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STRUCTURAL AND FUNCTIONAL INSIGHT OF HELICOBACTER PYLORI TRANSLATION INITIATION FACTOR IF1 (HPIF1) IN PROTEIN BIOSYNTHESIS AND RATIONAL DESIGN OF NEW ANTIMICROBIAL CANDIDATE

A Thesis

by

ASIF SHAHRIAR

Submitted in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Major Subject: Biochemistry and Molecular Biology

The University of Texas Rio Grande Valley

May 2023

STRUCTURAL AND FUNCTIONAL INSIGHT OF HELICOBACTER PYLORI TRANSLATION INITIATION FACTOR IF1 (HPIF1) IN PROTEIN BIOSYNTHESIS AND RATIONAL DESIGN OF NEW ANTIMICROBIAL CANDIDATE

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ABSTRACT

Shahriar, Asif, Structural and Functional Insight of Helicobacter pylori Translation Initiation Factor IF1 (HpIF1) in Protein Biosynthesis and Rational Design of New Antimicrobial Candidate Master of Science (MS), May, 2023, 69 pp., 13 figures, 1 table, references, 87 titles.

Helicobacter pylori (H. pylori) is a Gram-negative bacterium that colonizes the human gastric mucosa and is associated with peptic ulcer disease and gastric cancer. Bacterial protein synthesis is a fundamental metabolic process occurring in all bacteria and is a validated target of antibiotics; however, the precise structural mechanism of H. pylori protein biosynthesis remains unknown. Protein synthesis contains four stages, and translational initiation is the most important and highly regulated critical step, involving three initiation factors (IF1, IF2, IF3) and other key components. IF1 plays a vital role in regulating this step by binding to the 30S ribosomal subunit cooperatively with other initial components. However, H. pylori's IF1 structure is unknown. Using a computational model, this study investigated the interaction between H. pylori initiation factor IF1 and the 30S ribosomal subunit. Hp-IF1 was subcloned into pET24b vector for constructing recombinant DNA plasmid- pET24b-Hp-IF1 DNA plasmid. The recombinant DNA plasmid was used to express the Hp-IF1 protein by the E. coli expression system. The proteins were purified by column chromatography, which were used for NMR structural analysis. The Hp-IF1 protein structure was predicted and used for building the complex model binding with the 30S subunit. We elucidated the structure of the IF1-bound 30S complex, revealing the importance of a short α-helix in IF1 for ribosomal binding and function. A peptide derived from this α-helix Hp-IF1 was designed and tested by MIC assay showing broad-spectrum activity against Gram-negative and positive bacteria, including H. pylori. The peptide shows a low cytotoxicity effect against human embryonic kidney (HEK-293) cells. These results suggest Hp-IF1 peptide as a potential compound for the development of new antimicrobial agents.

DEDICATION

Completing my master's studies would not have been possible without the love and support of my family. My mother, father, wife, and mentor/supervisor have wholeheartedly inspired, motivated, and supported me to accomplish this degree. Thank you all for giving me all your love, motivation, and patience as I pursue and complete my educational goals. I also would like to thank my sibling, who is always pushing me to accomplish my goals and expand my career.

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CHAPTER I

INTRODUCTION

1.1 Statement of Problem

Helicobacter pylori (Hp) is a Gram-negative microaerophilic mobile bacillus that recolonizes the stomach and, therefore, can cause a wide range of infections, including those that can lead to the formation of peptic ulcers, atrophic gastritis, and gastric cancer, as well as gastric mucosa-associated lymphoid tissue (MALT) lymphoma, and can have a wide range of complications on gastric function. (Serena et al., 2018; Yang et al., 2022). Helicobacter pylori (H. pylori) is a type of bacteria that can infect the stomach and small intestine. It is estimated that around 50% of the world's population is infected with H. pylori, with a higher prevalence in developing countries, where the incidence can reach up to 90% of the population (Yang et al., 2022). Due to extreme genetical variance and heterogeneity, H. pylori acquire the ability to cause different diseases, detrimental and nondetrimental chronic infections (Blaser, 1997; Mi et al., 2021). Bacterial infection can be eradicated by antibiotic treatment over the years (Hasan et al., 2021; Shahriar et al., n.d.), However, antibiotic resistance among H. pylori strains has been rising steadily around the world over the past decade, and this disturbing sign presents a significant obstacle for the treatment of this fastidious bacterium (Hasan et al., 2021; Mascellino et al., 2017).

H. pylori-induced chronic gastritis is associated with an increased risk for a wide range ofclinical outcomes, including but not limited to peptic ulcer disease (gastric and duodenal ulceration), distal gastric adenocarcinoma, and gastric mucosal lymphoproliferative diseases like non-Hodgkin's-lymphoma. The typical function of the human stomach is to prevent bacterial colonization by a combination of gastric acidity and the contractions of the peristalsis muscle. H. pylori, on the other hand, has been endowed by natural selection with several strategies that enable it to circumvent these primary defenses and develop a permanent infection. These strategies include the capacity to endure an acidic gastric pH and motility.

H. pylori gastritis is characterized by invasion of the gastric mucosa from both acute (polymorphonuclear leukocytes) and chronic (inflammatory) cells (lymphocytes, plasma cells, and macrophages); and H. pylori invasion of the stomach mucosa elicits a host immunological response, which includes the recruitment of polymorphonuclear leukocytes (PMNs) as part of the acute inflammatory response and lymphocytes, plasma cells, and macrophages as part of the chronic inflammatory response (de Falco et al., 2015a). H. pylori has several characteristics, including genetic flexibility, to adapt to the specific conditions of each host, which modulates the response of the host immune system and develops several different virulence factors. However, factors are related to the host, such as genetic makeup or physiological and immunological conditions, particularly those that enhance or reduce the immune response to the infection by consumption of meat, high salt, dietary products, and smoking (de Falco et al., 2015a; Hnatyszyn et al., 2013; Sampieri, 2013).

1.2 Statement of the Purpose

By determining the structures of initiation factor 1 in h. pylori pathogen, we can further explore their viability as targets for new antibiotics as an alternative treatment. In this study, we sought to design peptide based on the amino acid sequence of H.pylori initiation factor 1. We tested our designed peptide against different gram-positive and gram-negative bacteria as well as confirmed the toxicity against human cells. We have also shown the complex mode of HpIF-1 peptide, which interacts the with 30S ribosomal subunits generated by computational analysis.

CHAPTER II

REVIEW OF LITERATURE

2.1 Mechanism of causing H. pylori infection

The host is modulated in a diverse range of ways via multiple different virulence factors, ultimately leading to the pathogenicity of H. pylori. However, the extent and severity of the injury to the host tissues are also determined by host variables. Cellular growth, inflammation, and apoptosis are just some biological impacts of H. pylori on the host cells (Yamaoka, 2010). Increased cellular proliferation of host cells has been associated with H. pylori infection. Expression of various cell cycle markers associated with the proliferation was checked in the host cells following the co-culture of gastric epithelial cells with bacteria (J. Kim et al., 2010). Alternatively, the host's immune response to H. pylori could encourage cell development. Helicobacter pylori cause increased gastrin production by mucosal G cells; hence it is more prevalent in infected people (Subhash & Ho, 2015). Some studies revealed that transcription of cyclin D1 in gastric cancer (AGS) cells were upregulated after co-culturing with Helicobacter pylori. The cag pathogenicity island was required for this cyclin

D1 activation, whereas vacA wasn't (Hirata et al., 2001). The transition from the G1 to the S phase is regulated by several cyclins, one of which is cyclin D1. Overexpression of cyclin D1 also has the additional effect of speeding up cell division and decreasing the duration of the G1 phase. The cell cycle regulating protein p27 is downregulated in H. pylori-epithelial cell co-cultures, resulting in G1 arrest in the epithelial cells (Chang et al., 2006; Hirata et al., 2001).

In addition, H. pylori are correlated to inflammation. The production of inflammatory prostaglandins is catalyzed by the pro-inflammatory cyclooxygenase (COX) enzymes Cox1 and 2. Co-culturing gastric epithelial cells with Helicobacter pylori increases COX-2 expression, which is stimulated by cytokines but is already present in the cells due to COX-1's constitutive expression (J. Kim et al., 2010).

2.2 Molecular Mechanism of H. pylori infection

The multi-step process by which H. pyloric causes disease in humans can be described as follows: first, the bacterium must enter the mucous layer (colonization) while avoiding the bactericidal activity of the gastric juices barrier; after that, it must adapt to and multiply in the conditions present in the gastric mucus (persistence) (de Falco et al., 2015a; Manente et al., 2008). Due to its 4-6 lipoprotein vesicles can penetrate the gastric mucosa and survive the harsh acid environment, unlike flagella. It uses chemotaxis to steer clear of places with low pH until settling in the antrum, which has no acid-producing cells (de Falco et al., 2015b; M. Valenzuela et al., 2003). Afterward, it uses the blood group antigen binding adhesin (BabA) to adhere to epithelial cells by attaching to ABO/Leb (Leb) group antigens and fucosylated carbohydrates expressed by stomach epithelial cells (Doohan et al., 2021; Kable et al., 2017; Matos et al., 2021). There

is a correlation between infection with H. pylori strains that express functional BabA and an increased risk of developing gastric cancer. A large volume of urease is produced, which converts the urea in the stomach into ammonia and carbon dioxide. This creates a pH-neutral environment suitable for bacterial growth (Ansari & Yamaoka, 2017). Several virulence factors, including the bacterial oncoprotein CagA and a type IV secretion system (T4SS), are encoded in the pathogenicity island (cagPAI) region of the Helicobacter pylori genome. The T4SS injects the CagA protein into gastric epithelial cells, where it can disrupt signaling pathways and cause alterations in cell structure, and accelerate cell proliferation that may eventually lead to cancer (Ansari & Yamaoka, 2020). Epithelial cell lines are particularly susceptible to the effects of VacA, which leads them to undergo significant vacuolation and, consequently, create pores in their membranes. Pathogenicity may also be associated with other bacterial components such as peptidoglycan, lipopolysaccharide (LPS), -glutamyl trans-peptidase (GGT), and protease HtrA (Pachathundikandi et al., 2013).

In addition, several host factors have been linked to the beginning stages of gastric cancer. Bacterial aspects in gastric cancer risk are also identified, and cellular and molecular biological approaches have been adopted to understand better the relationship between H. pylori and changing epithelial cells. H. pylori is a complex bacterial species with a wide range of genotypic and phenotypic variation, making it challenging to identify the essential bacterial components directly linked to etiopathogenesis. The mechanisms involved in this process result in significant cellular damage and DNA injury. Antioxidant defenses and damage-repair strategies become depleted when cells are subjected to persistent stress. Increased turnover or division of gastric epithelial cells can increase the

rate at which genetic errors, or mutations, accumulate. This is because the cell's genetic material (DNA) is replicated and distributed to the two daughter cells during cell division. However, errors can occur during replication, leading to daughter cell mutations (Wroblewski et al., 2010). The aggregation of these mutations in the overproduction of stomach cells leads to the formation of malignant tumors, which exhibit gastric cancer. As a result, the chance of developing cancer is enhanced by H. pylori infection due to oxidative damage to cells (Díaz et al., 2018; Elagan et al., 2020). H. pylori virulence factors, such as VacA and CagA, are inserted into stomach epithelial cells, which regulate the intrinsic production of reactive oxygen species (ROS).

2.3 Correlation of H. pylori and cancer

Infectious bacteria like H. pylori cause different types of stomach inflammation as well as other intestinal irritations. IBS (irritable bowel syndrome) is one of them. However, H. pylori infections mostly turn into gastric cancer, which may increase the risk of causing death. Specific biological pathways are triggered by H. pylori infection and the accompanying inflammation of stomach cells. Induction of oxidative and nitrosamine stress in the host cells has been identified as two of the most common biochemical processes involved in H. pylori-induced gastric carcinogenesis. Therefore, this occurs via several distinct molecular shifts (Butcher et al., 2017; Díaz et al., 2018). H. pylori colonizes the gastric epithelium using urease while residing in the stomach lumen. After H. pylori have bound to epithelial cells and CagA has been injected, the innate and adaptive immune systems have been activated, and IL-8 and other chemokines have been produced (Cadamuro et al., 2014). H. pylori is able to avoid being eliminated by the immune system because it has adapted ways to avoid being recognized by immune sensors, to dampen the activation of immune cells, and to hide from

immune effectors. Polymorphonuclear leukocytes, or PMNs (Portal-Celhay & Perez-Perez, 2006).

There was only limited support for a connection between H pylori and stomach cancer in the first studies to examine this possibility, which was undertaken in the 1980s and early 1990s (Elagan et al., 2020). Scientists have classified gastric cancer into two distinct classes: gastric cardia cancer (cancer of the upper inch of the stomach, where it meets the esophagus) and non-cardia gastric cancer (cancer in all other areas of the stomach). H. pylori infection is associated with an increased risk of developing both types of gastric cancer, particularly non-cardia gastric cancer. Factors such as smoking, alcohol consumption, and dietary habits are also associated with an increased risk of developing gastric cancer (Mukaisho et al., 2015). However, in other studies, the prevalence of H pylori infection was not noticeably higher than that observed in cancer-free controls; the stomach becomes less favorable to H pylori infection due to considerable intestinal metaplasia and hypochlorhydria, gastric cancer develops (Elagan et al., 2020). Three large cohorts with nested case-controls, where serum was banked from non-cancer patients and the cohort was monitored for a decade, offered more robust evidence for H pylori's role in stomach cancer (Moss, 2017).

Chronic infection of the stomach lining by the bacterium Helicobacter pylori has been identified as a major risk factor for the development of stomach cancer, particularly non-cardia gastric cancer, that link to the development of other cancers remains unknown. Evidence suggests that H. pylori infection is associated with a higher risk of pancreatic cancer. However, the results of the studies investigating this link are inconsistent (Elagan et al., 2020; Shin et al., 2010). There is no evidence yet to investigate that the risk factor of colorectal

adenocarcinoma or lung cancer is not associated with the risk of either type of cancer (Elagan et al., 2020; Shin et al., 2010).

2.4 Genetical Perspective and molecular biology of H pylori

The circular chromosomes of H. pylori strain 26695 and J99, which contain 1,643,831 and 1,667,867 base pairs, were used to sequence their individual genomes. The sizes of these organisms are comparable to those of Hemophilus influenzae and approximately one-third more diminutive than those of E. coli (Yamaoka, 2008).

