# MOLECULAR BIOLOGICAL CHARACTERIZATION OF THE VACUOLATING CYTOTOXIN A (VacA) FROM THAI CLINICAL ISOLATE *HELICOBACTER PYLORI*

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ABSTRACT

*H. pylori* vacuolating cytotoxin A (VacA) is an exotoxin that represents one of the most important virulence factors produced by *H. pylori.* Activities of VacA include formation of large cytoplasmic vacuoles in the host gastric epithelial cells as well as release of cytochrome c (Cyt c) from mitochondria resulting in cell apoptosis. VacA is present in all *H. pylori* strains, but its cytotoxic activity has been shown to occur in only 50% of *H. pylori* strains. Structurally, the mature VacA is an 88-kDa monomer that consists of two domains: p33, responsible for pore formation on plasma membrane and p55, which has an important role in binding to target host cells following its internalization into the cytosolic compartment. We aimed to study the sequence of Thai isolate VacA and compare it with model strains (60190) as well as to characterize the biological activities of this toxin on intestinal (T84) and kidney (MDCK) epithelial cell lines. We established molecular methods including PCR, gel electrophoresis, SDS-PAGE, Western blot analysis for cloning, sequencing, and expression of VacA in *E. coli*. Nuclear staining using DAPI was employed to detect apoptosis induced by purified VacA. Results showed that the Thai isolate protein is structurally similar to the *H. pylori* isolate s1m2 VacA strains: whereas homology to the 60190 model strain was found to be lower than expected due to the presence of extra amino acids in the mid region (m region) of the Thai isolate VacA protein. The m region has been found to be linked to the binding specificity of VacA to specific epithelial cells that are induced to undergo vacuolization. DAPI staining showed lower apoptotic effects of Thai isolate VacA on T84 cells while it has a higher apoptotic effect on MDCK cells. These findings suggest that the Thai isolate VacA could have biological activities on the host cells that may differ from those of the model strains.

KEY WORDS: *HELICOBACTER PYLORI*/ VACA/ APOPTOSIS/ CLONING

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| bp | base pair(s) |
| C-terminal | carboxy terminal |
| dNTP | deoxyribonucleotide triphosphate |
| *E. coli* | *Escherichia coli* |
| hr (s) | hour (s) |
| IMAC | immobilized metal affinity chromatography |
| IPTG | isopropyl-β-D-thiogalactopyranoside |
| Kb | kilobase pair (s) |
| kDa | kilodalton (s) |
| LB | Luria-Bertani (medium) |
| M | molar(s) |
| mA | milliampere(s) |
| min | minute (s) |
| mM | millimolar |
| N-terminal | amino terminal |
| OD | optical densities |
| PCR | polymerase chain reaction |
| RBS | ribosome binding site |
| Rpm | revolutions per minute |
| rVacA | recombinant vacuolating associated cytotoxin A |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| sec | second (s) |
| Tween-20 | polyoxyethylenesorbitan monostearate |
| U | unit (s) |
| VacA | vacuolating associated cytotoxin A |
| v/v | volume / volume |
| w/v | weight / volume |

# CHAPTER I
# INTRODUCTION

## *1.1. Helicobacter pylori*

*Helicobacter pylori,* a Gram-negative spiral-shaped bacterium belong to *Helicobacter* species. *H. pylori* persistently colonizes the human stomach for almost the entire life time of the host and causes a serious chronic transmissible infectious disease that damages gastric structure and function and is recognized as the causative agent in gastric atrophy, peptic ulcer disease, gastric adenocarcinoma and primary gastric cell lymphoma (1,2). The clinical outcome of *H. pylori* infection is determined by multiple factors; including *H. pylori* strain heterogenicity, host genetic predisposition and environmental factors such as dietary high salt intake (3).

### 1.1.1. Discovery of *Helicobacter pylori*

The first well-known report of gastric *Helicobacter* was in 1893 (4). Waaen and Marshall were able to culture a slow growing microaerophilic bacterium in the laboratory, by accidentally leaving agar plates containing samples from stomach biopsies in the incubator over Easter holidays (1). They named *Campylobacter pyloridis*, a name that was later changed to *Campylobacter pylori* and finally to the current name *Helicobacter pylori* (5).

### 1.1.2. Physiology and molecular biology

*H. pylori* has very unique characteristics with respect to physiology and molecular biology, such as microaerophily and nitrogen metabolism; therefore, the physiology and molecular biology of *H. pylori* is of a great interest for researchers in many fields. Specifically, the cell envelope components are most important since they are the first points of contact between bacterial factors and the host. These components include lipopolysaccharides, outer membrane proteins, and flagella. Many surface proteins of pathogenic bacteria are thought to have roles in adhesion, colonization and

immune response. Some outer membrane proteins also perform transport functions essential for metabolism. *H. pylori* colonizes the highly acidic environment of the gastric mucosa. Specifically, *H. pylori* can flourish acidic gastric niche by buffering its periplasm to near neutral using the mechanism of acid acclimation. This unique acid acclimation, in contrast to acid resistance or tolerance, is dependent on urease activity; therefore, it is believed that urease activity plays a central role in survival of *H. pylori* in stomach. Acid acclimation also is necessary for gastric colonization (116).

