well annotated genome sequence, strain HP-B128 originates from patients with gastric ulcer, its genome sequence is not complete but consists of 73 supercontigs. The strain HP-B128 is one of the strain most frequently found in infected patients and it is linked to infection progression towards gastric ulcer

and/or more serious pathologies such as gastric cancer, autoimmune gastritis and MALT lymphoma.

A comparative analysis of the genomic sequences of these two strains, and of the ORF portions that we found represented in their ORF filtering libraries, herein produced, was performed in order to understand if the *H. pylori* genetic diversity between these two strains could in some way introduce a bias into their ORF filtering libraries content and prevent their use as universal instrument for new specific diagnostic and prognostic markers discovery.

7.2. Assembly and phylogenetic analysis of HP-B128

Since the genome sequence of HP-B128 strain was not complete [145], before starting with the production of its ORF-filtering library, HP-B128 genome was re-sequenced in our laboratories. The reads obtained were then assembled and a comparative sequence analysis between HP-B128 strain new genome sequence and all the complete genome sequences of *H. pylori* strains available in public databases was performed.

In this way we estimated the level of genetic diversity that separates the HP-B128 strain from the HP-26695 reference strain to understand if the genome annotation of the reference strain could be used for the successive steps of functional analysis of the two ORF filtering libraries generated, and extended to the functional definition of the domains/antigens found enriched after sera selection.

7.2.1. HP-B128 genome assembly

The whole genome sequencing of HP-B128 isolate revealed that the chromosome size is around 1.66 Mb similar to others *H. pylori* strains. The genome also revealed a low G+C content of 38.76% which is another important characteristic of *H. pylori*. Detailed assembly metrics of the genome sequencing are reported in Table 4: we obtained a total of 61 contigs (>500nt) by assembling 180.967 reads produced with 454 GS-FLX+ platform (Roche). Afterwards 44 contigs were merged in a single FASTA file by the scaffolding

procedure, taking the HP-26695 genome as template, and a single scaffold of 1.664.509 bp length was obtained (see Appendix 10.1). The remaining 17 contigs were duplicates of parts of the 44 contigs assembled into the big scaffold, only repeated genomic regions were excluded from this scaffolding step. Thus we strongly improved the genome sequence of the HP-B128 strain previously available, that was composed of 73 contigs, and obtained a draft genome of this strain.

Sequencing Assembly of HP-B128	
Raw reads	180.967 SE
Total contigs	61
# contigs (>= 1000 bp)	34
# contigs (>= 5000 bp)	23
# contigs (>= 10000 bp)	21
# contigs (>= 25000 bp)	17
# contigs (>= 50000 bp)	9
Total contigs assembled	44
Largest contig	376433
Total length	1664509
GC (%)	38.76
N50	128427

Table 4 - Assembly metrics of HP-B8 strain. In this table are reported the number of raw reads (Raw reads), number of total contig (Total contigs), number of contig bigger that 1000,5000,10000,25000,50000 nt, total number of contigs assembled(Total contigs assembled), the most large contig assembled(Largest contig), total genome assembled (Total length), median of contig lengths (N50).

7.2.2. Analysis of HP-B128 genome sequence similarity

The HP-B128 draft genome obtained was automatically annotated by using RAST, 1711 CDSs were annotated, but their functional annotation was quite poor, about 25% of the CDSs were described as “hypothetical protein”. Thus to improve HP-B128 annotation I decided to compare its draft genome sequence to the genomic sequences of 52 strains available in the NCBI database. A similarity phylogenetic tree was constructed using Gegenees and Splitree software. The results of this phylogenetic comparison are shown in Figure 11 and the formation of distinct cluster (Green square), based on sequence similarity, between HP-B128 and HP-B8 strain can be clearly appreciated. HP-B128 draft genome showed a level of similarity of 96% with HP-B8 (NC_014256.1). The strain HP-B8 evolved from the human strain HP-B128 and was adapted to infecting gerbils, used as animal models for HP infection and gastric cancer development. This close phylogenetic association among HP-B128 and HP-B8 strain is in accordance with the comparative genome analysis of Farnbacher et al [145], in this work the authors found that about 98% of the whole genome sequence of strain B8, that they obtained, was covered by B128-supercontigs with a percentage of sequence similarity greater than 90%. They also performed a comparative analysis between the two strains revealing that more than 86% HP-26695 CDSs completely matched with the HP-B8 CDSs. At the same time it is possible to observe, from the phylogenetic tree, that even between the genome sequence of the strain HP-26695 and of the HP-B8 and HP-B128 respectively there is a very high level of similarity (80%).So after having verified the high level of similarity between our draft genome sequence of strain HP-B128 and the already published complete genome sequence of strain HP-B8 we associated the well curated genome annotation of this second strain to our draft genome. In this way we greatly improved HP-B128 CDSs functional annotation and made it comparable with the annotation of the whole HP-26695 CDSs set.

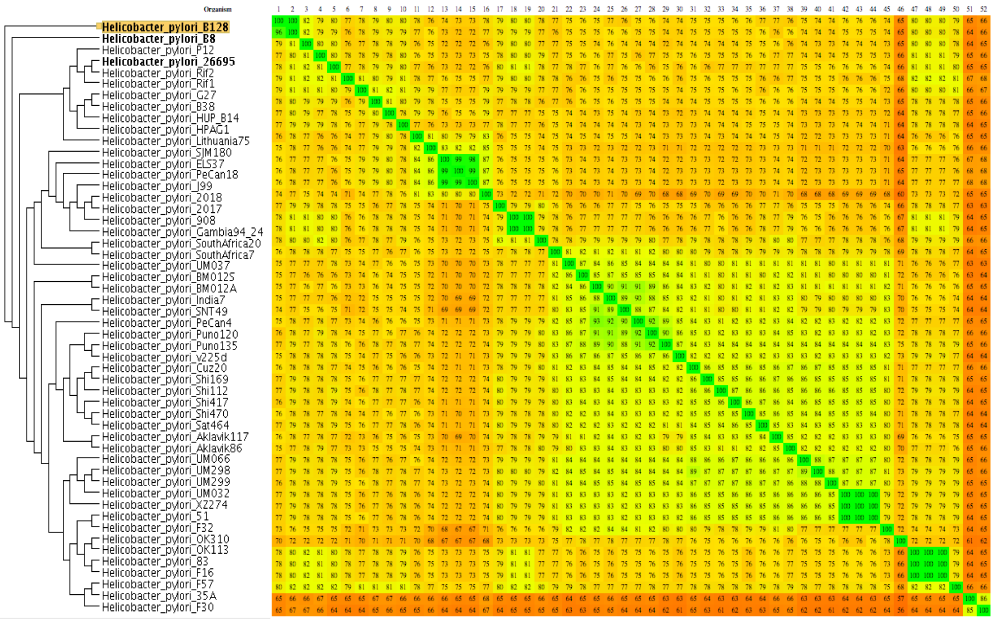


Figure 11- Gegenees heat-plot over a set of all complete *H. pylori* genomes**.** A fragmented alignment in BLASTn mode was performed with settings 200/200. The cutoff threshold for non-conserved material was 20%. A dendrogram was produced in SplitsTree 4 (using neighbor joining method) made from a distance matrix Nexus file exported from Gegenees.

7.2.3. Crossmapping comparison of HP-26695 and HP-B128 ORF-filtering libraries

As one of the main advantages of producing ORF-filtering libraries from intron-less genomes (i.e bacterial genomes) is the possibility to characterize their whole ORF-eome and/or Domain-ome, it is important first to evaluate if the ORFs/Domains filtered out by this technique can show relevant differences when ORF-filtering libraries are produced from genomes of different strains having high level of similarity, such in the case of HP-B128 and HP-26695 strains. This verification step is fundamental when the genomic ORF-filtering library, produced starting from the bacterial genome, should be used to perform selections/enrichments with sera from patients. Indeed in this case it is important to obtain a genomic ORF-filtering library that could be considered a universal instrument able to work equally with different sera regardless of the HP strain infecting patients.

To this purpose I performed a crossmapping comparison by aligning the reads obtained from the sequencing of both the genomic ORF-filtering libraries of HP-26695 and of HP-B128 against their respective genomic sequences of HP-29965 (NC_000915) and HP-B8 (NC_014256) and viceversa. Table 5 reports the metrics of all these crossmapping steps, both raw reads datasets were first trimmed then mapped with Bowtie2. In panel A, the 91,61% of the trimmed reads of HP-26695 ORF-filtering library aligned with its own reference genome while the 85,75% aligned to HP-B128 (HP-B8) sequence. In panel B the 84,7 %.of trimmed reads of HP-B128 ORF-filtering library aligned with the HP-B8 genome while again the 85,75% align with the HP-26695 genome. Considering that the cross-mapping metrics observed in particular for the HP-B128 ORF filtering library do not differ much when the reads are aligned against the HP-B8 and the HP-26695 genome sequences respectively, we can conclude that the genetic diversity that separates these two strains does not significantly affect the nature of ORFs/Domains sequences filtered out. So in the light of these results we decided to use as reference the HP-26695 genome and to use its CDSs annotation to functionally read out the results obtained after selection with sera from patients.

A) Reads mapping vs HP-26695 genome sequence		
sequencing metrics	**HP-26695 reads**	**HP-B128 reads**
Raw Reads	1.425.554	1.425.554
Reads after trimming	1.216.124	1.216.124
Mapping Reads	1.114.184	1.042.943
% of Mapping reads	91,61%	85,75%
Unmapping Reads	11.158	108.581
Multiple mapping reads	90.782	64.600
B) Reads mapping vs HP-B128(B8) genome sequence		
sequencing metrics	**HP-26695 reads**	**HP-B128 reads**
Raw Reads	1.031.956	1.031.956
Reads after trimming	954.895	954.895
Mapping Reads	746.325	802.021
% of Mapping reads	78,10%	84,70%
Unmapping Reads	158.301	92.896
Multiple mapping reads	50.269	59.978

Table 5 - Geonomic ORF filtering library crossmapping results for HP-26695 and HP-B128 strains. In the panel A are reported the mapping metrics of HP-26695 ORF-filtering library vs HP-26695 and HP-B8 strains. In panel B are reported the mapping metrics of HP-B128 ORF-filtering library vs HP-26695 and HP-B8 strains. Metrics are composed be number of Raw Reads (Raw Reads), number of Reads after trimming (Reads after trimming), number of Mapping reads (Mapping reads) and percentage (% Mapping reads), number of unmapped reads (Unmapping Reads) and number of reads that map in more than one point of genome (Multiple mapping reads).