Several enzymes, including urease, vacuolating cytotoxin (VacA), catalase, phospholipases, DNases, and protease, are produced by H. pylori. Urease breaks down urease into ammonium ions, neutralizing the stomach's acidic environment. Because of its importance in H. pylori metabolism and pathogenicity, its requirement for colonization of the gastric mucosa, and its role as a potent immunogen that triggers a robust immune response, urease has been identified as a promising target for the development of effective vaccines (Mobley et al., 2001).

Vacuolating cytotoxin (VacA) is the major virulence factor associated with H. pylori; an enzyme responsible for causing cell vacuolation and disrupting the normal functioning of infected cells (Foegeding et al., 2016). The death-inducing protein VacA is secreted by H. pylori via a type V secretion system. The N-terminal domain of VacA is responsible for binding to host cells, while the C-terminal domain is responsible for the enzyme's toxic activity. The insertion of the VacA pore follows the binding of VacA to host cells into the host cell’s plasma membrane, which leads to the formation of vacuoles in the cytoplasm of infected cells.

VacA is a highly toxic enzyme that damages the host cells by causing the formation of massive vac uoles, which are aberrant fluid-filled regions in the cytoplasm of infected cells (Foegeding et al., 2016). This toxic effect disrupts cellular activity and causes significant damage to the gastrointestinal epithelium, which can result in gastric ulcers, gastric cancer, and other gastrointestinal diseases.

The capacity to deactivate the noxious effects of the hydrogen peroxide produced by the acid-producing cells in the stomach is a significant feature in H. pylori's ability to stay in the stomach by producing the catalase enzyme. H. pylori can survive the oxidative stress caused by the cells in the stomach that create acid because it produces high quantities of catalase. This, in turn, permits the bacterium to survive for extended periods in the stomach, despite significant amounts of hydrogen peroxide. Cell membranes are composed of phospholipids, which phospholipases can degrade.

Phospholipases C and A2 are just two of the many phospholipases that H. pylori can produce. These enzymes are crucial to the bacterium's capacity to infiltrate and thrive in the stomach lining. H. pylori's phospholipases may also play a role in the bacterium's ability to evade the host's immune response. By breaking down the cell membrane of the stomach lining, the phospholipases can interfere with the host's ability to recognize and respond to the bacterium (Kusters et al., 2006). This damage can make the stomach lining susceptible to further injury and infection. Additionally, the enzymes can release fatty acids and other toxic substances that can cause further damage to the stomach lining (Wroblewski et al., 2010). H.pylori produces a wide range of enzymes, including DNases and proteases, which contribute to t he bacteria's capacity to survive in the stomach and cause illness (Wroblewski et al., 2010). The DNases and proteases in the stomach help bacteria by breaking down DNA

and proteins, respectively, providing a favorable environment for the bacteria to proliferate. H. pylori's DNases and proteases can contribute to disease in several ways, one of which is weakenin g the host's immune system. The mucus layer that shields the stomach lining, for instance, can be br oken down byenzymes, which makes the stomach lining more susceptible to injury and infection (Kusters et al., 2006; Wroblewski et al., 2010).

Further, the enzymes can weaken the host's defenses, making it harder for the immune system to er adicate the infection. A novel approach to treating H. pylori infection and preventing associated disorders could emerge from targeting or decreasing the activity of these enzymes.

H. pylori can regulate gene expression through various mechanisms, including transcriptional regulation, post-transcriptional regulation, and translation regulation (Mobley et al., 2001). H. pylori can adapt to the shifting conditions of the stomach by employing several pathways to counteract changes in pH, food availability, and the host immunological response. Since Helicobacter pylori and E. coli are gram-negative bacteria, their transcription and translation regulatory mechanisms are assumed to be comparable (Mobley et al., 2001).

Helicobacter pylori have several mechanisms at its disposal for regulating the activity of its genes. H. pylori, for example, uses transcription factors to control its gene expression. These transcription factors work as on/off switches, turning the expression of particular genes on and off. Non-coding RNAs, which can function either as repressors or activators of gene expression, are also used by H. pylori to control gene expression (de la Cruz et al., 2017; Mobley et al., 2001).

Furthermore, DNA methylation and histone modification are two epigenetic mechanisms that play a critical role in regulating gene expression and have been linked to the

onset of many illnesses, including cancer, which alter the DNA structure and are also used by H. pylori to modulate gene expression. It modifies the structure of genetic material without changing the actual DNA sequence. In addition, to regulate its gene expression, H. pylori uses quorum sensing, a technique by which bacteria can detect the presence of other bacteria in the environment and respond accordingly(Mobley et al., 2001). It takes a complex network of interconnected processes for H. pylori to adjust gene expression in response to changes in its environment. Regulation of genes in H. pylori can also occur post-transcriptionally, meaning that genes can be silenced or activated after transcribed. Modifications to the RNA molecule can regulate many aspects of the RNA-to-protein conversion process, including translation, mRNA stability, and others. Translation regulation in H. pylori is also vital for controlling gene expression. This can involve the control of the initiation of translation, the control of ribosome function, and the translation termination (Albrecht, 2020).

2.5 Immunology of H. pylori

It is possible to distinguish between an innate and an adaptive immune response when confronted with bacterial infections. Once bacteria invade, the body's first line of defense is an immediate and nonspecific reaction to several different bacterial chemicals that signal danger and ultimately lead to the death of the bacteria. In contrast, The innate immune response provides the first line of defense against pathogens and helps to shape the adaptive immune response, which is delayed, antigen-specific, and leads to the activation of T cells, B cells, and memory cells (Portal-Celhay & Perez-Perez, 2006). TLRs, also known as toll-like receptors, are expressed on antigen-presenting cells (APCs) such as monocytes

and DCs. They are responsible for mediating the innate immune system's identification of bacterial substances. This occurs when H. pylori colonize the stomach (dendritic cells) (Portal-Celhay & Perez-Perez, 2006). Mono-cytes and other antigen-presenting cells (APCs) are essential for initiating the adaptive immune response. When they come into contact with bacteria, they secrete pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1 beta, and interleukin (IL)-8, which help to activate T cells and B cells and initiate an immune response. These cytokines have been demonstrated to rise in concentration during an H. pylori infection, where they function as local chemo-attractants to bring about an influx of granulocytic cells (Crabtree, 1996; Portal-Celhay & Perez-Perez, 2006). A number of inflammatory cells and mediators are therefore activated, along with the transcription factor nuclear factor (NF)-B, pro- and anti-inflammatory cytokines, cell proliferation and survival factors, and other components of the host's immunological response (Cadamuro et al., 2014). Several studies sought that TLR4 is the specific 'pathogen-recognition molecule' (PRM) of Gram-negative LPS (lipopolysaccharide), which has been the focus of a significant portion of the research that has been conducted on innate immune responses to H. pylori in epithelial cells (Portal-Celhay & Perez-Perez, 2006; Su et al., 2003). The lipopolysaccharide (LPS) of Helicobacter pylori is primarily identified by toll-like receptor 4 (TLR4); however, structural alterations to the LPS can affect this recognition and inadequately trigger the human immune response, increasing the bacterial evasion and pathogenicity. When the immune system is activated in response to a bacterium, oxidative and genotoxic stress is raised, which can cause damage to cells, including DNA, and promote the development of mutations that can lead to carcinogenesis. This is due to the release of pro-inflammatory cytokines, reactive oxygen species, and other molecules that can cause damage to cells (Cadamuro et al., 2014); This damage can lead to mutations and genetic instability, which can, in turn, lead to the

development of cancer. Phosphorylation then dissociates protein complexes, and fi NF-kappa B (NF-kB) is translocated into the nucleus, activating the expression of genes associated with the inflammation response. As a virulence factor, CagA increases the production of cytokines like IL-1 and IL-8, which play a role in the inflammatory response (Cadamuro et al., 2014; Isomoto et al., 2010). Carcinogenesis-relevant NF-κB activationcan endow bacteriawith a proliferative phenotype which maystimulatethe production of growth factors and prevent cell death (apoptosis) (Cadamuro et al., 2014). Since CagA deregulates cell signaling pathways and promotes the development of oncogenic cells, it plays a significant role in the pathogenesis of H. pylori infection (Ding et al., 2010). Upon NF-kappaB activation and cytokine production, H. pylori infection triggers chemotaxis of monocytes/macrophages, infiltration of polymorphonuclear leucocytes, recruitment of neutrophils and lymphocytes, and induction of IL-8 and IL-10 production by macrophages (Cadamuro et al., 2014). Due to the low frequency with which H. pylori invades the stomach mucosa, the host response is predominantly driven by bacterial adhesion to epithelial cells. HSP (heat-shock protein), urease, and lipopolysaccharide (LPS) are only a few of the antigenic chemicals produced by the organism that can be taken up and processed by lamina propia macrophages and activate T-cells (Higashi et al., 2004). Cellular disruption, especially near tight epithelial junctions, promotes lamina propia antigen presentation and immunological stimulation along with increasing IL-1, IL-6, TNF-α, ad IL-8 production (Cadamuro et al., 2014; Higashi et al., 2004). Interleukins, including IL-1, IL-6, IL-10, and IL-12p40 (partially secreted as IL-23) are released by monocytes along this pathway. Similarly, dentritic cells (DCs) release IL-1, IL-6, IL-10, IL-12p40, IL-12p70, and IL-23. While M1 macrophages can regulate inflammation through the production of IL-1 and other cytokines, M2 macrophages, which cannot produce IL-10 but fewer pro-inflammatory cytokines, causing an ongoing inflammatory response (Cadamuro et al., 2014; Fehlings et al., 2012). Activated macrophages/dendritic cells

secrete TNF- and IL-6, which promote the differentiation of Th17 cells; in contrast, IL-12 and interferon (IFN)- stimulate the development of Th1 cells (Cadamuro et al., 2014). There is additional evidence that antigen-specific regulatory T lymphocytes are recruited to aid in long-term stomach colonization either by direct cell-to-cell contact or the secretion of immunosuppressive cytokines like transforming growth factor (TGF)-1 and IL-10 (Bornschein et al., 2010). A more robust mucosal Th1 response has been associated with progression to atrophic gastritis and cancer. This is thought to occur because Th1 cells are involved in the induction of chronic inflammation, which can lead to tissue damage and the promotion of carcinogenesis (Portal-Celhay & Perez-Perez, 2006). Th1 responses are triggered by the vast majority of internal microorganisms, while The majority of external pathogens elicit th2 responses. Since H. pylori do not invade host tissue and infection is accompanied by an intense humoral response, it stands to reason that the gastric mucosa colonized by H. pylori would primarily exhibit a Th2 response. In addition to Th1 cells, other immune cells such as T regulatory (Treg) cells and Th17 cells have also been implicated in Helicobacter pylori-induced atrophic gastritis and cancer pathogenesis. Treg cells can suppress the immune response and promote immune tolerance, allowing Helicobacter pylori to persist in the stomach and promote chronic inflammation. Th17 cells, on the other hand, can produce pro-inflammatory cytokines that contribute to tissue damage and cancer development.

Additionally, microRNAs (miRNAs) constitute a crucial class of immune regulators. Some microRNAs (miRNAs) have been shown to play a regulatory role in H. pylori infection and related illnesses, as reported recently (Cadamuro et al., 2014). Therefore, H. pylori bacteria both influence and are affected by miRNAs, which modulate the infection.

2.6 Epigenetic modifications of gastric epithelial cells induced by H. pylori

Epigenetic modifications describe alterations in gene expression that do not result from shifts in the DNA sequence but rather from alterations in DNA or the proteins that interact with it. Molecules such as cytokines, chemokines, free radicals (ROS and NOS), prostaglandins, growth factors, and matrix metalloproteinases are among the numerous potentially significant intermediates produced during inflammation (M. A. Valenzuela et al., 2015). DNA methylation and histone modifications are two examples of epigenetic alterations contributing to cancer's formation and progression. These modifications encourage leukocyte recruitment, neo-angiogenesis, proliferation, survival, invasion, and metastasis (M. A. Valenzuela et al., 2015; Yousefi et al., 2019).

It has been established that DNA methylation contributes to controlling genes implicated in inflammation and carcinogenesis when H. pylori are present. Alterations in histone modifications have been linked to H. pylori-induced alterations in gene expression, such as the upregulation of genes associated with the inflammatory response and the downregulation of genes involved in cell development and differentiation (M. A. Valenzuela et al., 2015). Moreover, H. pylori-mediated epigenetic regulation has been linked to non-coding RNAs such as microRNAs. Certain genes are involved in gastric cell proliferation, differentiation, and inflammation that can be targeted, and their expression is regulated by these short RNA molecules (M. A. Valenzuela et al., 2015).

Multiple genetic and epigenetic alterations have been identified in gastric carcinogenesis, including alterations in tumor suppressor genes, oncogenes, DNA repair genes, and genes involved in cell cycle regulation. These alterations can lead to uncontrolled

cell growth and division and contribute to gastric cancer's development and progression. Chromatin modifications, facilitated by chromatin-remodeling complexes and histone-modifying enzymes, and DNA methylation, steered by DNA methyltransferases, are two examples of epigenetic mechanisms that can function at the gene level (B. Yousefi et al., 2019). DNA methylation is an epigenetic mechanism that involves adding a methyl group to cytosine residues in DNA, typically in CpG dinucleotides. Methylation of promoter regions can block the binding of transcription factors and RNA polymerase, leading to gene silencing. This can be important for maintaining genome stability and preventing the transcriptional deregulation of neighboring genes, especially in regions with a high density of repetitive DNA sequences that can otherwise mediate illegitimate recombination events. Chromatin remodeling is a process where proteins interact with DNA and can alter gene expression. Histone modification is a process where histone proteins interact with DNA and can alter gene expression (B. Yousefi et al., 2019).

The involvement of microRNAs (miRNAs) has been widely documented in gastric cancer and H. pylori infection. miRNAs are short RNA molecules that regulate gene expression by binding to the 3' untranslated region (UTR) of target mRNAs and inhibiting their translation or promoting their degradation. Alterations in miRNA expression have been found in gastric cancer, and some miRNAs have been identified as oncogenes or tumor suppressors. Additionally, H. pylori infection has been shown to alter the expression of miRNAs in the gastric mucosa, potentially contributing to the development and progression of gastric cancer (Noto & Peek, 2012). To survive in the stomach, H. pylori must undergo a number of epigenetic modifications that can alter the expression of genes involved in inflammation, cell proliferation, and death.

2.7 Key molecular and cellular factors enhance the susceptibility of H. pylori infection

Several molecular factors can enhance the susceptibility of H. pylori infection, which is further help in colonization with gastric epithelial cells. The surface of the stomach epithelium expresses Lewis's antigens, which H. pylori bind to specifically. H. pylori infection is more common in people who exhibit the Lewis b antigen. Multiple adhesins bind to specific host cell receptors, including adhesin BabA and SabA, which bind to the Lewis b antigen on gastric epithelial cells and to sialyl-Lewis x antigens, respectively (Doohan et al., 2021). Adhesins can promote bacterial colonization and facilitate the delivery of virulence factors. Mutations in the IL-1β genes encoding cytokines found in the host may contribute to a higher vulnerability to H. pylori infection and related gastritis. H. pylori produces several virulence factors, such as vacuolating cytotoxin A (VacA) and the cag pathogenicity island, which can enhance its ability to colonize and infect the gastric mucosa (Chang et al., 2006). H. pylori expresses several Outer membrane proteins (OMPs) that enable it to attach to the gastric epithelium and evade host immune responses. OMP CagL is a component of the type IV secretion system (T4SS) that H. pylori use to inject the CagA protein into host cells. CagA can then manipulate host cell signaling pathways and contribute to H. pylori-induced inflammation (Ansari & Yamaoka, 2019; Radosz-Komoniewska et al., 2005).

Recent studies have suggested that non-coding RNAs (ncRNAs), such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), may also can enhance the susceptibility of H. pylori infection (C. Wang et al., 2021; L. Yousefi et al., 2021). The well-known miRNA, such as miR-155 and miR-223 is highly expressed in gastric epithelial cells infected with Helicobacter pylori. miR-155 has been demonstrated to increase H. pylori proliferation and the production of pro-inflammatory cytokines like interleukin-8 (IL-8). In

addition, miR-223 has been shown to inhibit the expression of CXCL12, a chemokine that promotes the recruitment of immune cells to the site of infection. Therefore, miR-155 and miR-223 overexpression contributes to gastric inflammation and increases susceptibility to H. pylori infection (C. Wang et al., 2021).

lncRNA GAS5 is downregulated in H. pylori-infected gastric epithelial cells whereas, lncRNA H19 is upregulated in H. pylori-infected gastric epithelial cells and has been shown to regulate the expression of genes involved in cell proliferation, apoptosis, and inflammation. Downregulation of GAS5 in H. pylori-infected cells can lead to increased cell proliferation and decreased apoptosis, Thus, upregulation of H19 can enhance the susceptibility of H. pylori infection and contribute to the development of inflammation in the stomach (Radosz-Komoniewska et al., 2005; L. Yousefi et al., 2021).

Several cellular factors can enhance the susceptibility of H. pylori infection, including Blood type, Cytokines, Gastric acid secretion, Mucin secretion, Cell surface receptors, Innate immune response, and T cell response. Research has shown that individuals with blood type O are more susceptible to H. pylori infection than individuals with other blood types (Chakrani et al., 2018). Interleukin-1 (IL-1), interleukin-8 (IL-8), and tumor necrosis factor-alpha (TNF-) are all cytokines produced by the gastric epithelium that has been demonstrated to increase susceptibility to H. pylori infection through their promotion of inflammation and cell growth (Su et al., 2003). Large glycoproteins called mucins function as a barrier in the stomach lining. H. pylori is able to attach to mucins and use them as a food supply. Those with abnormalities in mucin glycosylation patterns or lower mucin secretion may be more vulnerable to H. pylori infection. Defense against bacterial infections

begins with the body's innate immune system. By regulating the expression of Toll-like receptors and other genes involved in immunity, H. pylori can avoid being destroyed by the body's innate immune system (TLRs). Someone with a compromised innate immune response may be more vulnerable to H. pylori infection (Su et al., 2003). A robust T cell response can be triggered in the stomach lining by H. pylori infection. However, persistent H. pylori infection and chronic inflammation can occur in those with a compromised T cell response or an imbalance between T helper cell (Th) 1 and Th2 cytokines (Radosz-Komoniewska et al., 2005). Overall, the susceptibility of H. pylori infection is influenced by various factors, including molecular and cellular aspects. Identifying these factors can help develop new strategies to prevent and treat H. pylori infection.

2.8 Factors involved in H. pylori drug resistance

Bacterial drug resistance is a complex phenomenon that can involve a wide range of factors. The following are some of the most important aspects that can contribute to developing drug resistance in bacteria. The most widely increasing factor is overdeveloping of antibiotics can create selective pressure that favors the survival and growth of resistant bacteria (Shahriar et al., 2021), however, bacteria can acquire mutations in their DNA that confer resistance to specific antibiotics or multiple classes of antibiotics by exchanging their genetic material through processes such as conjugation, transduction, and transformation, which can lead to the spread of antibiotic resistance genes among different bacterial species (Hasan et al., 2020; Shahriar et al., 2021). The ability to produce biofilm is another factor involved in bacterial multidrug resistance. Embedded in a matrix of extracellular material, bacterial populations called biofilms can be quite resilient (Hasan et al., 2020; Shahriar et al., 2021). Biofilms can aid

horizontal gene transfer, and their physical barrier can reduce the effectiveness of antibiotics. In order to circumvent the effects of antibiotics, bacteria can employ an efflux pump mechanism to expel the medications from their cells before they can reach their intended targets (Moniruzzaman et al., 2022; Uddin et al., 2022). In addition, bacteria can produce enzymes that can modify or degrade antibiotics, rendering them ineffective, and can also change their cell walls or membranes to reduce the uptake of antibiotics. After invading the stomach mucosa, H. pylori secrete proteins, polysaccharides, extracellular DNA (eDNA), and other components to produce extracellular polymeric substances (EPS), which the bacteria then wrap around and adhere to each other to form biofilms (Hasan et al., 2020).

Several research was carried out to ascertain the frequency of resistance, its underlying mechanism, the various techniques for detecting it, as well as its implications for therapeutic practice. Clarithromycin- and metronidazole-resistant strains of H. pylori are the most prevalent types of antibiotic-resistant H. pylori strains; the prevalence of these strains is relatively high, although it varies from population to population (Megraud, 2004). Inheriting antibiotic resistance in H. pylori is a complex process involving multiple genetic factors. Multiple genes involved in the evolution of antibiotic resistance in H. pylori have been identified by genome-wide association studies (GWAS). A recent study conducted on 140 clinical H. pylori isolates found that there was more than 99% similarity between phenotypic antibiotic susceptibility testing (AST) results for clarithromycin, levofloxacin, and rifampicin and certain single nucleotide polymorphisms (SNPs) identified in the 23S rRNA, gyrA, and rpoB genes. This study used whole genome sequencing (WGS) to compare the AST results and the presence of the predicted genetic determinants. The results suggest that WGS can be used to predict antibiotic resistance in H. pylori (Lauener et al., 2019; Megraud, 2004).

2.9 Antibiotic Treatment against H. pylori / Therapeutics against H. pylori

Infections caused by H. pylori are notoriously challenging to treat successfully without antibiotics. Antibiotic treatments for H. pylori often involve a combination of two or more antibiotics and an acid-reducing medicine, such as a proton pump inhibitor. This happens because H. pylori are most effectively treated by inhibiting acid production in the stomach (PPI). Several antibiotics, including Clarithromycin, Amoxicillin, Metronidazole, and Tetracycline. The macrolide antibiotic clarithromycin is effective against the bacterium known as Helicobacter pylori. In most cases, a PPI and either amoxicillin or metronidazole will be prescribed alongside clarithromycin. Antibiotics like amoxicillin, which belong to the penicillin family, are frequently prescribed alongside clarithromycin and proton pump inhibitors. Clarithromycin and proton pump inhibitors (PPIs) are typical partners for the antibiotic metronidazole. But resistance to metronidazole is on the rise in several regions worldwide. At the same time, Tetracycline is an antibiotic frequently administered alongside a proton pump inhibitor (PPI) and metronidazole treatment. Standard treatment for Helicobacter pylori infection typically involves a combination of three drugs: a proton pump inhibitor (PPI), amoxicillin, and clarithromycin. However, due to the increasing prevalence of antibiotic-resistant strains of H. pylori, several other drug combinations are also used to treat the infection. These alternative regimens may include bismuth quadruple therapy, sequential therapy, or concomitant therapy. Treatment choice depends on factors such as local antibiotic resistance patterns, patient-specific factors, and drug availability. These include quadruple, sequential, and concurrent therapy regimens enhanced with metronidazole, clarithromycin, and levofloxacin (Goderska et al., 2018; S. Y. Kim et al., 2015). This treatment seems to last 14

days and consists of tetracycline, metronidazole antibiotics, bismuth, and a proton pump inhibitor (Goderska et al., 2018). This treatment is suggested as the primary therapeutic approach in areas where clarithromycin resistance is high. Furthermore, if the initial treatment using the standard triple therapy for H. pylori was unsuccessful, this treatment is favored as the subsequent treatment option (S. Y. Kim et al., 2015). This treatment is effective even without the use of clarithromycin, which is the antibiotic that presents the most significant challenge in terms of drug resistance. Levofloxacin, a broad-spectrum quinolone, is being utilized to replace clarithromycin in triple or sequential H. pylori eradication regimens because of the rise in clarithromycin resistance. In regions where levofloxacin resistance is low [less than 10%], levofloxacin-containing therapy may have an eradication rate of greater than 90% (Goderska et al., 2018).

In order to combat the spread of H. pylori strains that are resistant to standard treatment methods, physicians are adopting new approaches. To better eradicate bacteria and reduce antibiotic side effects, scientists have developed and used novel and more effective treatments, such as probiotics (Goderska et al., 2018; Shahriar et al., 2019). The stomach's acidity and the protective barrier provided by the gastric mucosa are the body's first line of defense against harmful pathogens. The gut mucosal barrier is the first line of defense against pathogens, and it was speculated that probiotics could strengthen this barrier by increasing the production of antimicrobial compounds that would compete with H. pylori for adhesion receptors, thereby bolstering mucin formation and ensuring the barrier's integrity (Goderska et al., 2018).

Probiotics can restrain the growth of H. pylori by producing antibacterial substances and short-chain fatty acids. In the process of breaking down sugars, probiotics produce short-chain fatty acids, including acetic acid, propionic acid, and lactic acid, which lowers the pH of the digestive tract. Scientists were the first to discover that one strain of Lactobacillus had an antagonistic impact on H. pylori via short-chain fatty acids (Bhatia et al., 1989). However, it is important to note that not all probiotics have the same effects on H. pylori. Further research is needed to determine which specific strains and formulations are most effective. Additionally, while probiotics may have the potential as a complementary therapy for H. pylori infection, they are not a substitute for standard medical treatment.

2.10 Current development of new drug compound/ antimicrobial peptide against H. pylori that inhibits H. pylori

Several ongoing research and development projects focus on developing new pharmacological compounds and antimicrobial peptides that target Helicobacter pylori. Several new drug compounds, including Talicia (RHB-105), PyloriX (XC005), Arikayce (amikacin liposome inhalation suspension), ACU-D1, and Nitazoxanide in development that are aimed at inhibiting H. pylori. Talicia is a medication comprising a proton pump inhibitor, omeprazole, in addition to two antibiotics, amoxicillin and rifabutin. It is currently in Phase 3 clinical trials and has shown promising results in eradicating *H. pylori*.

Talicia is a combination of rifabutin, amoxicillin, and omeprazole and is currently in Phase 3 clinical trials. The combination of the three drugs has shown promising results in eradicating H. pylori infections. Talicia is more effective than other treatments, such as triple therapy and bismuth-based quadruple therapy, and has fewer side effects (Gisbert, 2020; Saleem & Howden, 2020).

PyloriX is a small molecule inhibitor that targets the HtrA enzyme, which is essential for the growth of H. pylori. Preclinical research has shown that it is effective against several different H. pylori strains, and it is currently being tested in Phase 1 clinical trials. PyloriX works by blocking the activity of the HtrA enzyme, preventing the bacteria from being able to grow and survive in the stomach. This has the potential to be a safe and effective treatment for H. pylori infections (Sutton & Boag, 2019; Zarzecka et al., 2019).

ACU-D1 is a novel small chemical inhibitor that targets the protein FtsZ, which is involved in bacterial cell division. Preclinical research has demonstrated that it is effective against H. pylori and is currently being tested in Phase 1 of clinical trials. ACU-D1 works by blocking the activity of FtsZ, preventing the bacteria from being able to divide and replicate. This could provide a safe and effective treatment for H. pylori infections (Sutton & Boag, 2019). However, Nitazoxanide and Arikayce is an FDA-approved antiparasitic drug that has also shown activity against H. pylori in preclinical studies. It is currently being evaluated in clinical trials for this indication. The drug works by blocking the activity of the enzyme thiol reductase, which is necessary for the survival of H. pylori. This could provide a new and effective treatment for H. pylori infections.

A great deal of research is currently being done to develop new drug compounds and antimicrobial peptides that can inhibit H. pylori. Bactenecin-3 is a modified version of a naturally-occurring peptide that has been shown to have antibacterial and anti-inflammatory properties. This peptide has been studied for its potential use as an antimicrobial agent against various bacterial pathogens. Studies have shown that Bactenecin-3 effectively kills many bacteria, including Escherichia coli, Staphylococcus aureus, and Mycobacterium tuberculosis. Additionally, certain plant extracts, such as those from Eucalyptus, have been shown to have antimicrobial activity against H. pylori (Bonifácio et al., 2014; Y.-C. Wang, 2014). These compounds may have potential therapeutic applications for treating H. pylori infections. Another promising new drug compound is a synthetic peptide called HP-11, which is effective against both H. pylori and Helicobacter species. Additionally, research is being done on compounds derived from natural products, such as the lignin derivative lignin-2, which has been found to have antimicrobial activity in vitro (Bonifácio et al., 2014; Cardos et al., 2021). These compounds could be used to develop new treatments for H. pylori infections.

2. 11 Recent Perspective of AMPs against H. pylori

Antimicrobial peptides (AMPs) have been studied as a potential therapeutic option for Helicobacter pylori infections (H. pylori). Antimicrobial peptides (AMPs) are small molecules with antimicrobial activity and can treat bacterial infections. AMPs have been found to have promising antibacterial, antiviral, and antifungal properties (Chianese et al., 2022). Over the past few years, researchers have been investigating the possibility of using antimicrobial peptides (AMPs) instead of conventional antibiotics in order to treat Helicobacter pylori infections.