7.3. Development of a New Bioinformatic Data Analysis Pipeline

7.3.1 The Idea behind

As previously explained in the paragraph 4.3, the output generated by ORF-filtering libraries sequencing can't be analysed with the existing bioinformatics resources, indeed this kind of data do not completely resemble other sequencing data like ChIP-Seq or RNA-Seq. Up to date, there aren't dedicated pipelines able to reveal enriched domains from dataset generated by Interactome-Seq technology. So, in order to fill this gap and to correctly analyze the Interactome sequencing data herein produced, I designed a new dedicated data analysis pipeline.

Even if Interactome-seq data stand apart from ChIP-seq and RNA-seq data, they have some common features, with these two kind of data, that should be taken in consideration.

1. One feature in common with ChIPseq analysis is essentially the step of detection/calling of enriched domains. For ChIP-Seq experiments the input DNA is mandatory to model the background noise of the experiment before the Peaks Finding step, even in Interactome-Seq experiments, background is necessary both for Genomic ORF-eome definition and for building the statistical model required to identify the enriched putative domains.
2. The features in common with RNAseq are two. The first one is the necessity of normalizing data between samples. The normalization can be done with an approach similar to that used for RNA-Seq experiments, indeed my pipeline counts the reads per kilobase per million mapped (RPKM), this normalization step, usually adopted to normalize RNA-Seq data, consists of multiplying the raw counts for each gene in each sample by a factor incorporating both sequencing depth and putative domains lenght and facilitates a trasparent comparison for putative domains within and between the selections. The second feature in common with RNA-Seq experiments is the statistical model used to identify the enriched/differential domains. Like in RNA-Seq the data count is fitted as a binomial negative (NB) distribution and the false discovery rate (FDR) of p-values obtained are controlled with the Benjamini-Hochberg procedure [147].

The pipeline is divided into 4 main steps of analysis (Figure 12): in the first step, all alignment files are scanned for the detection of putative domains (Figure 12 Step 1). Starting from the genome origin of replication (OriC base N.1) the coverage depth for each genome position is calculated, then start and end coordinates of each domains are defined. The starting coordinate of a putative domain is fixed on the first genome position having a coverage depth equal or greater than 1, while the ending coordinate of the same putative

domain is the next postion having a coverage depth equal to 0. The second step of the analysis is: the annotation inside CDSs of the putative domains previously determined (more than one domain can be associated to one CDS) and the determination of domains enrichment in the selections respect to their representation in the genomic phage library, which is used as reference/background for the step of “differential analysis” (Figure 12 Step 2). In the third and fourth steps of the analysis the lists of domains, significantly differentially enriched in the selections respect to the reference genomic phage library, are compared, and a subtractive step allows the identification of those domains/antigens specific of selections with the sera of sick patients (Figure 12 Step 3). Thus, all the antigens equally recognized by antibodies of healthy patients and of patients affected by Gastric Cancer, Autoimmune gastritis, and MALT lymphoma are removed, and lists of antigens specific infection progression are obtained (Figure 12 Step 4). These lists of specific antigens are given in output as ranking lists associated with statistical values (FDR) thus allowing a guided selection of the best targets for validation (i.e. top lists antigens).

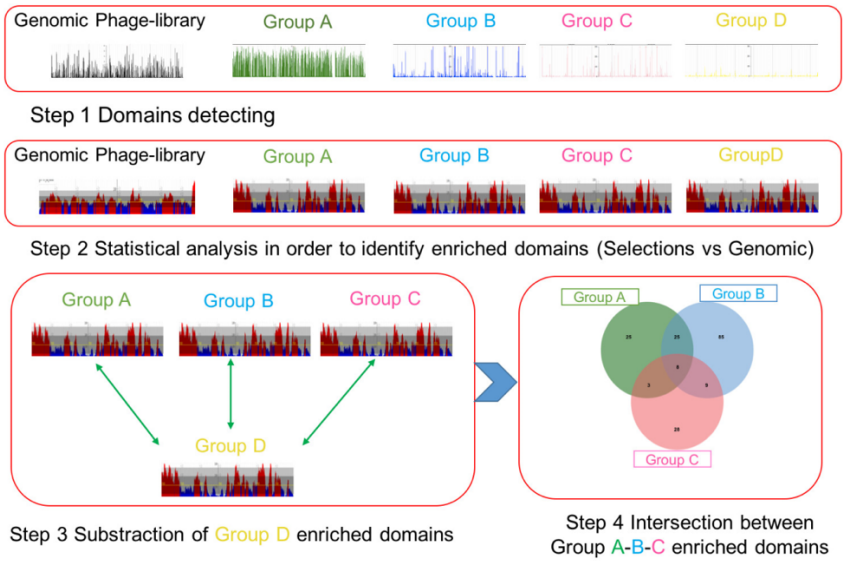


Figure 12 - Workflow of Interactome-Sequencing analysis. Analysis workflow is composed by four steps, Step 1 and 2 allow the detection of putative domains and the identification of only those that are enriched compared to the Genomic Phage library used as background. Steps 3 and 4 allow the identification of the enriched putative domains specific for each Selection group by consecutive subtraction of all the domains common to the Group D selection (healty patients) and intersection of the specific antigens lists obtai

7.4. Overview of Interactome-Seq webtool

7.4.1 Input description

Concerning input data type, Interactome-Seq pipeline can handle Roche-454 reads in FASTA format, Illumina Fastq or alignment files (BAM), single end and paired-end reads are supported. The pipeline is flexible, more than one selection can be analysed, but Genomic dataset input is mandatory, and it can be also analysed alone, if the focus of research is the identification of all potential soluble domains on a genomic scale (Figure 13).

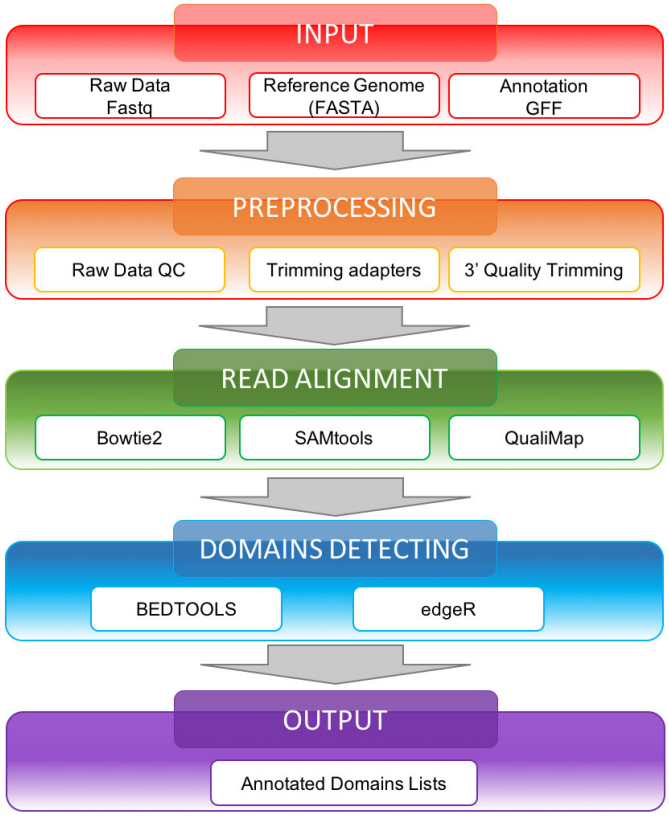


Figure 13 - Schematic steps of Interactome-Sequencing pipeline.

7.4.2. Pre-processing step

As shown in Figure 13 during the pre-processing step raw reads are analysed and quality checked using FastQC and Cutadapt tools. Reads with no identifiable adapters are discarded. During the pre-processing the dataset undergoes also quality trimming, a sliding window trimming is performed, this means that when the average quality within the window falls below a predetermined threshold (Q<30), the remaining part of the read is discarded.

7.4.3. Read alignment step

After the trimming reads are aligned with Bowtie2 to the genome sequence.
I used this mapping tool because it is optimized for working with the read length and error modes yielded by recent sequencers (Illumina, Roche 454, and Ion Torrent platforms). Users can decide the maximum number of mismatches allowed; the default mismatch option is set at 2% of reads length, only uniquely mapping reads are used for downstream analysis. A SAM file is generated and only reads with quality score greater than 30 (Q>30) are processed using SAMtools, and converted into a BAM file.

7.4.4. Putative domains Detection

The Interactome-Seq pipeline after the alignment step invokes bedtools to filter reads that overlap at least 80% inside CDSs, for each portion of CDSs covered by mapping reads, the coverage is calculated, and also max depth and focus values are calculated in according with NGS-Trex tool definition [131].
The coverage is the total number of sequences assigned to a gene; the depth is the maximum number of reads covering a specific genic position; the focus is an index obtained from the ratio between max depth and coverage, and its range is between 0 and 1.
Then, if the focus is higher than 0.8 and the coverage is higher than the average coverage observed for all mapping regions in the BAM file, the CDS portion is considered a putative domain/epitope.

When selections against sera or other interactors are available, domains lists are then statistically tested one at a time against the genomic phage library alignment file. Differential enrichment is calculated by using the R-package edgeR, in order to detect only domains that are enriched after the selections. The differential analysis is always performed testing the BAM files of the selections phage libraries against the BAM file of the genomic phage library that can be considered as the analysis background. .

7.4.5. Output parsing

The last step of the Interactome-Seq pipeline is the output parsing: the list of putative domains, resulted enriched in selection phage libraries respect to the genomic one, are annotated and a simple tabular file (.tab; .csv; .xls) containing the following fields is generated: Chromosome, Domain Start, Domain End, Gene Name, Focus and Annotation.

7.5. Overview of Interactome-Seq webtool

I decided to included Interactome-Seq pipeline in a web-tool to enable users without any bioinformatics or programming skills to perform Interactome-Seq analysis directly loading their reads through the graphical interface and to obtain their results in an easy and user friendly format.

7.5.2. Webtool design

Interactome-Seq webtool is available online at http://cerbero.ba.itb.cnr.it/cricket/ITBHelicobacter.