AMPs are naturally occurring peptides that are part of the innate immune system and can rapidly kill or inhibit the growth of a wide range of pathogens, including H. pylori. They have several advantages over conventional antibiotics, including broad-spectrum activity, low propensity for inducing resistance, and low toxicity to host cells (Liang et al., 2022). Several AMPs, including cathelicidins, defensins, and histatins, have demonstrated intense antibacterial action against H. pylori in vitro (Gopal et al., 2014; Liang et al., 2022). In vivo experiments with animal models have shown that AMPs are effective against an H. pylori infection. However, there have only been a limited number of clinical trials conducted to determine the efficacy of AMPs in treating H. pylori infection in humans (Liang et al., 2022; Zhang et al., 2021).

One clinical trial evaluated using a synthetic AMP called WLBU2 in patients with H. pylori infection. The study found that WLBU2 was well-tolerated and had a significant bactericidal effect against H. pylori, reducing the bacterial load in the stomach (Elsalem et al., 2022). In addition, patients with H. pylori infection were studied using lactoferricin B, an AMP produced from bovine lactoferrin. According to the results, H. pylori might be eradicated from the body more effectively using combo therapy than with antibiotics aloe (Okuda et al., 2005; Wronowski et al., 2021). AMPs can disrupt the bacterial cell membrane, leading to bacterial death. AMPs can also modulate the host immune response, leading to increased clearance of the bacteria. Furthermore, AMPs effectively reduce inflammation and decrease the concentration of harmful toxins produced by H. pylori.

Cecropin-like amino-terminal polypeptide (CLAMP) is a type of antimicrobial peptide initially identified in insects (Erviana et al., 2021; FINK et al., 1989; Mobley et al., 2001). However, a recent study reported the presence of a cecropin-like peptide with antibacterial activity in the ribosomal protein RpL1 of H. pylori. In this study, the researchers found that the amino-terminal region of RpL1 from H. pylori exhibited significant antimicrobial activity against several bacterial strains, including H. pylori (Erviana et al., 2021). Further investigation revealed that the antimicrobial activity of the amino-terminal region was due to the presence of a cecropin-like peptide, which the researchers named CLAMP. It has exhibited potent bactericidal activity against antibiotic-sensitive and -resistant strains of H. pylori and other bacterial species (Mobley et al., 2001). The mechanism of action of CLAMP was found to involve disruption of the bacterial cell membrane, leading to cell death.

AMPs (antimicrobial peptides) are naturally occurring peptides found in many different organisms, including humans, and have antimicrobial properties. They have been proposed as a promising alternative to conventional antibiotics for treating various bacterial infections, including Helicobacter pylori (H. pylori) infection. Though further clinical trials are needed to evaluate their efficacy and safety in humans, the preclinical data and early clinical trials suggest that AMPs may offer a new approach to managing H. pylori infection.

2.12 Challenges of H. pylori treatment

Several promising therapies against H. pylori have been developed but still have some challenges to eradicating the infection of H. pylori. It is well established that H. pylori are capable of developing antibiotic resistance, which can make treatment more challenging. In certain areas, resistance to clarithromycin, one of the antibiotics utilized in the conventional

treatment regimens for H. pylori, is particularly widespread (Bagirova et al., 2017; Guevara & Cogdill, 2020). Though antibiotics used to eradicate H. pylori have negative effects, including diarrhea, constipation, and stomach pain. Due to the potential severity of these adverse effects, medication may need to be terminated, which may be another challenge in treating H. pylori Infection. Patients successfully treated for H. pylori may still be at risk for reinfection if they consume contaminated food or drink after completing their treatment. Other challenges include difficulty in diagnosing the infection due to its slow-growing nature and difficulty in determining the success of the treatment, as the infection can recur after treatment is completed (Guevara & Cogdill, 2020).

Despite the potential of AMPs as a new class of therapeutics for the treatment of H. pylori infection, several challenges need to be overcome. One of the challenges is the production of AMPs on a large scale. Another challenge is the development of delivery systems that can effectively target the bacteria in the stomach. Additionally, the potential toxicity of AMPs needs to be carefully evaluated.

CHAPTER III

MATERIALS AND METHODS

3.1. Bacterial Strains and Cell Culture

Alcaligenes faecalis and Proteus vulgaris were acquired from the Microbiology Laboratory, UTRGV. Staphylococcus epidermitis (ATCC 12228), Mycobacterium smegmatis (ATCC 14468), Bacillus cereus (ATCC 14579), Helicobacter pylori (NCTC 11639), and human embryonic kidney cells (HEK-293) were purchased from American Type Culture Collection (ATCC). Recombinant Escherichia coli BL21 (DE3) strain was obtained by Novagen.

3.2 Culture Conditions

H. pylori strains were cultured in Helicobacter Pylori Agar/ Broth Medium (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China) supplemented with 7% FBS and antimicrobial agents (10 mg/l vancomycin, 5 mg/l trimethoprim, 5 mg/l cefclidine, 5 mg/l amphotericin B) at 37 °C under microaerobic condition created by sealing the plates or test tubes in an anaerobic box using by an AneroPak environment generator (Mitsubishi Gas Chemical) for 48–72 h. E. coli BL21 (DE3)-CagA was cultured with kanamycin (50 µg/ml) in nutrient agar/broth media. IPTG (1 mM) was added to induce the expression of HpIF-1 for subsequent experiments. HEK-293 cells were cultured in DMEM medium supplemented with 10% FBS at 37 °C in a humidified atmosphere of 5% CO_2.

3.3 Hp-IF1 Peptide and Reagents

The peptide derived from the short α-helix (NH2-CRIS GKMRMHYIRI ALGAHISGKMRKNYIRILTG-COOH) of H. pylori IF1, was purchased from APeptide Co. Ltd. (China). The peptide was chemically synthesized and purified by a preparative HPLC method. HPLC purification was performed using SinoChromCore 120 C18ODS-BP (5 µm, 250 × 4.60 mm) column. The peptide was eluted using a gradient of buffer A (0.1% trifluoroacetic acid in 100% acetonitrile) and B (0.1% trifluoroacetic acid in 100% Water) with a flow rate of 1 mL/ min as a single peak (UV Absorbance at 220 nm) via HPLC with >95% purity. The peptide was verified by ESI-MS (SHIMADZU LCMS-20210EV) with the experimental value (2005.481958.38) close to the theoretical mass (2005.491958.36).

3.4 Gene Synthesis and PCR

The oligonucleotide assembly method has been used to synthesize genes in P. aeruginosa (Valdez et al., 2021). Genomic DNA of H. pylori 43504, ATCC, RPH 13487 has been used in this study. Oligonucleotides of Hp-IF1 from Invitrogen (Forward: cat#A15612-UPXKMYZ; Reverse cat#A15612-UPYRGJX) has been used to amplify the gene of interest. To transform an H. pylori strain, a total of 1 mL of 100 mM forward and reverse oligonucleotides comprising the genes to be synthesized was combined with vector fragments generated by polymerase chain reaction. The concentrations of PCR-amplified DNA were measured using a Qbit fluorometer (Molecular Probes, Eugene, OR, USA), and PCR confirmed the DNA sequence. DNA amplification of the region and DNA sequencing was performed by Functional Bioscience (Madison, WI).

3.5 Preparation of H. Pylori IF1

The H. pylori gene encoding IF1 was amplified by PCR (Bio-Rad MJ Mini Thermo Cycler) from H. pylori (ATCC 43504) genomic DNA using the forward primer (5'-G GCT AGC ATG GCA AGA GAT GAT GTT ATA G-3') which added a NheI restriction site to the 5' end of the gene contained (29 bp, 44.8% GC) and the reverse primer (5'- C CTC GAG TTT ATA TCT AAA AGT TAT CCG ACC C -3') which added an XhoI restriction site to the 3' end of the gene contained (32 bp, 40.6% GC). The PCR product was inserted into a pET-24b(+) plasmid (**Figure 1**) (Novagen) digested with NheI/XhoI placing the gene upstream of a sequence encoding six histidine residues (Y. Hu et al., 2016; Valdez et al., 2021).

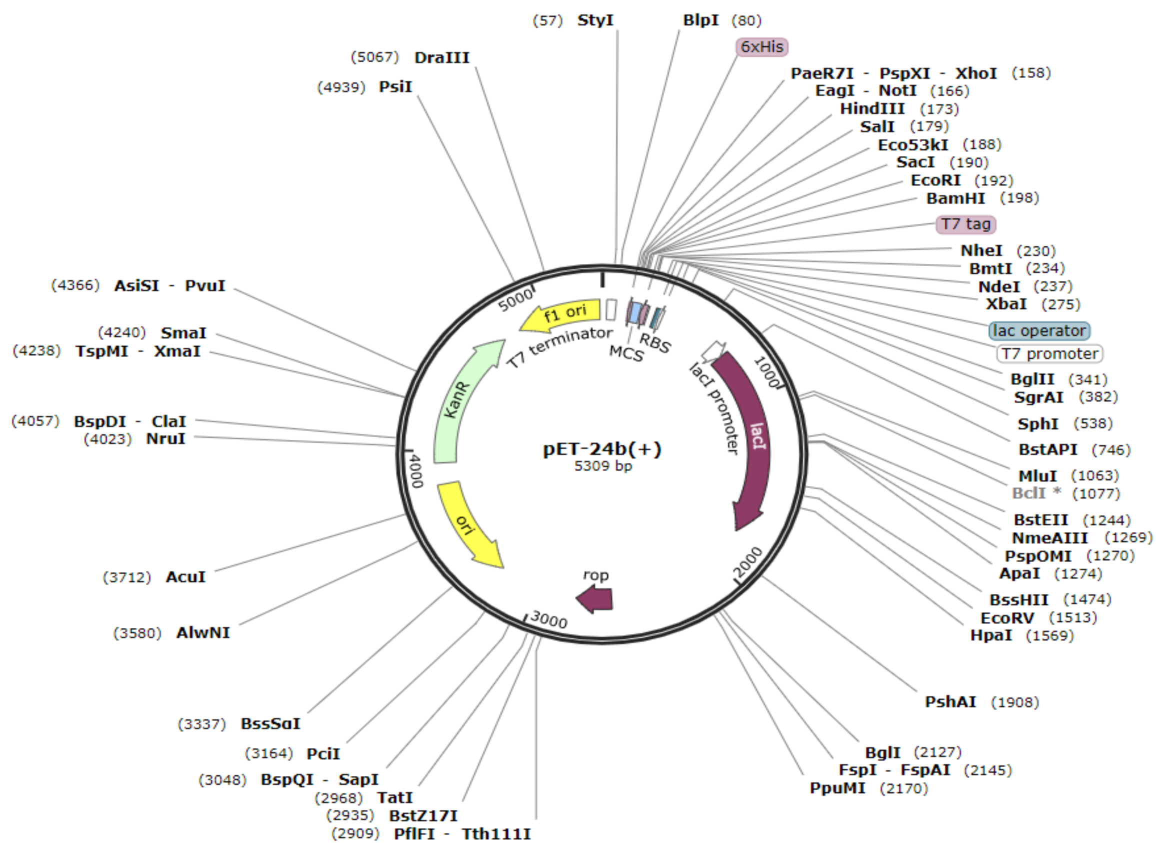


Figure 1: pET-24b(+) plasmid, which is Kanamycin gene resistant.

3.6 Recombinant HpIF-1 Protein Expression and Purification

Constructed Recombinant DNA Plasmid was subcloned and subsequently transformed into E. coli Rosetta 2(DE3) competent cells (Novagen) for overexpression and preparation of the HpIF-1 protein. To produce IF1 for use in structural studies,15N-labeled or 13C/15N-labeled Pa-IF1 was prepared using the high cell density method (Sivashanmugam et al., 2009). Using a previously established technique, 15N-labeled HpIF-1 was overexpressed uniformly (Sivashanmugam et al., 2009). The cells were grown in 2 L of LB Broth supplemented with 50 L/ml kanamycin until they reached an optical density (A600) of 0.8–1.0. After being centrifuged at 6000 rpm for 15 minutes at 4 °C, the cells were resuspended in 2 liter of freshly made M9 minimal media to grow for 30 min at 37 °C. Isopropyl β-D-1-thiogalactopyranoside (IPTG), with a final concentration of 0.5 mM, was shown to be the most effective in inducing the expression of the target protein. Bacterial cultures were harvested by centrifugation after 2 hours of postinduction of incubation at 37 °C. When the cells were lysed, the HpIF-1 proteins were initially precipitated for the purification process by adding ammonium sulfate at a concentration of 40%, as was previously described (Palmer et al., 2013). The precipitated proteins were extracted by centrifugation around 17500g for 1 hour at 4 °C, redissolved, and then purified using nickel-nitrilotriacetic acid affinity chromatography. HpIF-1 was purified to >98% homogeneity, determined by SDS-PAGE.

3.7 Protein Purification Size-Exclusion Chromatography (SEC)

As we described previously, during the plasmid construction (Y. Hu et al., 2016), when our protein of interest sequence inserted into pET24b(+) vector; it adds these three amino acids first and histidine tag on the end. When we added the protein (HpIF-1) in equilibrated resin with nickel ion and the targeted protein only can binds which has histidine tag residues only. Further we washed the column with wash buffer to elute any protein that do no contains histidine tag. And lastly we used elution buffer that has 500mM of imidazole (mimic of histidine tag) to elute the protein contained histidine tag residues and prepared the samples for FPLC (First Protein Liquid Chromatography). We loaded HpIF-1 eluted samples into the loop, which goes into the column along with the buffer of choice (PBS Buffer, Stable pH 7.4).

3.8 NMR Spectroscopy

NMR samples of Hp-IF1 (1.0 mM, unlabeled, 15Nor 15N/13C-labeled) were prepared in 90%/10% H2O/ D2O or 100% D2O with 20 mM phosphate (pH 5.1), 100 mM KCl. A D2O-exchanged sample was prepared for H-D exchange experiments by freezing the Hp-IF1 sample followed by lyophilization and suspension in 99.9% D2O. All NMR experiments were performed at 298 K on a Bruker AVANCE III Ultrashield Plus 600 MHz spectrometer equipped with a double resonance broad band room-temperature probe (BBO), or a Bruker AVANCE 700 MHz or 600 MHz spectrometer, both equipped with a four channel interface and triple resonance cryogenic probe (TXI) with triple-axis (X,Y,Z) pulsed field gradients. Backbone and side-chain NMR chemical shift assignments were obtained by analyzing the following spectra: HNCACB, CBCA(CO)NH, HNCO, HBHA(CO)NH, and 15N-HSQC-TOCSY (mixing time of 60 ms) spectra.

3.9 Computational Analysis of HpIF-1 and their Complex Model of 30s subunit

The structure model of Hp-IF1 in complex with the 30S was created based on computational analysis and the crystal structure of IF1 from T. thermophilus bound to the 30S (Carter et al., 2001). Hp-IF1 predicted structure was used to replace T. thermophilus IF1 in the complex structure (PDB 1HR0) by PyMOL (Version 2.4.0a0 Open-Source) (Valdez et al., 2021).

3.10 Antimicrobial activity of HpIF-1 peptide against Bacteria (MIC)

The 96-Well Microtiter Microplates from Thermo Fisher Scientific were utilized in order to test the effectiveness of a HpIF-1-derived peptide in inhibiting the growth of bacteria. The representative bacteria for the test included Alcaligenes faecalis, Proteus vulgaris, Staphylococcus epidermitis (ATCC 12228), Mycobacterium smegmatis (ATCC 14468), Bacillus cereus (ATCC 14579), and Helicobacter pylori (NCTC 11639). The E. coli strain was BL21(DE3) from Invitrogen (One Shot BL21(DE3), cat. no. C600003), which is descended from the E. coli B strain and commonly used for high-level expression of recombinant proteins. The other two strains, A. faecalis and P. vulgaris, were obtained from The Microbiology Laboratory at Department of Biology, The University of Texas Rio Grande Valley, which have been used in General Microbiology Lab Course. The Microtiter plate was set up by adding 50 μL bacterial cultures (LB broth with initial OD600 value ~0.1) into each well, which contained different concentrations of the HpIF-1 peptide or one of the control substances (DMSO, ampicillin, and/or kanamycin). Serial 2-fold dilutions from the first well across the plate diluted the HpIF-1 peptide. DMSO and ampicillin and kanamycin (0.01 g/mL (28.6 mM) in each well) were used as the negative and positive control, respectively. The microtiter plate was incubated at 37 °C overnight and further analyzed by measuring the absorbance.

3.11 Cytotoxicity assay of HpIF-1 peptide

The toxic effect of HpIF-1 peptide on the growth of human cell cultures was tested using human embryonic kidney cells (HEK-293). Before the assay, HEK-293 cells in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and Penicillin-Streptomycin Solution were plated in 96-well plates with 14,000 cells per well. The plates were incubated in a CO2 incubator under standard tissue culture conditions (5% CO2 and 37 °C) for 18 hours. The peptide was dissolved in DMSO and diluted to yield a final concentration from 3 mg/ml (1.5 mM) to 1 µg/ml (0.5 µM) in the cell cultures for the assay. Cells were treated with the peptide, dichlorodiphenyltrichloroethane (DDT, a potent inhibitor of human cell culture growth as a comparator), or DMSO alone for 18 h. Next, the Trevigen TACS MTT Cell Proliferation Assay Kit (Gaithersburg, MD) was used to assess human cell proliferation and/or viability impacts. MTT reagent (10µL) was added to each well and incubated under 5% CO2 at 37 °C for another 4 h. Finally, 100 µL of detergent reagent was loaded into each well and cells were incubated for additional 4 hours. Absorbance at 570 nm was measured using a BioRad iMark microplate absorbance reader. Samples were done in duplicate, and two-tiered T-tests were utilized to assess the statistical significance.

3.12 Statistical Analysis

All data obtained during this study were presented as Mean ±Standard error of mean (SEM). Statistical analyses were done using unpaired, two-tailed student's *t*-test and one-way analysis of variance (ANOVA) with the level of significance set at *p*-values < 0.05 with the levels of significance represented respectively as follows *p< 0.05, **p<0.01, ***p<0.001. All graphs were generated using GraphPad Prism (5.03, GraphPad Software, Inc., La Jolla, CA, USA).

CHAPTER IV

RESULTS

4.1 pET24b-HpIF1 Plasmid DNA Profiling

Agarose gel electrophoresis of HpIF-1 plasmid DNA preparations revealed that crystal producing isolate carried plasmids. A common characteristic of most of the isolate was the presence of a plasmid band above 200, 225 bp (Figure 2). The genomic DNA of HpIF-1 has amplified the oligonucleotides (both forward and reverse primer) and carried the intended plasmid in the pET-24b (+) vector. In previous studies, pET24b plasmid was used to identify genes expressed in C. glutamicum and P.aeruginosa (Y. Wang et al., 2022). Various genes were expressed using this plasmid, including genes for antibiotic resistance, metabolic enzymes, and fluorescent genes proteins. Their previous study found that the genes were integrated into the C. glutamicum and P.aeruginosa genomes in a stable manner. The results of this study demonstrate that pET24b plasmid DNA profiling is a valuable tool for identifying genes expressed in C. glutamicum and P.aeruginosa(Valdez et al., 2021; Y. Wang et al., 2022). In our study, we also used pET24b plasmid to amplify HpIF-1 gene, which is Kanamycin resistant. Interestingly, the gene was amplified, leading to subcloning with BL21(DE3) E.coli competent cells.

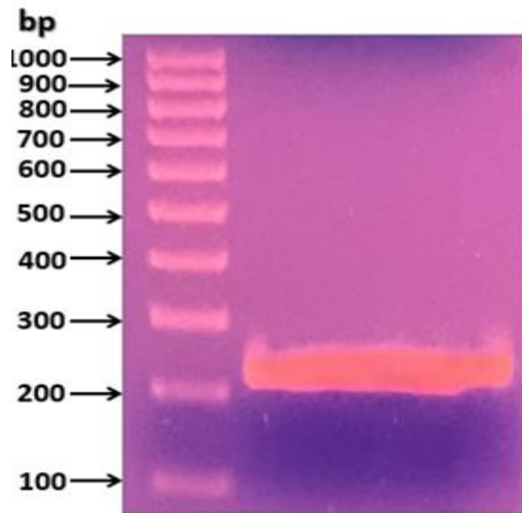
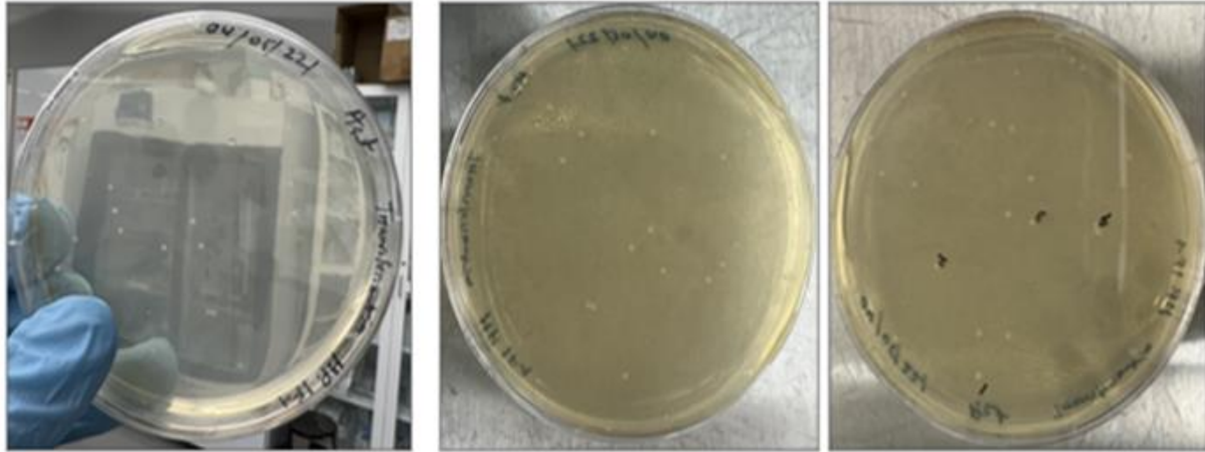


Figure 2: Photograph of the gel under UV illuminator after gel electrophoresis.

4.2 Molecular Subcloning

pET-24b HpIF-1 DNA plasmid cloned into BL21(DE3)E. coli competent cells and harboring on Luria-Bertani (LB) agar plate containing 50 uL of kanamycin and after 15 hours incubation at 37 °C CO_2 incubator, single colony contained HpIF-1 plasmid was observed (Figure 3). In addition, Cells harboring the recombinant plasmid are overexpressed in LB and M9 minimum medium.



Clone Contains Recombinant Plasmid

Figure 3: Bacterial Clone contains Recombinant Plasmids. LB Agar plates showing DH5alpha growth with pET24b-HpIF1 DNA plasmids and BL21(DE3).

4.3 HpIF1 Purification by FPLC

pET24b-HpIF1 protein has been separated from the eluted samples, can be assumed where it will be separated on the column. Smaller protein are elutes last, whereas, heavier molecular weight protein elutes first (Y. Hu et al., 2016; Valdez et al., 2021). Based on the concentration (224uM) by absorbing the ultraviolet (UV) light at a wavelength of 280nm, we decided to collect protein fractions between 31 to 43 (Figure 4), where was highest peak assuming that is purified protein.

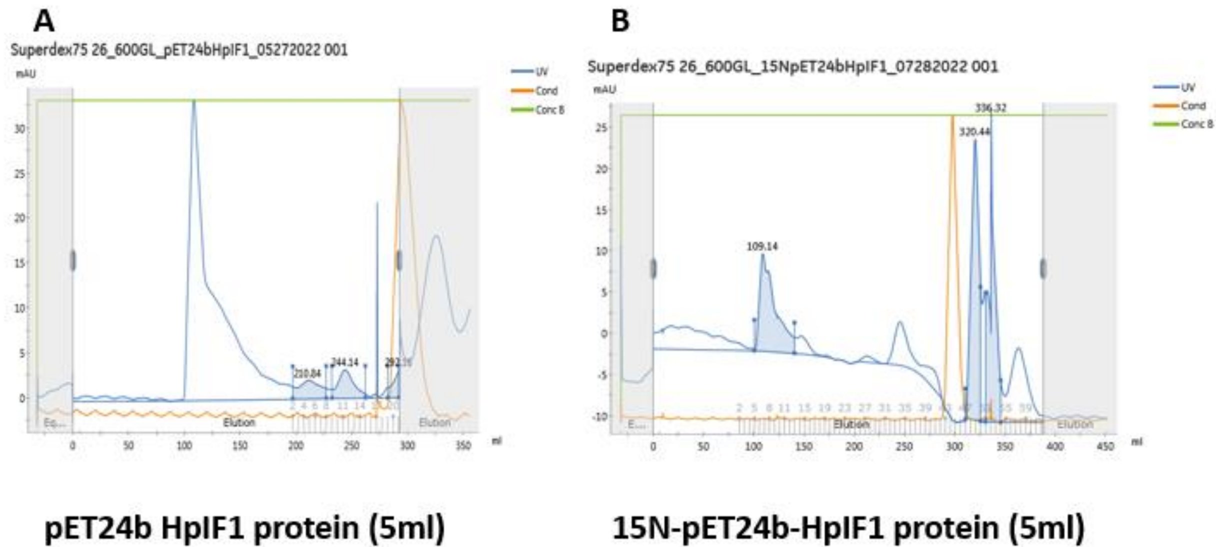


Figure 4: Size-Exclusion Chromatography (SEC) concentration of HpIF-1 protein. (A) Fraction containing concentration of highest peak in 2 LB medium whereas, (B) Fractions contains concentrated proteins based on the molecular weight in 15N contained M9 medium.

4.4 HpIF-1 Protein Expressed before and after purification

The analysis of purification and FPLC fractions with before IPTG and after IPTG as well as cell lysate and elution flow through around 10 kilo Dalton. And we can also observe that binding and washing elution has no expressions (Figure 5). Interestingly, around 34, 42, and 52 kilo Dalton overexpression was observed comparing with before and after IPTG as well as concentrated , purified flow through, respectively in Figure 5A and Figure 5C). However, binding and washing elution have no expressions due to washing out the histidine tag from the purified protein.

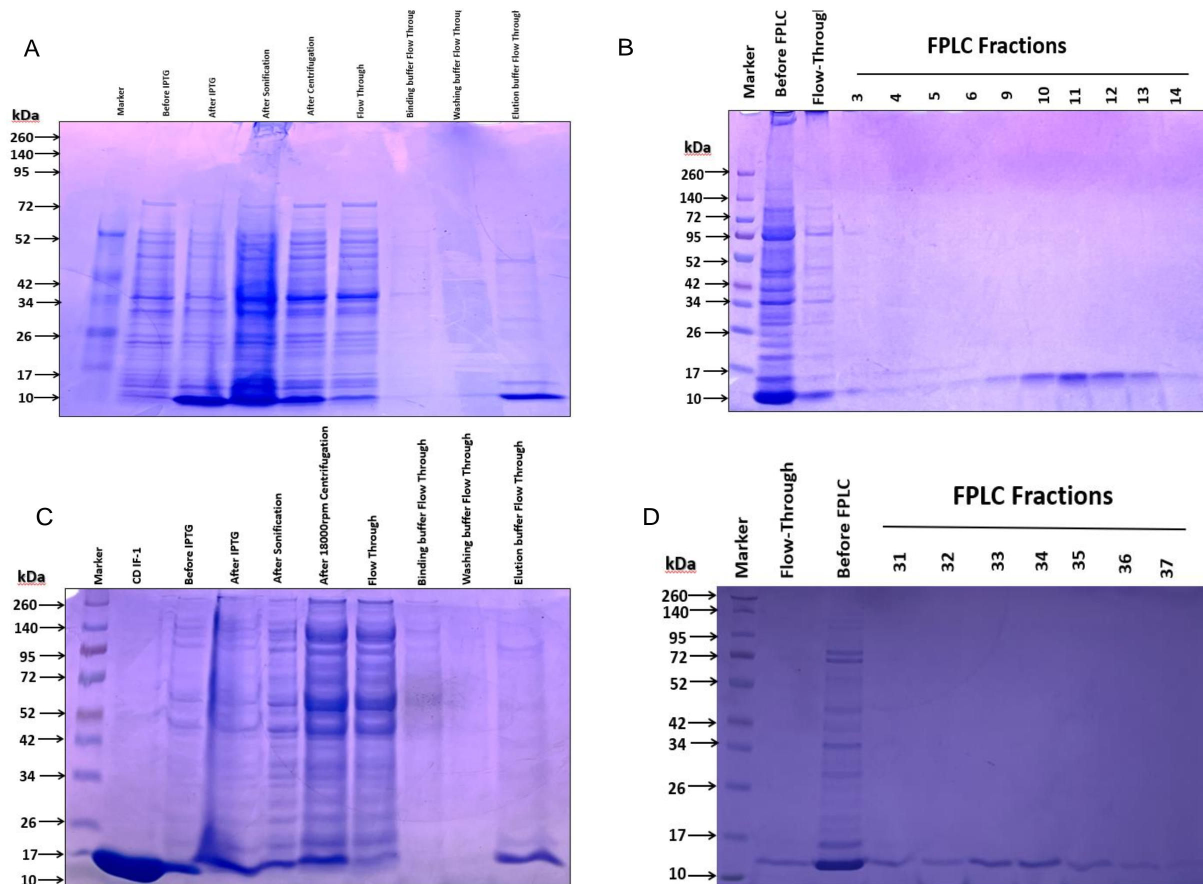


Figure 5: **SDS-Page** data has shown the expression of **pET24b HpIF-1** protein in 2L LB Broth Medium (**A,B**) and in M9 Medium (**C,D**). Both mediums were able to aberrantly overexpress the HpIF-1 protein after adding **IPTG** and purification (**A,C**); However, significant expression was found in the fractions of FPLC (**B,D**).