This webtool requires the following inputs:

1. **Genome sequence**: The Bowtie2 index of the genome of interest can be selected from a database of pre-loaded genomes. The default genome is set to Hp-26695 for the example.
2. **Raw reads file(s):** Users can choose to upload raw reads formatted files in two different ways: (i) a single file in '.fastq'

(Illumina platform) or '.fasta' (Roche 454 platform) format, (ii) alignment files ('.bam' or '.sam'). Alternatively, the user can also select an example file to run the analysis.

3. **Annotation.** User can upload custom annotation or use standard annotation by interrogating the internal database.
4. **Setting Parameters.** Number of Mismatches and sequences of Trimming adaptors should be set before launching the analysis.

There are two main components of the web-tool: the first one is the execution of Genomic analysis and the second is the performance of Case Sample Files. Genomic analysis is mandatory, a user can decide to perform only this step of the analysis if his/her objective is the definition of the ORF-eome of a intron-less genome. The analysis on Case Sample files can be performed only if Genomic analysis has been executed, and users can include one or more Case Samples in the analysis. The web-tool provides a progress bar that is located at the bottom of the web page and shows the processing step of a job in real time (Figure 14).

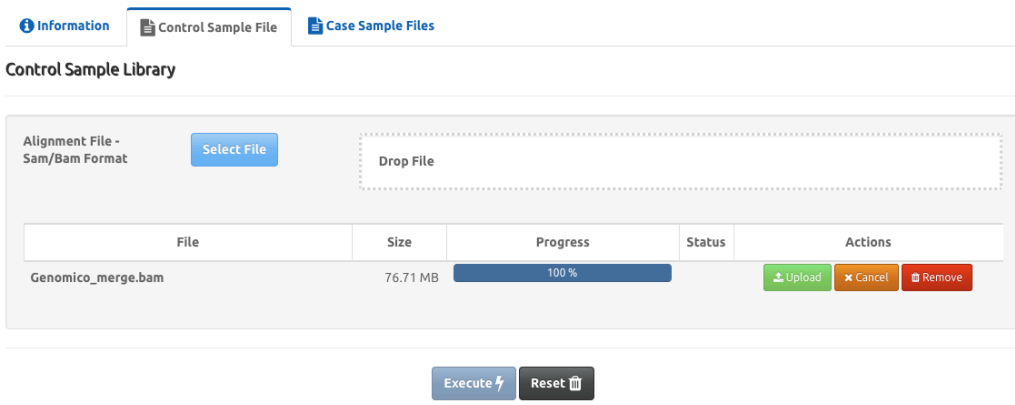


Figure 14 - Screenshot of Interactome-Sequencing webtool input. Input files allowed are FastQ single or paired end, Fasta or alignment file in format BAM. Others input files mandatory for the execution are the genome sequence and annotation file.

7.5.3. Output visualization and description

As shown in Figure 15 the output result of an analysis, performed with the Interactome-Seq web-tool, is a tabular file in which all the putative domains detected and/or found statistically enriched, respect to the Genomic Sample, are reported. The output file is organized in eight columns containing the following information and values (Figure 15):

- Chromosome
- Start of putative domain
- End of putative domain
- Strand
- Gene (ID or name of the gene associated with domain)
- Description (Gene Annotation)
- Q-Value
- Fold Change

These two last values can be used to order domains and/or filter out only statistically significantly enriched domains (for example: Q-Value<0.05 and Fold Change>2 or <0.5) The web-tool allows to sort lists for each of the fields previously described and to download files in tab separated format.

The Interactome-Seq web-tool that I implemented will be useful for scientists without any programming skills, and will provide a fast method to quickly analyse Interactome-seq datasets.

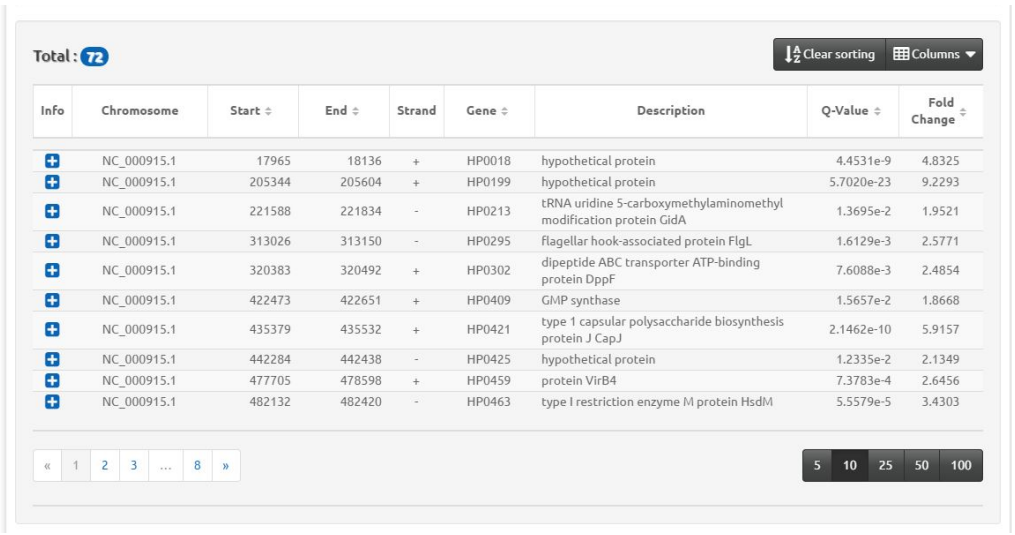
Previously, as I mentioned in the Introduction part of this Thesis, the tool used up to date, for interactome sequencing data analysis, was NGS-Trex [131], but this web-tool was originally created for analysing RNAseq data thus resulting quite inconsistent for the analysis of phage libraries sequencing data, being also no longer suitable to work with Illumina reads..

Recently a paper, in which an approach similar to our Interactome sequencing analysis was applied, has been published [145]. In this paper phage diplay was coupled with NGS and data analysis was performed. A statistical approach, similar to the one that we here describe, based on a two sided

parametric t-test, was applied. Sequencing data deriving from an experiment in which a phage library that displays random 7-mer peptides was challenged against suspensions of *M. tuberculosis,* were analysed to identify peptides that bind mycobacteria.

I used the statistical approach described in this recent paper as a scaffold to implement my pipeline and to integrate all the consecutive data analysis steps previously described.

The result is a new pipeline statistically consistent and highly flexible that, thanks to its integration into the web-tool will be available to a wide plethora of users.


Total : 72

Clear sorting | Columns

Info	Chromosome	Start	End	Strand	Gene	Description	Q-Value	Fold Change
+	NC_000915.1	17965	18136	+	HP0018	hypothetical protein	4.4531e-9	4.8325
+	NC_000915.1	205344	205604	+	HP0199	hypothetical protein	5.7020e-23	9.2293
+	NC_000915.1	221588	221834	-	HP0213	tRNA uridine 5-carboxymethylaminomethyl modification protein GidA	1.3695e-2	1.9521
+	NC_000915.1	313026	313150	-	HP0295	flagellar hook-associated protein FlgL	1.6129e-3	2.5771
+	NC_000915.1	320383	320492	+	HP0302	dipeptide ABC transporter ATP-binding protein DppF	7.6088e-3	2.4854
+	NC_000915.1	422473	422651	+	HP0409	GMP synthase	1.5657e-2	1.8668
+	NC_000915.1	435379	435532	+	HP0421	type 1 capsular polysaccharide biosynthesis protein J CapJ	2.1462e-10	5.9157
+	NC_000915.1	442284	442438	-	HP0425	hypothetical protein	1.2335e-2	2.1349
+	NC_000915.1	477705	478598	+	HP0459	protein VirB4	7.3783e-4	2.6456
+	NC_000915.1	482132	482420	-	HP0463	type I restriction enzyme M protein HsdM	5.5579e-5	3.4303

« 1 2 3 ... 8 »

5 10 25 50 100

Figure 15 - Screenshot of Interactome-Sequencing webtool output. The figure shows an example of output generated, for each putative domains the information reported is Chromosome, that is the name of chromosome used for alignment of the reads,Start,End,Strand,Gene Name that are fields with information about coordinates of putative domains and CDS associated, Q-Value and Fold Change are fields derived from statistical testing. Output can be downloaded or sorted using the arrows near the colums name.

7.6. Novel biomarker identification

The web-tool previously described was used to analyse data deriving both from genomic phage libraries and from selections phage libraries sequencing.

7.6.1. Analysis of the two genomic phage libraries

The two genomic phage libraries constructed, starting from the two strains HP-26695 and HP-B128, were analysed with my new Interactome–seq pipeline in order to define the Domains filtered out by our filtering strategy and to compare them between the two strains.

In Table 6 the sequencing metrics for all the phage libraries obtained are reported. More than 1.4 million and more than 1 million reads were produced for HP-26695 and for HP-B128 phage libraries respectively, thus reaching a total genome coverage of more than 76X and 77X for HP-26695 and HP-B128(B8). When analysing the coverage inside CDSs we found that more than 93% and 89% of the total CDSs of HP-26695 and of HP-B128 where represented in their respective genomic phage libraries, and the percentages of nucleotide covered by reads inside CDSs were respectively 73.5% and 76.8%. The first step of the Interactome-seq pipeline (i.e. Putative Domain Detection) identified a total of 1761 Domains represented into the HP-26695 phage library and 1237 Domains in the HP-B128 phage library (Table 7) (see APPENDIX 10.3); it should be noted that the number of domains detected can be higher than the number of total CDSs of the genome because more than one domain can be found inside a single CDS.

To understand if the domains, found in common CDSs, represented inside the two phage libraries have the same features, in terms the portions of the CDS filtered out, I compared their aminoacidic sequences by blasting all the HP-26695 domains against all the HP-B128 domains using BLASTx (search protein databases using a translated nucleotide query).The blasting step was performed by imposing the following parameters: aminoacidic sequence identity>30% and length overlapping>50%. Among the 1268 common domains 98,82% have an aminoacid sequence identity more than 30% and 60% of

them have an overlap of the aminoacid sequence lenght higher than 50%. Thus after this preliminary comparative evaluation we can conclude that the two instruments constructed (i.e. HP-26695 and HP-B128 phage libraries) can be considered equally representative of *H. pylori* domainome and can be used both separated or mixed together for the selection steps with sera from patients.