After adding IPTG protein expression by triggering the transcription of Lac Operon, we also checked the protein expression by different time manners. After collecting the cell lysates every one hour after adding IPTG and then running the SDS-Page, we can observe after one hour of IPTG expression; protein has less expression but after two to four hours the expression was constantly same (Figure 6).

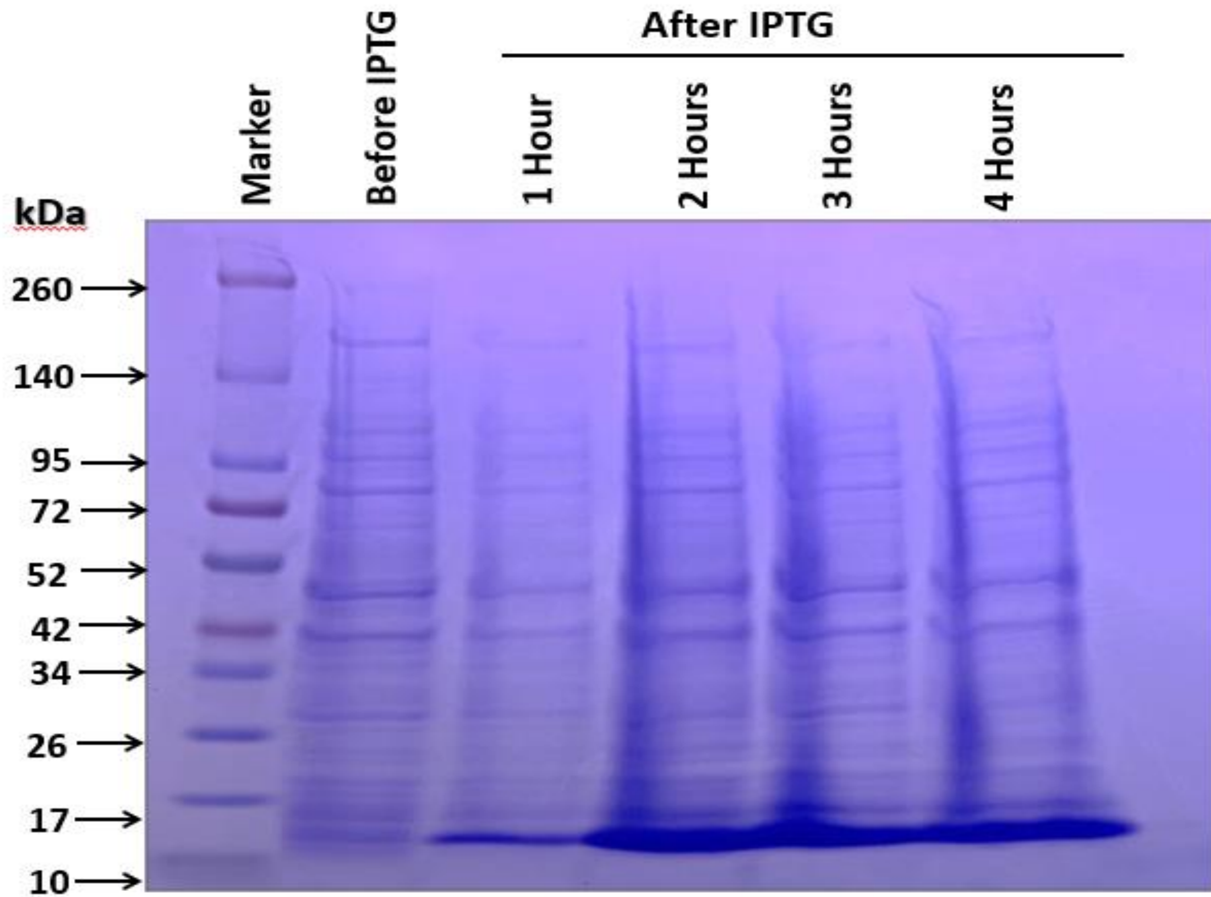


Figure 6: Protein Expression by Different Time Manners. There was no protein expression observed after 2 to 4 hours of adding IPTG.

After transforming the pET24b plasmid into the DE3 (BL21) E.coli competent cells, the HpIF-1 protein has overexpressed and comparing with the size of protein and molecular weight, identify the intended protein overexpression after adding IPTG (Figure 6).

4.5 1H-15N Heteronuclear Single Quantum Correlation (HSQC) Spectrum of HpIF-1

By performing NMR titration of HpIF-1 into 15N-labeled HpIF-1 solution, we checked the binding of HpIF-1 to 30S ribosomal small subunit. The1H−15N heteronuclear single quantum correlation (HSQC) NMR spectrum of HpIF-1 in free form exhibited characteristics of a well-folded protein (Figure 7), including highly dispersed backbone amide cross peaks, uniform peak intensities, and narrow peak shapes. Complex structure model of Hp-IF1 and the 30S ribosomal subunit (Hp-IF1 binding to the A-site of the 30S ribosomal subunit to regulate initiator tRNA binding to the A-site. High concentration of IF1 would prevent initiator tRNA binding to the A-site) (Figure 7). These peaks are uniform and, with a chemical shift dispersion, indicates a well-folded protein.

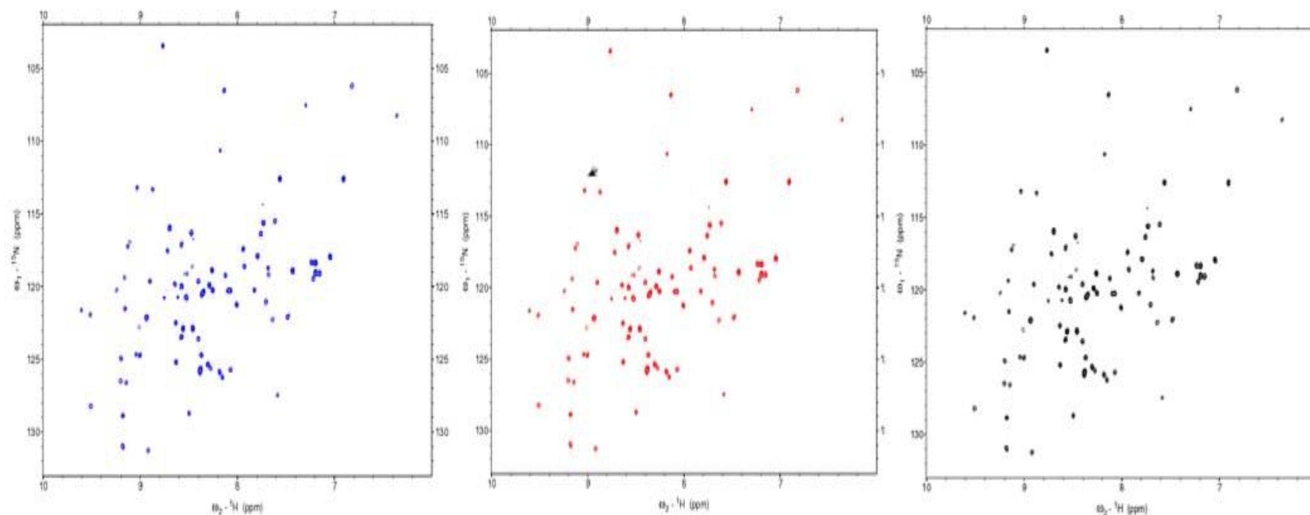


Figure 7: Analysis of 1H−15N HSQC titrations of 15N-labeled Pa-IF1 into reactions H. pylori 30S ribosomal subunit. It indicates the highest pure protein where is the highest peak observed.

4.6 Structural comparison of bacterial IF1 proteins and sequence alignment

Alignment (Clustal Omega) of the primary sequence of Hp-IF1 with those of other bacterial homologs from: E. coli (PDB 1AH9), M. tuberculosis (PDB 3I4O), S. pneumoniae (PDB 4QL5), B. thailandensis (PDB 2N3S), T. thermophilus (PDB 1HR0) etc. The region comprising most amino acid residues of our peptide is highly conserved in other bacteria, indicating a very functional and important region among the other bacterial homologs (Figure 8A). In previous studies, amino acid sequence alignment represented IF-1 from all kingdoms and showed all secondary residual elements strictly conserved. Aligned residues were functionally important, and their side chain had special interactions with 30S ribosome (Hatzopoulos & Mueller-Dieckmann, 2010). On the other hand, another study found that amino acid sequence alignment among the prokaryotes was highly conserved and indicated a high specific region to binding on the A site with 30S ribosome (Y. Hu et al., 2016).

Structural-based alignment is also shown in (Figure 8C), indicating the peptide has a shorter alpha helix than the full-length one. The secondary structure elements of Hp-IF1 are indicated schematically above the sequence (Figure 8C). These structures are highly similar concerning the oligonucleotide/oligosaccharide binding fold (OB-fold); however, there are significant differences in the structure in the C-terminal region.

A

```
                    β1          β2      β3     α1
                 1     10          20      30     40
T.thermophilus   MAKEKDTIRTEGVVTEALPNATFRVKLDSGPEILAYISGKMRMHYIRILPGDRVVVEITP   60
M.smegmatis      MAKKDGAIEVEGRVIEPLPNAMFRIELENGHKVLAHISGKMRQHYIRILPEDRVVVELSP   60
M.tuberculosis   MAKKDGAIEVEGRVVEPLPNAMFRIELENGHKVLAHISGKMRQHYIRILPEDRVVVELSP   60
S.pneumoniae     -MAKDDVIEVEGKVVDTMPNAMFTVELENGHQILATVSGKIRKNYIRILAGDRVTVEMSP   59
S.aureus         -MAKQDVIELEGTVLDTLPNAMFKVELENGHEILAHVSGKIRMNYIRILPGDKVTVEMSP   59
B.cereus         -MAKDDVIEVEGTVLETLPNAMFKVELENGHVVLAHVSGKIRMNFIRILPGDKVTVELSP   59
H.pylori         -MARDDVIEVDGKVIEALPNATFKVELDNKHVVLCRISGKMRMHYIRIALGDRVKLELTP   59
B.thailandensis  -MAKDDVIQMQGEVIENLPNATFRVKLENGHVVLGHISGKMRMHYIRILPGDKVTVELTP   59
A.faecalis       -MAKDDVIQMQGQVLENLPNATFRVKLENGHVVLGYISGKMRMHYIRILPGDKVTVELTP   59
P.aeruginosa     -MSKEDSFEMEGTVVDTLPNTMFRVELENGHVVTAHISGKMRKNYIRILTGDKVRVELTP   59
E.coli           -MAKEDNIEMQGTVLETLPDTMFRVELENGHVVTAHISGKMRKNYIRILTGDKVTVELTP   59
P.vulgaris       -MAKEDNIEMQGTVLDTLPNTMFRVELENGHVVTAHISGKMRKNYIRILTGDKVTVELTP   59
                    ... :. :* * : :*:: * ::*:.   :   :***:* ::***    *:* :*::*

                 β4    β5
                 50 60   70
T.thermophilus   YDPTRGRIVYRK-      72
M.smegmatis      YDLSRGRIVYRYK      73
M.tuberculosis   YDLSRGRIVYRYK      73
S.pneumoniae     YDLTRGRITYRFK      72
S.aureus         YDLTRGRITYRYK      72
B.cereus         YDLNRGRITYRFK      72
H.pylori         YSLDKGRITFRYK      72
B.thailandensis  YDLSRARIVFRAK      72
A.faecalis       YDLSRARIVFRSK      72
P.aeruginosa     YDLSKGRITYRAR      72
E.coli           YDLSKGRIVFRSR      72
P.vulgaris       YDLSKGRIIFRSR      72
                 *.  :.** :*
```

B

C-terminus

N-terminus

H. Pyroli IF-1
(HpIF-1)

C-terminus

N-terminus

E.Coli IF-1
(EcIF-1)

C

Figure 8: (A) Alignment (Clustal Omega) of the primary sequence of HpIF-1 with those of other bacterial homologs from: E. coli(PDB 1AH9), M. tuberculosis(PDB 3I4O), S. pneumoniae (PDB 4QL5), B. thailandensis (PDB 2N3S), T. thermophilus (PDB1HR0) etc. (C) Overlay of HpIF-1 (secondary structures in magenta) and IF1 from E. coli (PDB 1AH9), secondary structures in sky-blue).

4.7 Antimicrobial activity of HpIF1 peptide compared with broad-spectrum antibiotics

Hp-IF1-derived peptide from the short alpha-helix of IF1 structure inhibits bacterial growth, including Gram-negative and Gram-positive (MIC results) but less toxic to HEK-293 cells. HpIF-1 was tested against gram-positive and gram-negative bacteria compared to negative (DMSO) and positive inhibition controls (ampicillin and kanamycin) (Figure 9). The HpIF-1 exhibited inhibitory activity with a 6 mg/mL concentration against all bacteria except *P.vulgaris.* Furthermore, HpIF-1 found inhibitory activity against P.vulgaris with a concentration of 20 gm/mL (Figure 9E), indicating that to eliminate P.vulgaris growth, HpIF-1 required a higher concentration than the other gram-negative and gram-positive bacteria. Valdez *et. all*, previously showed that PaIF-1 peptide inhibited the growth of P.aeruginosa and P.vulgaris with the lowest concentration of 0.047 mg/mL and 2.1 mg/mL respectively (Valdez et al., 2021).