7.6.2. Analysis of the three selections phage libraries

The selections against sera from patients who developed: Gastric Cancer (selection A), Autoimmune Gastritis (selection B) and MALT lymphoma (Seleciton C) (Table3), were performed by using both genomic phage libraries previously obtained and described. The two genomic phage libraries of HP-26695 and of HP-B128 were mixed up in equimolar concentration and then the pool of the two libraries was challenged against pools of sera from patients with the three different pathological outcomes. In the three selection steps we pooled together respectively 2 gastric cancer (sel A), 3 autoimmune gastritis (sel B), 2 MALT lymphoma (sel C) sera from patients HP positive, and 6 sera from healthy patients both HP positive or negative.

The pooling of the two phage libraries was aimed at exploiting at maximum level their potentiality in terms of HP domains repertoire and it was validly justified by the previously described comparative analysis that demonstrated their complete functional similarity. The pooling of sera was necessary to reduce the inter-individual variability of antibody titer. In Table 6 the sequencing metrics for all the three selection phage libraries are reported.

A total of more than 9 million paired end reads of 250 bp in length, were produced by sequencing the four selection phage libraries. For each selection library we generated 2.169.178 reads for Control, 815.891 reads for Selection A, 3.737.010 reads for Selection B and 1.633.626 reads for Selection C. After the alignment step, we mapped respectively 1.294.576 (71,97%), 503.401 (73,94%), 2.563.391 (81,37%), 961.686 (75,22%) reads for the Control and for the three Selections A, B and C, and we observed that the total number of

CDSs covered at least by 10 reads were 1225, 1130, 1331, 1263 respectively. However, even if the number of CDSs represented inside the four selection phage libraries was high, and comparable with that obtained for the genomic phage library (Table 6), the percentage of nucleotides covered inside those CDSs decreased to 48,44% (control), 61,86% (sel A), 52,01%(sel B) and 44,97% (sel C). This result shows that the diversity of the libraries obtained after selections with patients' sera was significantly reduced demonstrating that specific enrichment of precise portions of CDSs/Domains occurred, and that probably specific epitopes have been recognized by the antibodies, presents inside the sera from patients, as expected.

	HP-26695	HP-B128	Control	SelA	SelB	SelC
Raw Reads	1.425.554	1.031.956	2.169.178	815.891	3.737.010	1.633.626
Reads after trimming	1.216.124	954.895	1.798.722	680.764	3.149.939	1.278.335
Mapping Reads	1.114.184	746.325	1.294.576	503.401	2.563.391	961.686
% of Mapping reads	91,61%	89,10%	71,97%	73,94%	81,37%	75,22%
Unmapping Reads	11158	158301	166,831	113,397	257,481	128,346
Mean Coverage	76,81X	76,81X	96,13X	31,19X	155,82X	56,97X
CDS covered	1372	1597	1225	1130	1331	1263
% CDS covered	93,46%	93,80%	83,44%	76,97%	90,66%	86,03%
Nucleotides covered inside CDS	1068020	1164096	703739	898694	755447	653331
% of Nucleotide covered inside CDS	73,51%	76,80%	48,44%	61,86%	52,01%	44,97%

Table 6 - Summary of mapping metrics of Genomic phage library and Selection libraries. The metrics reported are the total number of reads sequenced (Raw reads), number of reads after trimming step (Reads after trimming), number of mapping reads (Mapping reads) and percentage of mapping compare to the total number of reads (% of Mapping reads), number of unmapped reads (Unmapped reads), mean coverage is a parameter derived from number of reads * read length / genome size (Mean Coverage), the number of CDS covered is the number of CDS with almost 10 reads mapping, the percentage of CDS covered is number of CDS covered * 100/ total number of CDS annotated,number of nucleotides is the number of bases with depth different of 0 and the percentage of nucleotide covered inside CDS is the Nucleotides covered inside CDS* 100/total CDS nucleotide length.

7.6.2. Identification of common and/or specific biomarkers for HP infection progression

The Interactome–seq data analysis pipeline identified 535, 627, 780 and 652 putative domains respectively present in the Control and A, B, C selection phage libraries (Table 7). Afterwards through the second step of the analysis the domains differentially enriched (Q-value<0.05 and Focus>0.8) in the selection phage libraries respect to the genomic phage libraries were determined. As shown in Table 7, the number of domains found significantly enriched are 115, 125, 183 and 117 respectively for the Control, A, B and C selections. Then the last step of the analysis was applied and output lists were parsed to generate sub-lists of domains/epitopes specific for the pathological outcomes and absent in the healthy controls. This step was carried out in order to eliminate poly-reactive domains and consists of a simple subtractive comparison between the domains lists of Selections A, B and C and the list of the Control selection

	HP-26695	Control	SelA	SelB	SelC
Putative Domains	1761	535	627	780	652
DEG Domains	###	115	125	183	117
Domains subtracted Control	###	###	61	127	49

Table 7 - Overview of putative domains detected, enriched and specific for selections. The first line of table shows the total of Putative domains that are detected for HP-26695 ORF-filtering library, Control and all Selections, the second line of table shows the total number of domains of Contol and Selections that are enriched compare to Genomic HP-26695 ORF-filtering library, in the third line are reported the domains of Selection A, B and C that are resulting after the exclusion of that domains in common with Control Selection. DEG: Differentially Expressed Gene

The Venn diagram in Figure 16 shows the result of this last part of the analysis. It is evident that the most of the enriched domains, found after selections against sera (in total 138), are specific for the three pathological outcomes: Selection A (25), Selection B (85) and Selection C (28), while only 45 enriched domains are common to two or to all the selections.

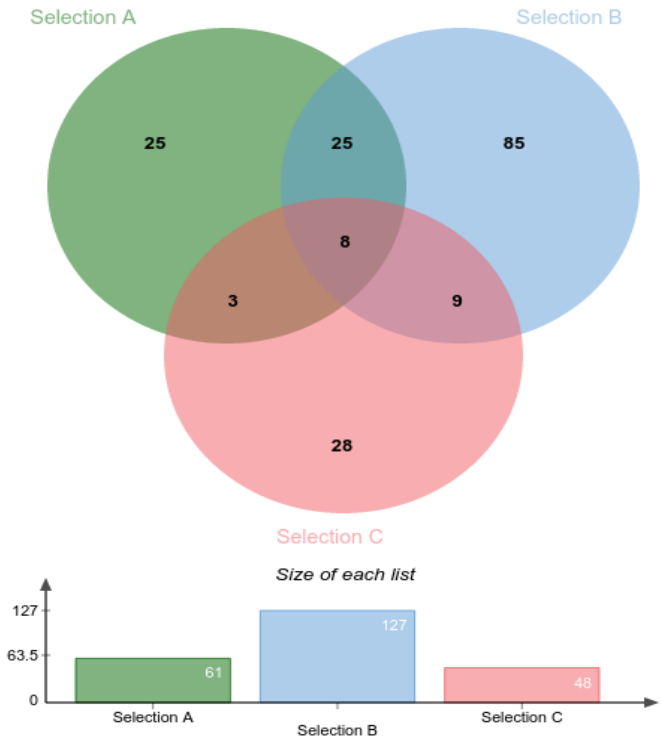


Figure 16 - Venn diagram of specific and common putative antigens. The Venn diagram shows the number of different CDS shared between the libraries. Group A represents sera from individuals affected by gastric adenocarcinoma, Group B represents sera from individuals affected by autoimmune gastritis and group C is sera from patients affected by MALT lymphoma.

This result is really promising, indeed our Interactome-Seq approach coupled with Interactome web-tool data analysis, allowed the identification of many putative domains specifc for the three different pathological outcomes under study.

7.6.3. Validation of one target for HP infection progression

As previously elucidated, it is very important to find novel biomarkers that can provide a “disease signature” for different pathological outcomes, or at least biomarkers associated to HP infection progression towards serious diseases.
Among the sub-lists of domains/epitopes specific for Gastric Cancer, Autoimmune Gastritis, MALT lymphoma and absent in the Healthy controls we selected the top list ones, having the highest Fold Change values (see APPENDIX 10.3-10.4-10.5-10.6-10.7-10.8-10.9) as putative targets for validation with ELISA assay.
Our attention was, from the beginning, captured by the HP0527 gene domains filtered out by our approach and resulting significantly enriched after the selections. This gene also named cagY/cag7 encodes for a large protein of 1927 aminoacids that has been described by Liu et al [149] as one of the main components of *H. pylori cag* T4SS-associated pilum. Interestingly, CagY has an unusual domain structure, in which a number of direct repeats is predicted to cause rearrangements that invariably yield in-frame insertions or deletions [150]. Recent infection studies in murine and non-human primate models have shown that the rearrangements in CagY are sufficient to cause gain or loss of function in the *H. pylori* T4SS and are driven by the host immune system [151]. It was therefore proposed that CagY may function as a sort of molecular switch that alters the function of the T4SS and tunes CagA injection and host pro-inflammatory responses. A majority of *H. pylori*-positive MALT lymphoma patients were serum positive for CagA (9, 10), leading to the hypothesis that CagA might be one causative factor in MALT lymphoma. This observation could be supported by our results, as a matter of fact we find a portion of CagY highly enriched among the top list domains specific for the MALT selection (sel C). At the same time we found highly enriched also other portions of the CagY gene among the top list domains specific for the Gastric Cancer selection (sel A) but also among the top list domains common to all the three pathological conditions.

So we selected two different portions of this gene corresponding to:

1. the domain identified between aminoacidic positions 600 and 853
2. the domain identified between aminoacidic positions 344 and 396

The first one resulted commonly enriched and differential in all the three selections (Figure 17 panel A red box) while the second one is specific for the MALT selection (sel C) and has a very high Fold Change of 7,71 and a length of 761 bp (Figure 17 panel A blue box).

Both these domains were excised from the phagemid vector and cloned into a compatible pGEX-FLAG [130] vector, creating for each output of selection a library for the expression of the selected domain as GST-fusion product (see materials and methods section 6.6)(Figure 17 panel B).

Up to date it was possible to purify the correct recombinant protein only for the first of the two domains, the second one seems to be less soluble and further attempts for purification are ongoing.

To investigate possible cross-reactivity and specificity of the first domain an antibody-capture ELISA assay was implemented (Figure 17 panel B) and a large number of sera, from HP positive and negative Healthy patients (51 sera), from patients with Gastric cancer (29 sera), Autoimmune gastritis (10 sera) and MALT lymphoma (6 sera) were tested (Figure 17 panel C).

It should be noted that the number of patients affected by Autoimmune Gastritis and MALT lymphoma, that was possible to recruit, is much less than the number of patients affected by Gastric Cancer because these two outcomes are rare diseases.

ELISA results show that all the Control Sera (healthy patients) are non-reactive independently from being HP positive or negative, so from this first result we can infer that the domain tested is specific for infection progression towards serious outcomes and not simply related to HP infection. Then looking at the results obtained with the sera from sick patients we found that almost all the sera of HP negative ill patients resulted non-reactive while for the sera of HP positive patients we obtained very high percentages of positive reactivity: 90%

for Gastric Cancer (9 over 10 sera); 75% for Autoimmune Gatsritis (3 over 4 sera) and finally 83% for MALT Lymphoma (5 over 6 sera) (Figure 17 panel D). Thus after ELISA validation we can conclude that this first CagY domain could become a good new biomarker for HP infection progression towards serious pathologies. We expect to obtain much more specific results for the second CagY domain, that we are trying to purify, especially for MALT sera, because, as it can be appreciated from Figure 17 panel A blue box, this portion of the gene seems to be specifically enriched after selection with MALT sera (Sel C).
We have already started to purify other specific domains indentified from our analysis for further ELISA validation assays.
In the next future we will increase the number of sera of patients, that will be recruited over time; furthermore, we will continue with ELISA test validation on other new markers specific for gastric adenocarcinoma, autoimmune gastritis and MALT lymphoma.

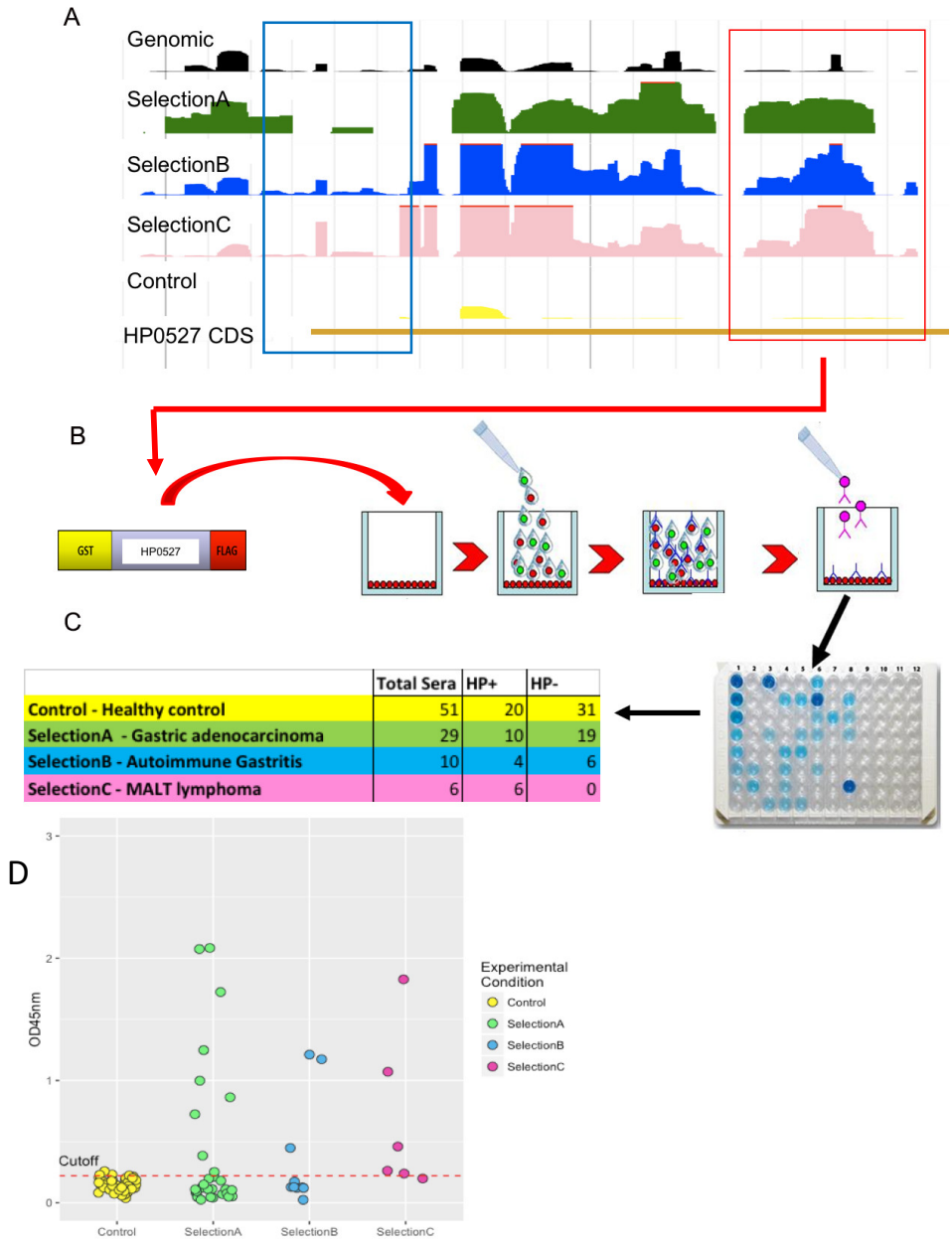


Figure 17 - Schematic overview of HP0527 ELISA validation. Panel A shows genomic browser tracks of HP0527 for genomic and all selections. Panel B shows domains that were excised from the phagemid vector and cloned into a compatible pGEX-FLAG vector. Panel C shows ELISA test sera number used for validation. Panel D shows results of ELISA assay.

8. CONCLUSION AND FUTURE DIRECTIONS

In this section I will summarize the results obtained during the Thesis work and the future perspectives.

The diagnosis of Gastric Cancer and MALT lymphoma is not easy because patients do not show notable symptoms of illness in early onset stages, at the same time they progress rapidly. The possibility of doing an early diagnosis increases the opportunity to save time for active therapy and to improve the survival rate of patients. Many of the previous studies, aiming at biomarkers discovery, were hypothesis-driven, limiting the identification of clinically significant antigens to those directed against proteins that had been already described in pathological outcomes associated with *H. pylori* infection. The Interactome Sequencing approach, here proposed and described, is an ideal unbiased and powerful high-throughput strategy to identify novel antigenic targets. We have exploited the huge data output generated by high throughput sequencing to investigate samples having great complexity, specifically sera samples from patients with different pathological outcomes.

In this thesis work I was able to identify lists of new putative common and specific antigens by: i) constructing ORF genomic filtered libraries from two strains of *H. pylori,* HP-26695 and HP-B128; ii) developing a dedicated web-tool for Interactome-seq data analysis iii) selecting the genomic phage libraries with sera from patients affected by gastric adenocarcinoma, autoimmune gastritis and MALT lymphoma and iv) validating one enriched protein domain specific for infection progression.

Overall, we produced a panel of putative specific domains for three different pathological outcomes, these targets could be validated in the next future comprehensibly associated to the onset of serious diseases related to HP infection.

The new data analysis pipeline here developed and the web-tool implemented has been designed taking into consideration several factors: first of all it is compatible with all sequencing platforms outputs, secondly the “blind” detection of putative domains is very important for reducing the noise

background typical of approaches based on phage display coupled with NGS. Compared to softwares previously used for performing this kind of analysis (NGS-TRex), the Interactome-seq pipeline allows the identification of more than one putative domain/epitope from each protein, thus enabling to discriminate, for example, the portion of CagY specifically recognized by MALT sera from the portion commonly reactive by selections performed with all the three sets of sera tested.

In order to make public and accessible this new pipeline a a web-tool was implemented; this is a user-friendly web interface that doesn't require any programming skills, thus allowing every user to perform Interactome-Seq analysis starting from raw-sequencing data, to sort and visualize the outputs and if needed to download the domain lists in tabular format for interpolation with other results.

In conclusion this thesis work demonstrated that the Interactome-Seq technology is an easily available and powerful strategy, which can help deciphering the molecular mechanisms involved in modulating host immune response.

Providing a new web-tool for data analysis the way to detection of new biomarkers by applying innovative approaches based on interactome sequencing strategy was open.

The release of this tool is timely and relevant for the scientific and clinical community as the importance of using new unbiased experimental and data analysis approaches to discover new biomarkers, for improving early diagnosis of *H. pylori* infection progression towards serious pathological outcome, is among the WHO priorities.

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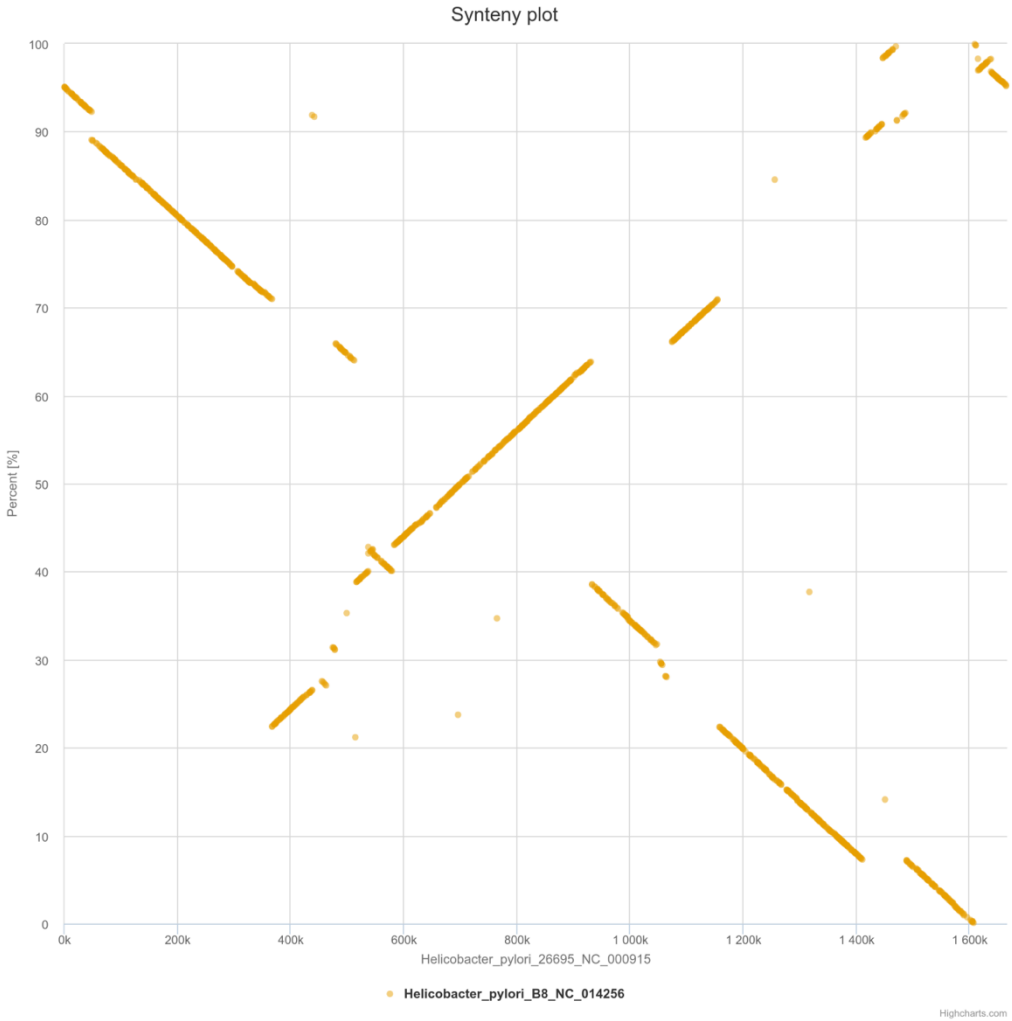
10. APPENDIX