In our recent study we found HpIF-1 has the highest inhibition against *E. coli, A. faecalis, P. vulgaris, S. epidermitis, M. smegmatis and B. cereus* with a concentration of 0.3 mg/mL, 0.15 mg/mL, 1.0 mg/mL, 0.037 mg/mL, 0.3 mg/mL and 0.037 mg/mL respectively (Table 1).

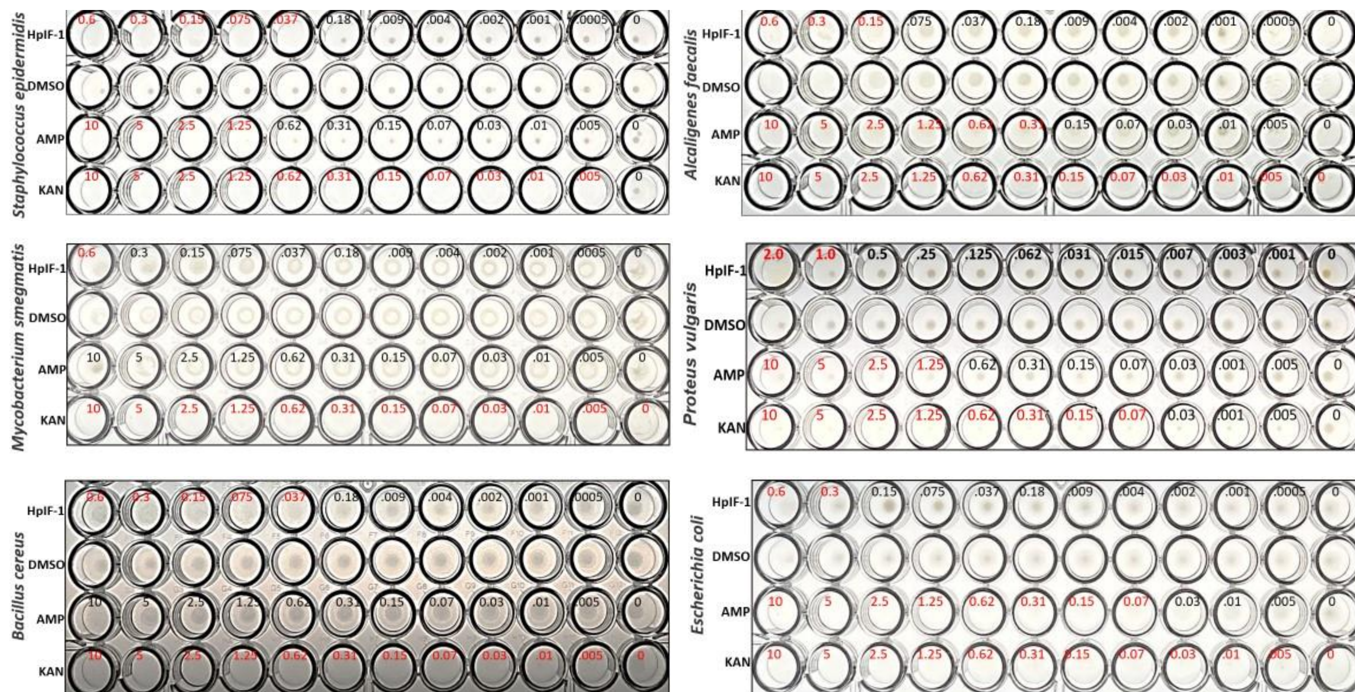


Figure 9: (**A-F**) Peptide was tested against gram-positive and gram-negative bacteria compared to negative (DMSO) and positive inhibition controls (ampicillin and kanamycin). The peptide exhibited inhibitory activity against all bacteria except *M.smegmatis* and *P.vulgaris.* HpIF-1 peptide inhibited the bacterial growth with the lowest concentration of 0.037 mg/ml against *S.epidermidis*; 0.037 mg/ml against *B.cereus,* 0.15 mg/ml against *A.faecalis*; 0.15 mg/ml against *E.coli. HpIF-1 peptide was tested in comparison with ampicillin and kanamycin. Interestingly, the peptide was found with an inhibitory activity better than ampicillin against S.epedermidis, B.cereus, and A. faecalis growth, but Kanamycin has the highest inhibitory activity against all bacteria except P. vulgaris.* But while increasing the HpIF-1 concentration, the MIC was found around 1.0 mg/ml.

4.8 Minimum Inhibitory Concentrations (MIC)

Peptide was tested in comparison with Ampicillin & Kanamycin (positive controls), and DMSO (negative control) against several Gram-negative and Gram-positive bacteria (Table 1).

Table 1: Minimum Inhibitory Concentrations (MIC) of HpIF-1

Species Name	Strain	Media*	Peptide Stock Concentration in DMSO	MIC (mg/ml)
Escherichia coli	BL21(DE3)	LB agar/broth	6 mg/ml	0.6
Alcaligenes faecalis	Obtained from The Microbiology Laboratory, UTRGV	LB agar/broth	6 mg/ml	0.15
Proteus vulgaris	Obtained from The Microbiology Laboratory, UTRGV	LB agar/broth	20 mg/ml	1.0
Staphylococcus epidermitis	ATCC 12228	Nutrient agar/broth	6 mg/ml	0.037
Mycobacterium smegmatis	ATCC 14468	LB agar/broth	6 mg/ml	0.3
Bacillus cereus	ATCC 14579	Nutrient agar/broth	6 mg/ml	0.037

- **The best growth conditions observed for each strain were chosen to move forward within the MIC.**

AS HpIF-1 peptide was tested in comparison with ampicillin andkanamycin. Interestingly, the peptide was found with an inhibitory activity better than ampicillin.

4.9 Compound HpIF-1 was not toxic to human embryonic kidney cells (HEK-293)

Antibiotic therapies that specifically target bacterial translation could potentially be toxic to mammalian cells given that both cytoplasmic and mitochondria GLuRS are contained within the eukaryotic cells and each share biochemical similarity with bacteria GluRS. MTT assays were performed to examine whether compound HpIF-1 was cytotoxic to mammalian cells. HEK-293 cells were treated with HpIF-1 for 24 hours under standard tissue culture condition in duplicate. The compound was not observed to be toxic to cells at any concentration tasted (Figure 10). Therefore, no cytotoxic effect was observed, suggesting that this compound would be amenable as therapeutics.

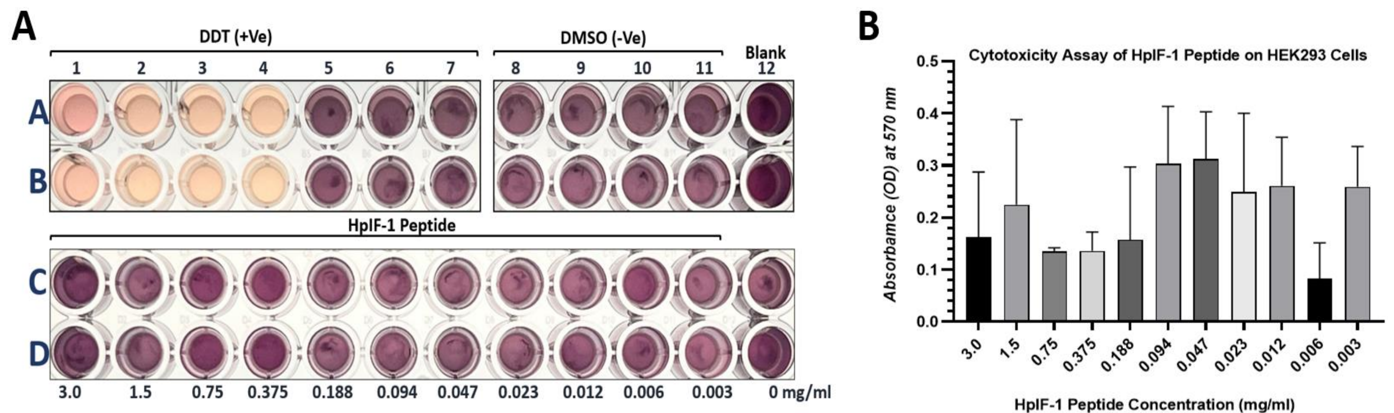


Figure 10: MTT Assay of HpIF-1 peptide to test the Toxicity against HEK-293 (Human Embryonic Kidney Cells); (A) toxic effect of HpIF-1 peptide on the growth of HEK-293 cells with Dichlorodiphenyltrichloroethane (DDT) (positive control); DMSO (negative control). (B) absorbance measured by OD 570 nm; data clearly shows the less toxic against cells with both low to high concentrations. There is no significant change between them. So based on this assay, we can demonstrate that our peptide HpIF-1 is less toxic against human cells.

4.10 Predicted HpIF-1 protein interact with 30S Ribosome

we submitted the amino acid sequence of our HpIF-1 protein to trRosetta and AlphaFold server and the predicted structure that we obtained for the protein with the optimate TM score 0.43 that contains five stranded Beta sheets and one stranded alpha-helix, and the majority of alpha-helix regions consist of hydrophobic and acidic residues (Figure 11 A, B). TM score is commonly used in protein structure prediction and model validation. TM score (Template Modeling score) is a widely used measure for the similarity between two protein structures. It is a normalized root-mean-square deviation (RMSD) of the distances between the C-alpha atoms in two structures, weighted by the length of the protein chains and the average distance between corresponding C-alpha atoms in the two structures. The TM score ranges from 0 to 1, with 1 indicating a perfect match between the two structures. A TM score of 0.5 is often considered as the threshold for the meaningful structural similarity between two proteins, although this can vary depending on the specific application (Price et al., 2022; Xu & Zhang, 2010). Based on the significance of our predicted structure, shown mostly similar to the full-length one along with an optimized TM score 0.4. This alignment Indicates that both structures have mostly similar alpha helix regions (Figure 11C).

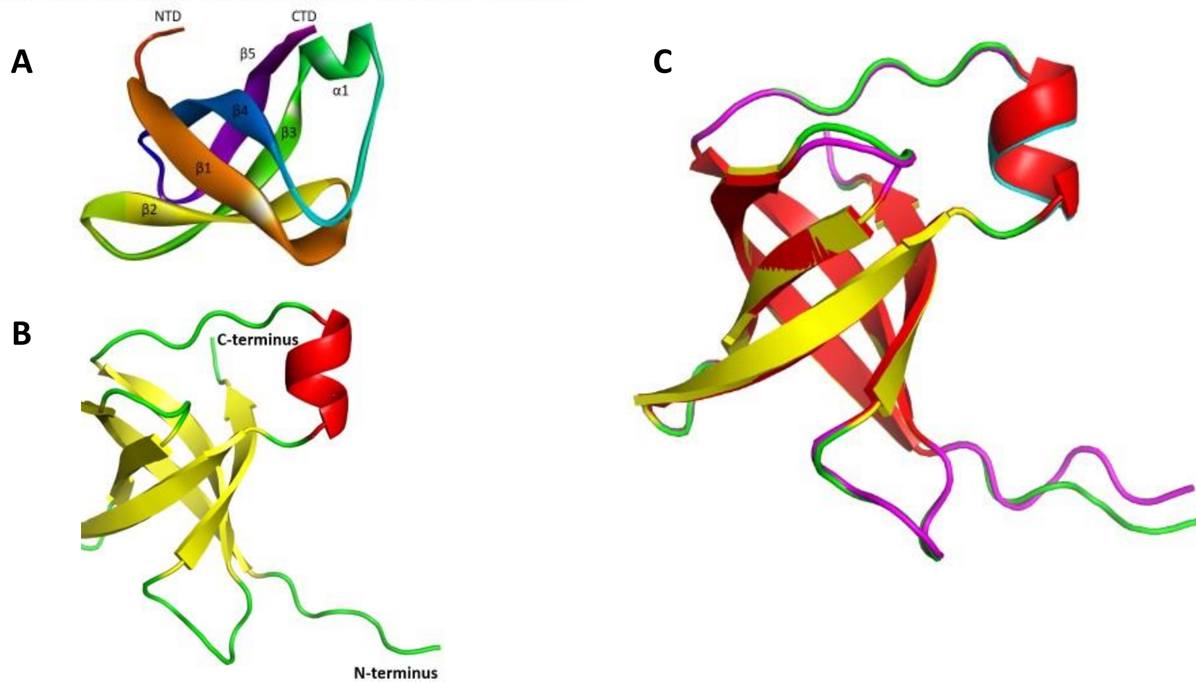


Figure 11: Predicted Structure of HpIF-1 Peptide. The Sequence of HpIF-1 was submitted to the trRosetta server for sequence-based structure prediction. (A,B) The predicted structure of HpIf-1 contains five stranded Beta sheets and one stranded alpha-helix, TM-score 0.433. The majority of alpha-helix regions consist of hydrophobic and acidic residues. (C) The structural alignment of HpIF-1 predicted structure from trRosetta and AlphaFold by using PyMoL. HpIF-1 from trRosetta structure is (secondary structures in magenta) and from AlphaFold structure is (secondary structures in yellow).

The structural model of IF-1 bind to the 30S ribosome, It was found that the alpha helix IF-1 bind to the 30S ribosome and it's the main interaction of A site of 30S ribosome; and the region that contained the most significant binding interaction of this short alpha helix,

specifically the residues histidine 45, leucine 55, glycine 38, methionine 40, arginine 46 and 74, and alanine 2 which highlights the importance of this regions the ribosome binding to A site (Figure 12).