10.1. Synteny plot of HP-26695 and HP-B128



10.2. HP-26695 Genomic Domains

CHR	START	END	GENE_ID
NC_000915	2750	3018	HP0005
NC_000915	9339	9574	HP0011
NC_000915	16862	17165	HP0018
NC_000915	20596	20890	HP0021
NC_000915	27157	27358	HP0026
NC_000915	27642	28180	HP0027

NC_000915	28900	29293	HP0028
NC_000915	31960	32344	HP0031
NC_000915	34508	34722	HP0033
NC_000915	41162	41382	HP0043
NC_000915	43242	43482	HP0045
NC_000915	47004	47542	HP0048
NC_000915	50497	50927	HP0051
NC_000915	52551	53065	HP0053
NC_000915	61618	61828	HP0057
NC_000915	67353	67792	HP0063
NC_000915	75066	75320	HP0071
NC_000915	84358	84667	HP0080
NC_000915	87828	88141	HP0082
NC_000915	98567	98901	HP0092
NC_000915	100684	101444	HP0096
NC_000915	101645	102356	HP0097
NC_000915	112851	113200	HP0105
NC_000915	115793	115996	HP0108
NC_000915	124752	125086	HP0116
NC_000915	128034	128489	HP0118
NC_000915	128534	128964	HP0118
NC_000915	135989	136323	HP0123
NC_000915	143701	144650	HP0133
NC_000915	144688	144835	HP0133
NC_000915	149234	149334	HP0138
NC_000915	152748	153301	HP0141
NC_000915	153513	153628	HP0141
NC_000915	156608	156999	HP0144
NC_000915	160724	161118	HP0150
NC_000915	167975	168396	HP0159
NC_000915	178792	178988	HP0172
NC_000915	181234	181434	HP0174
NC_000915	181498	181717	HP0174a
NC_000915	184797	185017	HP0178
NC_000915	185865	186178	HP0179
NC_000915	187996	188197	HP0181

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9.3

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NC_014256	13346	15741	HPB8_10_13346_15741
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NC_014256	23485	23755	HPB8_18_23485_23755
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NC_014256	32928	33357	HPB8_27_32928_33357
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NC_014256	1577513	1577723	HPB8_1608_1577513_1577723
NC_014256	1578468	1579295	HPB8_1611_1578468_1579295
NC_014256	1582399	1584420	HPB8_1615_1582399_1584420
NC_014256	1584765	1586291	HPB8_1616_1584765_1586291
NC_014256	1586394	1586651	HPB8_1616_1586394_1586651
NC_014256	1586778	1586864	HPB8_1617_1586778_1586864
NC_014256	1591958	1592274	HPB8_1625_1591958_1592274
NC_014256	1592733	1593476	HPB8_1626_1592733_1593476
NC_014256	1593579	1593691	HPB8_1627_1593579_1593691
NC_014256	1594223	1594618	HPB8_1629_1594223_1594618
NC_014256	1594713	1594991	HPB8_1630_1594713_1594991
NC_014256	1595090	1595878	HPB8_1631_1595090_1595878

NC_014256	1596725	1597737	HPB8_1633_1596725_1597737
NC_014256	1599661	1600668	HPB8_1638_1599661_1600668
NC_014256	1601274	1601676	HPB8_1639_1601274_1601676
NC_014256	1603301	1603515	HPB8_1641_1603301_1603515
NC_014256	1604522	1605271	HPB8_1644_1604522_1605271
NC_014256	1605381	1606470	HPB8_1645_1605381_1606470
NC_014256	1609811	1611876	HPB8_1652_1609811_1611876
NC_014256	1612375	1613190	HPB8_1655_1612375_1613190
NC_014256	1613336	1613932	HPB8_1656_1613336_1613932
NC_014256	1614173	1614508	HPB8_1657_1614173_1614508
NC_014256	1614619	1614865	HPB8_1657_1614619_1614865
NC_014256	1614996	1615132	HPB8_1657_1614996_1615132
NC_014256	1615372	1616024	HPB8_1658_1615372_1616024
NC_014256	1616063	1616216	HPB8_1658_1616063_1616216
NC_014256	1616402	1617568	HPB8_1659_1616402_1617568
NC_014256	1618823	1619149	HPB8_1663_1618823_1619149
NC_014256	1619307	1621134	HPB8_1664_1619307_1621134
NC_014256	1621450	1621751	HPB8_1665_1621450_1621751
NC_014256	1621918	1622715	HPB8_1665_1621918_1622715
NC_014256	1637672	1638103	HPB8_1678_1637672_1638103
NC_014256	1638181	1638488	HPB8_1679_1638181_1638488
NC_014256	1643029	1643823	HPB8_1682_1643029_1643823
NC_014256	1643879	1644890	HPB8_1683_1643879_1644890
NC_014256	1645812	1646823	HPB8_1686_1645812_1646823
NC_014256	1646954	1647721	HPB8_1688_1646954_1647721
NC_014256	1648341	1649277	HPB8_1689_1648341_1649277
NC_014256	1649353	1649877	HPB8_1690_1649353_1649877
NC_014256	1650001	1650334	HPB8_1690_1650001_1650334
NC_014256	1651543	1652027	HPB8_1692_1651543_1652027
NC_014256	1652172	1653656	HPB8_1693_1652172_1653656
NC_014256	1653699	1654534	HPB8_1694_1653699_1654534
NC_014256	1654720	1655428	HPB8_1696_1654720_1655428
NC_014256	1656572	1657225	HPB8_1699_1656572_1657225
NC_014256	1657360	1658488	HPB8_1700_1657360_1658488
NC_014256	1658692	1659647	HPB8_1701_1658692_1659647
NC_014256	1660005	1662516	HPB8_1702_1660005_1662516
NC_014256	1662620	1663327	HPB8_1703_1662620_1663327
NC_014256	1663620	1666264	HPB8_1704_1663620_1666264
NC_014256	1666391	1668838	HPB8_1705_1666391_1668838
NC_014256	1669787	1669959	HPB8_1706_1669787_1669959

10.3. SelectionA - Gastric Adenocarcinoma Unique Domains

CHR	START	END	GENE_ID	FOLDCHANGE	ANNOTATION	FLAG
NC_000915	432871	432986	HP0418	9,40	hypothetical protein	Sel A unique
NC_000915	749410	749497	HP0696	8,29	N-methylhydantoinase	Sel A unique
NC_000915	880251	880485	HP0828	5,06	ATP synthase F0F1 subunit A	Sel A unique
NC_000915	555864	556076	HP0527	4,79	cag pathogenicity island protein cag7	Sel A unique
NC_000915	1051051	1051229	HP0988	4,51	IS605 transposase TnpA	Sel A unique
NC_000915	356709	356816	HP0349	4,41	CTP synthetase	Sel A unique
NC_000915	1194612	1194688	HP1132	3,45	ATP synthase F0F1 subunit beta	Sel A unique
NC_000915	70004	70177	HP0066	3,10	ATP-binding protein	Sel A unique
NC_000915	271337	271508	HP0262	2,93	hypothetical protein	Sel A unique
NC_000915	1061927	1062105	HP0998	2,89	IS605 transposase TnpA	Sel A unique
NC_000915	312869	313125	HP0295	2,55	flagellar hook-associated protein FlgL	Sel A unique
NC_000915	1563976	1564163	HP1490	2,50	hypothetical protein	Sel A unique
NC_000915	979186	979349	HP0921	2,49	glyceraldehyde-3-phosphate dehydrogenase	Sel A unique
NC_000915	320256	320492	HP0302	2,45	dipeptide ABC transporter ATP-binding protein DppF	Sel A unique
NC_000915	951212	951394	HP0898	2,35	hydrogenase expression/formation protein HypD	Sel A unique
NC_000915	846725	846835	HP0790	2,28	anti-codon nuclease masking agent PrrB	Sel A unique
NC_000915	1063457	1063650	HP1001	2,25	hypothetical protein	Sel A unique
NC_000915	588313	588527	HP0553	2,21	hypothetical protein	Sel A unique
NC_000915	106698	106847	HP0100	2,17	hypothetical protein	Sel A unique
NC_000915	5875	6023	HP0009	2,07	hypothetical protein	Sel A unique
NC_000915	1243612	1243805	HP1177	2,06	hypothetical protein	Sel A unique
NC_000915	999864	1000073	HP0939	1,91	amino acid ABC transporter permease	Sel A unique
NC_000915	176025	176197	HP0169	1,89	collagenase PrtC	Sel A unique
NC_000915	1047284	1047501	HP0983	1,88	hypothetical protein	Sel A unique
NC_000915	1372726	1372975	HP1301	1,78	50S ribosomal protein L15	Sel A unique