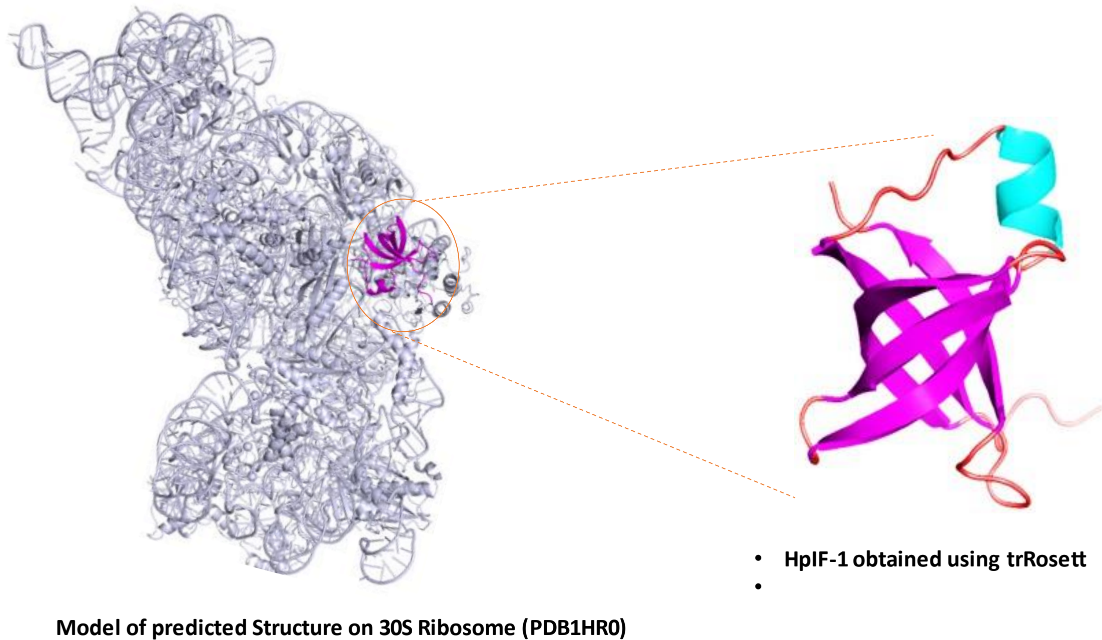


Figure 12: Structure Complex Model of HpIF-1 with 30S Ribosome.

Considering the importance of the short α-helix in IF1/ ribosomal binding, we theorized that it might be utilized as an IF1 functional mimic.

For additional structural insight into the binding of Hp-IF1 with the 30S, a complex structure model of Hp-IF1-bound 30S was developed. IF1 binds at the A-site on the ribosome and makes close contact with helix 44, loop 530, and protein S4 in the 30S (Figure 13A,B), similar to the crystal structure of T. thermophilus IF1 and the 30S (PDB 1HR0) with a RMSD of 2.4 Å.

As analyzed by pymol, the face of IF1 interacting with the 30S positively charged region is rich in basic amino acids, whereas most of the acidic residues forming the negatively charged face are on the solvent-exposed surface. The short α-helix between strands 3 and 4, which caps one end of the β-barrel toward the 30S head, is buried in the cleft between helix 44 and 530 loop, where residues D63, E15,A16, and K29 tightly interact with the phosphate backbone of 530 loop (nucleotides C420, C523, and G498) via electrostatic and H-bonding interactions (Figure 13D), and residues- R53 and R70 make tight interaction with flipped-out bases, A1454 and A1480. The β-turn between strands 1 and 2 loop inserts into the minor groove of helix 44 and forms contacts with the backbone of several nucleotides. The other end of the β-barrel pointing toward the ribosome body forms interactions with ribosomal protein S4, in which L56, H30, Y60 were significantly perturbed in the computational analysis. The overall structure model shows that the short α-helix forms the main contacts with the 30S and anchors IF1 at the A site of the subunit.

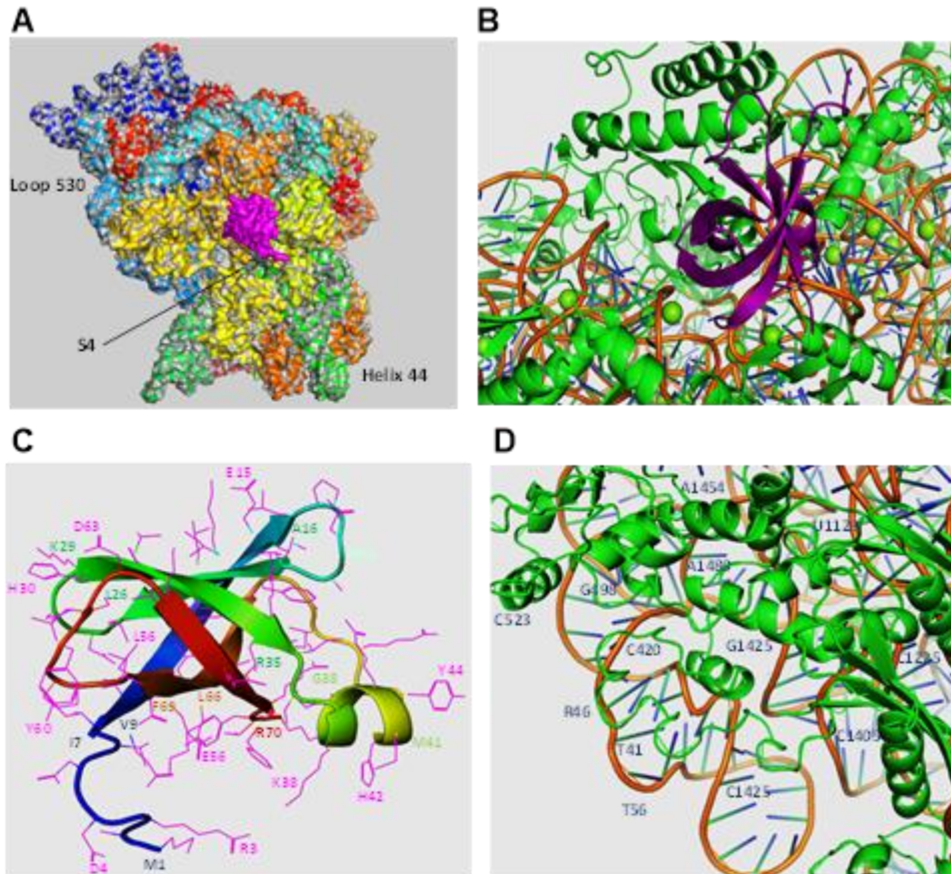


Figure 13: Structure model of Hp-IF1 bound to the 30S ribosomal subunit (PDB 1HR0) generated according to the predicted H.pylori initiation factor 1 amino acid sequence structure. (A) Surface representation of the 30S with Hp-IF1 (magenta) bound at the A (aminoacyl) site making contact with the 530 loop (cyan), ribosomal protein S4 (green) and helix 44 (orange). (B) Close-up of Hp-IF1 (deep pink) bound at the A site of the 30S in ribbon diagram. (C) The binding interface on the 30S surface. The highlights in green are the residues directly contacting with Hp-IF1, including the nucleotides in 530 loop (cyan) and helix 44 (orange) and the amino acids in protein S4 (green). (D) The key amino acids of Hp-IF1 on the binding interface with direct contact to the 30S are represented by a stick model with the residue names and numbers labeled. The residues are highlighted in cyan, orange, and green, indicating direct contact with ribosomal helix 44, 530 loop, and protein S4.

CHAPTER V

DISCUSSION AND CONCLUSION

Bacterial infection with H.pylori, one of the most dangerous and deadliest types of infections, has no specific drugs for early diagnosis and shows a feeble response to available therapeutic regimens. H. pylori infections further lead to causing peptic ulcers, gastritis, and stomach cancers. Various key molecular and genetic alterations, such as a mutation in TP53, CDH1, APC and KRAS, occur, which are frequently linked to gastric cancer.

In addition to these genetic events, desmoplastic tumor microenvironment (TME) containing various immune cell populations in gastric cancer by H. pylori infection can be affected by a variety of genetic events, including mutations in oncogenes or tumor suppressor genes, chromosomal abnormalities, and DNA damage, which can affect the behavior of immune cells in the TME. On the other hand, Certain strains of H. pylori may possess virulence factors, such as CagA, VacA, or OipA, that can modulate the immune response and promote chronic inflammation in the gastric mucosa, leading to the development of gastric cancer. Overall, the complex interplay between the tumor cells, immune cells, and microenvironmental factors in gastric cancer by H. pylori infection is undefined. Therefore, it is an empirical need to identify new molecular targets and therapy for managing this disease.

Bacteria require to survive ribosomes to synthesize proteins. Ribosomes are complex structures composed of RNA and proteins that read the genetic code and link amino acids to form a protein chain. Bacteria also require regulatory mechanisms to ensure that protein synthesis occurs at the correct time and at the correct rate. These regulatory mechanisms may involve controlling gene expression or modifying enzymes involved in protein synthesis. However, studies still need to investigate the role of initiation factor-1 (IF-1) protein biosynthesis in H.pylori. In our study, we have explored the inhibition pattern of the Hp-1F peptide against different gram-positive and gram-negative bacterial growth in vitro. The therapeutic efficacy of HpIF-1 peptide against gram-positive and gram-negative bacteria compared to negative (DMSO) and positive inhibition controls (ampicillin and kanamycin). The peptide exhibited inhibitory activity against all bacteria except M.smegmatis and P.vulgaris. HpIF-1 peptide inhibited bacterial growth with the lowest concentration of 0.037 mg/ml against S.epidermidis; 0.037 mg/ml against B.cereus, 0.15 mg/ml against A.faecalis; 0.3 mg/ml against E.coli compared with ampicillin and kanamycin. Interestingly, the peptide was found with an inhibitory activity better than ampicillin against S.epedermidis, B.cereus, and A. faecalis growth, but Kanamycin has the highest inhibitory activity against all bacteria except P. vulgaris. HpIF-1 found inhibitory activity against P.vulgaris with a concentration of 1.0 gm/mL (Figure 9E), indicating that to eliminate P.vulgaris growth, HpIF-1 required a higher concentration than the other gram-negative and gram-positive bacteria. Nevertheless, the MIC values in similar to the bacterial counterpart initiation factor 1 (IF-1) (Lomakin & Steitz, 2013; Valdez et al., 2021). Additionally, the shortα-helical region in gram-positive and gram-negative bacteria shows low sequence similarity to the HpIF-1 peptide, which consists of most amino acid residues of HpIF-1 and is highly conserved in other bacterial homologs.

Recently, Antimicrobial peptides (AMPs) have recently attracted renewed interest due to their unique ability to treat bacterial infections and their low potential for developing resistance (Heinbockel et al., 2013; Liu et al., 2021). Antimicrobial peptides (AMPs), which are being investigated as potential alternatives to traditional antibiotics, possess exceptional structural andfunctional diversity. They have the capability to target the cell membrane and cause permeabilization through pore formation or interrupt intracellular processes, including protein and nucleic acid synthesis (Kumar et al., 2018). Inhibitory efficacy against Gram-negative and Gram-positive bacteria growth was observed for our rationally created peptide, suggesting it may constitute a novel antibiotic due to it's low cytotoxicity effect against HEK-293 human embryonic kidney cells. Previous study has been demonstrated that PaIF-1 has potent inhibitory activity against gram-negative bacteria with low toxicity (Valdez et al., 2021). In another study nephrotoxic compounds, including cisplatin, paraquat and ibuprofen, have showed the toxic effect against duct (mIMCD3) cells and human embryonic kidney cells (HEK293) (Park et al., 2008).

HEK293 cells are commonly used in cytotoxicity testing because they are human embryonic kidney cells that can be easily grown in the laboratory and have a high proliferation rate. Additionally, HEK293 cells have been extensively characterized and are well understood, making them a reliable model for cytotoxicity testing. Cytotoxicity testing involves exposing cells to a substance of interest to determine its potential toxicity (J. Hu et al., 2018; Mordwinkin et al., 2013). The HEK293 cell line is particularly useful for cytotoxicity testing because it is sensitive to a wide range of toxins and can be used to evaluate the toxicity of both small molecules andbiological agents due to easy transfect, which allows testing the

toxicity of specific genes or proteins(J. Hu et al., 2018). However, it is important to note that no single cell line can perfectly represent the complexities of human physiology, and other cell types or in vivo studies may be necessary to fully evaluate the safety and efficacy of a new drug. Therefore, no cytotoxic effect was observed with HpIF-1, suggesting this compound would be amenable as therapeutics.

Recent studies have shown that, a peptide derived from the α-helix of P. aeruginosa initiation factor 1 (IF1) was tested and displayed a high ability to inhibit bacterial growth (Valdez et al., 2021). Interestingly, our designed peptide showed the highest inhibition against drug-resistant bacteria, which needs further screening to derive potential therapeutic agents. In order to determine the full-length structure of HpIF-1 protein, we tried NMR titration with the purified protein, but the protein was not highly concentrated on getting a full-length structure while it required a minimum concentration of 500 uM to 1 mM. We further used a computational model to get the complex model of HpIF-1 to 30S ribosome showing binding interactions. To prove the potential structural analysis of HpIF-1 by PymoL to interact with 30S ribosome and the amino acid sequence of our HpIF-1 peptide to trRosetta server and this is the predicted structure that we obtained for the peptide with the optimized TM score 0.43 that contains five stranded of Beta sheets and one stranded of alpha-helix, and the majority of alpha-helix regions consist of hydrophobic and acidic residues. However, the structural model of IF-1 bind to the 30S ribosome, It was found that the alpha helix IF-1 bind to the 30S ribosome and it's the main interaction of A site of 30S ribosome; and the region that containedthe most significant binding interaction of this short alpha helix. The previous study sought the importance of the short α-helix in IF-1/ ribosomal binding, which might be utilized as an IF1 functional mimic (Valdez et al., 2021).

The binding of aminoacyl-tRNA (a tRNA molecule carrying an amino acid) to the A-site of the 30S ribosome is a crucial step in protein synthesis. This binding is facilitated by the decoding center of the 30S subunit, which recognizes the codon on the mRNA and ensures that the correct aminoacyl-tRNA is selected. There are several mechanisms by which aminoacyl-tRNA binding to the 30S ribosome can be inhibited. One common mechanism is through the action of antibiotics that target the ribosome. For example, the antibiotic tetracycline binds to the A-site of the 30S ribosome and prevents aminoacyl-tRNA binding. Similarly, the antibiotic streptomycin can cause a misreading of the mRNA, leading to errors in protein synthesis. In addition to antibiotics, regulatory mechanisms within the cell can inhibit the binding of aminoacyl-tRNA to the 30S ribosome. For example, the protein IF3 can bind to the 30S subunit and prevent aminoacyl-tRNA binding to the A-site. This helps to regulate the rate of protein synthesis and prevent translation errors.

Taken together, our study provides a strong clue to the dysregulation of IF-1 protein biosynthesis in different gram-positive and gram-negative bacteria, including H.pylori. HpIF-1 is a non-toxic potential anti-microbial agent that inhibits bacterial protein synthesis. Furthermore, the use of HpIF-1 peptide could be explored for its therapeutic efficacy in the relevant in-vivo study as a further screening process.

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BIOGRAPHICAL SKETCH

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