10.4. SelectionB - Autoimmune Gastritis Unique Domains

CHR	START	END	GENE_ID	FOLDCHANGE	ANNOTATION	FLAG
NC_000915	1004123	1004218	HP0943	8,98	D-amino acid dehydrogenase DadA	Sel B unique
NC_000915	864151	864245	HP0809	8,55	flagellar basal body protein FliL	Sel B unique
NC_000915	1491923	1492043	HP1422	8,18	isoleucyl-tRNA synthetase	Sel B unique
NC_000915	512074	512214	HP0487	5,27	hypothetical protein	Sel B unique

NC_000915	1073160	1073202	HP1010	4,71	polyphosphate kinase	Sel B unique
NC_000915	945751	945866	HP0892	4,57	hypothetical protein	Sel B unique
NC_000915	373124	373195	HP0363	3,99	protein-L-isoaspartate O-methyltransferase	Sel B unique
NC_000915	918695	918813	HP0867	3,76	ipid-A-disaccharide synthase	Sel B unique
NC_000915	1574664	1574835	HP1502	3,73	hypothetical protein	Sel B unique
NC_000915	1158327	1158505	HP1096	3,60	IS605 transposase TnpA	Sel B unique
NC_000915	1028615	1028881	HP0969	3,47	cation efflux system protein CzCA	Sel B unique
NC_000915	140452	141127	HP0130	3,34	hypothetical protein	Sel B unique
NC_000915	555284	556076	HP0527	3,12	cag pathogenicity island protein cag7	Sel B unique
NC_000915	475825	476089	HP0457	3,06	hypothetical protein	Sel B unique
NC_000915	749410	749655	HP0696	3,05	N-methylhydantoinase	Sel B unique
NC_000915	1564206	1564417	HP1490	3,01	hypothetical protein	Sel B unique
NC_000915	1558353	1558552	HP1486	2,96	hypothetical protein	Sel B unique
NC_000915	576113	576186	HP0544	2,87	cag pathogenicity island protein cag23	Sel B unique
NC_000915	106624	106910	HP0100	2,84	hypothetical protein	Sel B unique
NC_000915	721767	722123	HP0671	2,80	hypothetical protein	Sel B unique
NC_000915	406342	406513	HP0396	2,79	hypothetical protein	Sel B unique
NC_000915	811715	811947	HP0758	2,79	hypothetical protein	Sel B unique
NC_000915	1627113	1627477	HP1547	2,75	leucyl-tRNA synthetase	Sel B unique
NC_000915	882360	883007	HP0830	2,74	aspartyl/glutamyl-tRNA amidotransferase subunit A	Sel B unique
NC_000915	627603	628013	HP0593	2,69	adenine-specific DNA methyltransferase	Sel B unique
NC_000915	1399532	1399604	HP1340	2,67	biopolymer transport protein ExbD	Sel B unique
NC_000915	852233	852399	HP0795	2,63	trigger factor	Sel B unique
NC_000915	278658	278779	HP0269	2,60	(dimethylallyl)adenosine tRNA methylthiotransferase	Sel B unique
NC_000915	1338786	1338916	HP1266	2,58	NADH dehydrogenase subunit G	Sel B unique
NC_000915	146944	147190	HP0136	2,56	bacterioferritin comigratory protein Bcp	Sel B unique
NC_000915	143701	144124	HP0133	2,55	serine transporter SdaC	Sel B unique
NC_000915	477705	478199	HP0459	2,52	protein VirB4	Sel B unique
NC_000915	328396	328584	HP0312	2,49	ATP-binding protein	Sel B unique
NC_000915	1374888	1375034	HP1306	2,34	30S ribosomal protein S14	Sel B unique
NC_000915	902944	903171	HP0851	2,32	hypothetical protein	Sel B unique
NC_000915	519533	519950	HP0493	2,32	phospho-N-acetylmuramoyl-pentapeptide-transferase	Sel B unique
NC_000915	552880	553202	HP0525	2,31	virB11-like protein	Sel B unique
NC_000915	10215	11080	HP0012	2,28	DNA primase	Sel B unique
NC_000915	1583199	1583444	HP1509	2,27	glycerol-3-phosphate acyltransferase PlsY	Sel B unique
NC_000915	1382149	1382490	HP1321	2,27	hypothetical protein	Sel B unique

NC_000915	951075	951395	HP0898	2,27	hydrogenase expression/formation protein HypD	Sel B unique
NC_000915	907649	907838	HP0855	2,26	alginate O-acetylation protein AlgI	Sel B unique
NC_000915	60676	60803	HP0056	2,23	delta-1-pyrroline-5-carboxylate dehydrogenase	Sel B unique
NC_000915	1026447	1026625	HP0967	2,20	virulence associated protein D vapD	Sel B unique
NC_000915	497151	497374	HP0475	2,16	molybdenum ABC transporter ATP-binding protein ModD	Sel B unique
NC_000915	397190	397438	HP0387	2,12	primosome assembly protein PriA	Sel B unique
NC_000915	320109	320326	HP0302	2,09	dipeptide ABC transporter ATP-binding protein DppF	Sel B unique
NC_000915	1284797	1284992	HP1207	2,08	hypothetical protein	Sel B unique
NC_000915	1334949	1335478	HP1262	2,06	NADH dehydrogenase subunit C	Sel B unique
NC_000915	1545372	1545572	HP1472	2,03	type IIS restriction enzyme M protein	Sel B unique
NC_000915	1107752	1108034	HP1045	1,99	acetyl-CoA synthetase	Sel B unique
NC_000915	798735	798907	HP0743	1,99	rod shape-determining protein MreB	Sel B unique
NC_000915	1570699	1570993	HP1498	1,98	hypothetical protein	Sel B unique
NC_000915	1354475	1354543	HP1279	1,98	bifunctional indole-3-glycerol phosphate synthase isomerase	Sel B unique
NC_000915	835626	835696	HP0781	1,95	hypothetical protein	Sel B unique
NC_000915	972820	972958	HP0916	1,95	iron-regulated outer membrane protein FrpB	Sel B unique
NC_000915	1117318	1117518	HP1055	1,94	hypothetical protein	Sel B unique
NC_000915	511175	511376	HP0487	1,92	hypothetical protein	Sel B unique
NC_000915	1332564	1332994	HP1258	1,91	hypothetical protein	Sel B unique
NC_000915	880169	880334	HP0828	1,90	ATP synthase F0F1 subunit A	Sel B unique
NC_000915	536705	536926	HP0510	1,89	dihydrodipicolinate reductase	Sel B unique
NC_000915	298023	298185	HP0289	1,87	toxin-like outer membrane protein	Sel B unique
NC_000915	837963	838354	HP0783	1,87	hypothetical protein	Sel B unique
NC_000915	1656737	1657203	HP1577	1,86	ABC transporter permease	Sel B unique
NC_000915	709780	709958	HP0661	1,85	ribonuclease H	Sel B unique
NC_000915	170854	171136	HP0162	1,83	hypothetical protein	Sel B unique
NC_000915	329641	329835	HP0313	1,82	nitrite extrusion protein NarK	Sel B unique
NC_000915	1536901	1537293	HP1466	1,82	hypothetical protein	Sel B unique
NC_000915	803140	803421	HP0747	1,81	tRNA (guanine-N(7)-)-methyltransferase	Sel B unique
NC_000915	1176168	1176502	HP1114	1,77	excinuclease ABC subunit B	Sel B unique
NC_000915	16862	17194	HP0018	1,76	hypothetical protein	Sel B unique
NC_000915	1557886	1558193	HP1485	1,72	hypothetical protein	Sel B unique
NC_000915	1374255	1374313	HP1304	1,70	50S ribosomal protein L6	Sel B unique
NC_000915	21826	21995	HP0022	1,69	lipid A phosphoethanolamine transferase	Sel B unique
NC_000915	237376	237722	HP0228	1,68	hypothetical protein	Sel B unique
NC_000915	32679	32942	HP0033	1,64	ATP-dependent C1p protease	Sel B unique

					ClpA	
NC_000915	855368	855623	HP0799	1,62	molybdenum cofactor biosynthesis protein MogA	Sel B unique
NC_000915	78559	78755	HP0074	1,61	lipoprotein signal peptidase	Sel B unique
NC_000915	61634	61828	HP0057	1,58	hypothetical protein	Sel B unique
NC_000915	854493	854659	HP0797	1,57	flagellar sheath adhesin hpaA	Sel B unique
NC_000915	524327	524527	HP0498	1,56	sodium- and chloride-dependent transporter	Sel B unique
NC_000915	1544120	1544616	HP1471	1,53	type IIS restriction enzyme R protein	Sel B unique
NC_000915	84756	85321	HP0080	1,52	hypothetical protein	Sel B unique
NC_000915	983327	983850	HP0922	1,52	toxin-like outer membrane protein	Sel B unique
NC_000915	1172395	1172634	HP1111	1,51	ferrodoxin oxidoreductase subunit beta	Sel B unique

10.5. SelectionC - MALT lymphoma Unique Domains

CHR	START	END	GENE_ID	FOLDCHANGE	ANNOTATION	FLAG
NC_000915	556305	557066	HP0527	7,71	cag pathogenicity island protein cag7	Sel C unique
NC_000915	777672	777742	HP0724	5,24	anaerobic C4-dicarboxylate transporter	Sel C unique
NC_000915	1585441	1585851	HP1512	5,04	iron-regulated outer membrane protein FrpB	Sel C unique
NC_000915	204394	204591	HP0197	4,12	S-adenosylmethionine synthetase	Sel C unique
NC_000915	17965	18272	HP0018	3,45	hypothetical protein	Sel C unique
NC_000915	712341	712488	HP0665	3,18	coproporphyrinogen III oxidase	Sel C unique
NC_000915	1317869	1317975	HP1243	3,10	hypothetical protein	Sel C unique
NC_000915	274274	274470	HP0264	3,01	ATP-dependent protease binding subunit ClpB	Sel C unique
NC_000915	477730	477960	HP0459	2,91	protein VirB4	Sel C unique
NC_000915	203274	203353	HP0196	2,82	UDP-3-O-[3-hydroxymyristoyl] glucosamine N-acyltransferase	Sel C unique
NC_000915	1626372	1626445	HP1547	2,72	leucyl-tRNA synthetase	Sel C unique
NC_000915	1461955	1462126	HP1400	2,62	iron(III) dicitrate transport protein FecA	Sel C unique
NC_000915	983602	984010	HP0922	2,55	toxin-like outer membrane protein	Sel C unique
NC_000915	422464	422818	HP0409	2,35	GMP synthase	Sel C unique
NC_000915	1024311	1024404	HP0965	2,31	hypothetical protein	Sel C unique
NC_000915	1249755	1250123	HP1181	2,21	multidrug-efflux transporter	Sel C unique
NC_000915	221588	221643	HP0213	2,12	tRNA uridine 5-carboxymethylaminomethyl modification protein GidA	Sel C unique
NC_000915	484059	484302	HP0464	2,11	type I restriction enzyme R protein HsdR	Sel C unique
NC_000915	1332625	1332798	HP1258	2,04	hypothetical protein	Sel C unique
NC_000915	827888	827994	HP0775	1,98	penta-phosphate guanosine-3'-pyrophosphohydrolase SpoT	Sel C unique
NC_000915	1241938	1242043	HP1175	1,96	hypothetical protein	Sel C unique
NC_000915	411221	411357	HP0400	1,94	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	Sel C unique

NC_000915	1019626	1019879	HP0961	1,90	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	Sel C unique
NC_000915	1206023	1206430	HP1143	1,85	hypothetical protein	Sel C unique
NC_000915	933394	933665	HP0884	1,79	hypothetical protein	Sel C unique
NC_000915	1428027	1428285	HP1366	1,77	type IIS restriction enzyme R protein	Sel C unique
NC_000915	795181	795399	HP0740	1,74	UDP-MurNac-pentapeptide presynthetase MurF	Sel C unique
NC_000915	1584941	1585092	HP1512	1,60	iron-regulated outer membrane protein FrpB	Sel C unique

10.6. Common Domains between Selection A-B-C

CHR	START	END	GENE_ID	FOLDCHANGE	ANNOTATION	FLAG
NC_000915	607582	607662	HP0577	5,33	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase	Common between Sel A-B-C
NC_000915	1592925	1592985	HP1517	3,03	type IIS restriction enzyme R and M protein	Common between Sel A-B-C
NC_000915	241192	241296	HP0231	2,49	hypothetical protein	Common between Sel A-B-C
NC_000915	414784	414866	HP0402	2,21	phenylalanyl-tRNA synthetase subunit beta	Common between Sel A-B-C
NC_000915	1396381	1396472	HP1335	2,09	tRNA-specific 2-thiouridylase MnmA	Common between Sel A-B-C
NC_000915	577358	577581	HP0544	2,03	cag pathogenicity island protein cag23	Common between Sel A-B-C
NC_000915	1018926	1019099	HP0960	1,65	glycyl-tRNA synthetase subunit alpha	Common between Sel A-B-C
NC_000915	1642827	1643112	HP1561	1,64	iron(III) ABC transportersubstrate-binding protein CeuE	Common between Sel A-B-C

10.7. Common Domains between Selection A-C

CHR	START	END	GENE_ID	FOLDCHANGE	ANNOTATION	FLAG
NC_000915	437431	437598	HP0422	4,18	arginine decarboxylase	Common between Sel A-C
NC_000915	195103	195230	HP0189	2,65	hypothetical protein	Common between Sel A-C
NC_000915	1345616	1345804	HP1272	2,16	NADH dehydrogenase subunit M	Common between Sel A-C

10.8. Common Domains between Selection A-B

CHR	START	END	GENE_ID	FOLDCHANGE	ANNOTATION	FLAG
NC_000915	1031857	1031904	HP0971	9,41	hypothetical protein	Common between Sel A-B
NC_000915	1051051	1051229	HP0988	5,57	IS605 transposase TnpA	Common between Sel A-B

NC_000915	1555522	1555630	HP1481	5,40	hypothetical protein	Common between Sel A-B
NC_000915	168551	168654	HP0159	4,95	lipopolysaccharide 1,2-glucosyltransferase RfaJ	Common between Sel A-B
NC_000915	1614604	1614782	HP1535	4,63	IS605 transposase TnpA	Common between Sel A-B
NC_000915	1552411	1552496	HP1479	4,55	hypothetical protein	Common between Sel A-B
NC_000915	454329	454507	HP0437	4,28	IS605 transposase TnpA	Common between Sel A-B
NC_000915	769890	769985	HP0715	4,13	ABC transporter ATP-binding protein	Common between Sel A-B
NC_000915	1061927	1062105	HP0998	4,04	IS605 transposase TnpA	Common between Sel A-B
NC_000915	614191	614470	HP0583	3,61	hypothetical protein	Common between Sel A-B
NC_000915	951646	951852	HP0898	3,60	hydrogenase expression/formation protein HypD	Common between Sel A-B
NC_000915	1030389	1030578	HP0970	3,52	nickel-cobalt-cadmium resistance protein NccB	Common between Sel A-B
NC_000915	1380289	1380602	HP1319	3,47	50S ribosomal protein L3	Common between Sel A-B
NC_000915	1187149	1187265	HP1121	3,21	cytosine specific DNA methyltransferase	Common between Sel A-B
NC_000915	797809	798321	HP0743	3,09	rod shape-determining protein MreB	Common between Sel A-B
NC_000915	1579060	1579204	HP1506	2,86	glutamate permease GltS	Common between Sel A-B
NC_000915	512849	513141	HP0488	2,70	hypothetical protein	Common between Sel A-B
NC_000915	1278378	1278463	HP1201	2,61	50S ribosomal protein L1	Common between Sel A-B
NC_000915	1410757	1410897	HP1350	2,60	protease	Common between Sel A-B
NC_000915	731951	732085	HP0681	2,48	hypothetical protein	Common between Sel A-B
NC_000915	11805	12001	HP0013	2,33	hypothetical protein	Common between Sel A-B
NC_000915	51391	51625	HP0052	2,30	hypothetical protein	Common between Sel A-B
NC_000915	79748	80106	HP0075	2,10	phosphoglucosamine mutase	Common between Sel A-B
NC_000915	657675	658335	HP0613	1,76	ABC transporter ATP-binding protein	Common between Sel A-B
NC_000915	1295199	1295391	HP1218	1,71	glycinamide ribonucleotide synthetase PurD	Common between Sel A-B

10.9. Common Domains between Selection B-C

CHR	START	END	GENE_ID	FOLDCHANGE	ANNOTATION	FLAG
NC_000915	524080	524265	HP0498	3,72	sodium- and chloride-dependent transporter	Common between Sel B-C
NC_000915	890884	891033	HP0839	3,41	outer membrane protein P1 ompP1	Common between Sel B-C
NC_000915	1037115	1037287	HP0974	2,74	phosphoglyceromutase	Common between Sel B-C
NC_000915	1243612	1243988	HP1177	2,60	hypothetical protein	Common between Sel B-C
NC_000915	1034744	1034877	HP0973	2,07	hypothetical protein	Common between Sel B-C
NC_000915	1317869	1317975	HP1243	1,90	hypothetical protein	Common between Sel B-C
NC_000915	999625	1000073	HP0939	1,76	amino acid ABC transporter permease	Common between Sel B-C
NC_000915	1584941	1585092	HP1512	1,73	iron-regulated outer membrane protein FrpB	Common between Sel B-C
NC_000915	1184140	1184277	HP1118	1,54	gamma-glutamyltranspeptidase	Common between Sel B-C

9.10. List of strains used for comparative analysis

Strain Name	Strain ID
H. pylori strain ausabrJ05	CP011485
H. pylori strain PNG84A	CP011487
H. pylori strain ML3	AP014712
H. pylori strain ML3	AP014713
H. pylori strain ML2	AP014711
H. pylori strain ML1,	AP014710
H. pylori strain L7	CP011482
H. pylori strain K26A1	CP011486
H. pylori strain DU15	CP011483
H. pylori strain CC33C	CP011484
H. pylori strain BM013B	CP007606
H. pylori strain BM013A	CP007604
H. pylori strain BM012B	CP007605
H. pylori strain 29CaP	CP012907
H. pylori oki828	CP006826
H. pylori oki673	CP006825
H. pylori oki422	CP006824
H. pylori oki154	CP006823
H. pylori oki128	CP006822

H. pylori oki112	CP006821
H. pylori oki102	CP006820
H. pylori XZ274	CP003419
H. pylori UM299	CP005491
H. pylori UM298	CP006610
H. pylori UM066	CP005493
H. pylori UM037	
H. pylori UM032	CP005490
H. pylori SouthAfrica7	CP002336
H. pylori SouthAfrica20	CP006691
H. pylori Shi470	NC_010698
H. pylori Shi417	CP003472
H. pylori Shi169	CP003473
H. pylori Shi112	CP003474
H. pylori Sat464	CP002071
H. pylori SNT49	CP002983
H. pylori SJM180	NC_014560
H. pylori Rif2	CP003906
H. pylori Rif1	CP003905
H. pylori Puno135	CP002982
H. pylori Puno120	CP002980
H. pylori PeCan4	NC_014555
H. pylori PeCan18	CP003475
H. pylori P12	NC_011498
H. pylori OK310	AP012601
H. pylori OK113	AP012600
H. pylori NY40	AP014523
H. pylori Lithuania75	CP002334
H. pylori J99	NC_000921
H. pylori J166	CP007603
H. pylori India7	CP002331
H. pylori Hp238	CP010013
H. pylori HUP-B14	CP003486
H. pylori HPAG1	NC_008086
H. pylori Gambia94/24	CP002332
H. pylori G27	NC_011333
H. pylori F57	AP011945
H. pylori F32	AP011943
H. pylori F30	AP011941
H. pylori F16	AP011940
H. pylori ELS37	CP002953
H. pylori Cuz20	CP002076
H. pylori BM012S	CP006889
H. pylori BM012A	CP006888
H. pylori B8	NC_014256

H. pylori B38	NC_012973
H. pylori Aklavik86	CP003476
H. pylori Aklavik117	CP003483
H. pylori 908	CP002184
H. pylori 83	CP002605
H. pylori 52	CP001680
H. pylori 51	CP000012
H. pylori 35A	CP002096
H. pylori 26695-1MET	CP010436
H. pylori 26695-1CL	AP013356
H. pylori 26695	NC_000915

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