#### 1.1.3. *H. pylori* genetic diversity and host colonization

*Helicobacter pylori* are characterized by a high level of genetic diversity. Genes reported as variable in populations but not found as such in other population isolates. The genetic diversity is important for the adaptation to the host stomach and for the clinical outcome of the infection. Differences in gene content among *H. pylori* isolates in patients with various gastric pathologies, including cancer, showed patterns of disease *H. pylori* associated genes (6). The plasticity of the *H. pylori* genome derives from its natural competence for transformation by exogenous DNA, from recombination and from mutations. These properties are at the origin of an extensive allelic diversity occurring even in a single host (7). It was proposed that a novel mechanism provided through competition between repair and antirepair pathways leads to generate strain diversity and to maximize fitness at the bacterial population level (8). After natural transformation of *H. pylori*, the import of short DNA fragments interrupted by short stretches of recipient sequence interspersed (ISR) within the imported regions. The mean length of ISR was 82 bp was shown to result in the formation of complex mosaic alleles (9).

## 1.2. Pathogenesis of *Helicobacter pylori* infection

### 1.2.1. *Helicobacter pylori* virulence factors

#### 1.2.1.1. Resistance to acid

*H. pylori* exhibit significant sequence diversity in multiple genes including those that encode urease (10). *Helicobacter pylori* are well to the

highly acidic conditions encountered in the stomach adapted as it possesses a highly potent urease enzyme, which has a *K*m value for urea of 0.8 mM. This means that the urease of *H. pylori* binds substrate with a much higher affinity than the ureases of other bacterial species and is able to hydrolyze the limited amounts of urea available in the stomach to generate ammonia and $CO_2$, which increases the pH (11). In a study by which sought to determine the contribution of surface-associated and/or extracellular urease to acid resistance of *H. pylori,* it was observed that *H. pylori* cells with surface-associated and/or extracellular and cytoplasmic urease activity survived in an acid environment (10). It has been proposed that presence of surface urease able to produce a cloud of ammonia around the bacteria that protects it against the acidity of the environment. However, it is thought that under the buffered conditions that are found in the stomach at low pH, intrabacterial neutral urease rather than surface bound urease is essential for survival of the organism. Internal urease has been shown to be regulated by external pH, to defend against gastric acidity by increasing periplasmic pH and membrane potential and to stimulate protein synthesis at acidic pH (13). *In vit*ro studies that *H. pylori* are able to survive for several hours at pH 1 in the presence of urea (14). Urease activity intra bacterial cytoplasm is regulated by a proton-gated urea channel, an integrated membrane protein named UreI (15). Research suggests that if the secretion of acid is powerfully suppressed, *H. pylori* in the presence of urea will increase the pH of its local environment to alkaline values and will be unlikely to survive in a culturable form. These results may explain why *H. pylori* has been isolated only rarely from other nonacidic sites in the body rather than stomach. At acidic pH, integral membrane protein may be involved in a transport process essential for *H. pylori* survival *in vivo* (16) and at acidic pH integral membrane protein is activated and urea diffusion into the cytoplasm is increased, allowing for maximal production of ammonia and $CO_2$. Integral membrane protein is not active at neutral (pH 8), thus avoiding alkalinization, which is lethal to *H. pylori* (17). The periplasmic α-carbonic anhydrase activity of *H. pylori* is essential for acid acclimation (18). Following urea entry into the periplasm, urea enters the cytoplasm via activated UreI. Then intrabacterial urease produces $2NH_3$ and $CO_2$ gases that diffuse rapidly into the periplasm. One of the $NH_3$ molecules can neutralize entering acid, and the other forms $NH_4HCO_3^-$ due to the rapid production of $HCO_3^-$ by carbonic anhydrase. Buffering of

the periplasm to a pH consistent with viability depends, not only on $NH_3$ efflux from the cytoplasm, but also on the conversion of $CO_2$, produced by urease, to $HCO_3^-$ by the periplasmic a carbonic anhydrase. Activity of this enzyme could be an additional requirement for gastric colonization by the organism (18). In addition to urease, *H. pylori* also possess other ammonia-producing enzymes, including two aliphatic amidases. Aliphatic amidases are enzymes that hydrolyze short-chain amides to produce ammonia and the corresponding organic acid the production of these enzymes is regulated to maintain intracellular nitrogen balance in *H. pylori*. Amidases have only been reported previously in environmental bacteria and their presence in *H. pylori* underlies the importance of ammonia to the survival of this organism (16). *Helicobacter pylori* also produce an arginase encoded by *rocF*. The *rocF* gene encodes the urea cycle enzyme arginase of the *H. pylori* urea cycle hydrolyzes L-arginine to L-ornithine and urea. It is involved in acid resistance but is not essential for colonization of mice or for urease activity (19). \

#### 1.2.1.2. Outer membrane proteins and adherence

Analysis of the completed *H. pylori* genomes for strain J99, isolated from a patient with duodenal ulcer disease has confirmed the presence of five major outer membrane proteins (OMP) families (20). It has been demonstrated that several OMPs in the largest family act as adhesins, and these include the blood group antigen binding adhesins (Baba: HopS), sialic acid binding adhesins (SabA: HoP), adherence associated lipoprotein (AlpA and AlpB: HopBN and HopC, respectively), outer inflammatory protein (Oip: HopH) and HopZ (21).

#### 1.2.1.3. Outer membrane proteins families

OipA is a phase-variable outer membrane protein of *H. pylori* linked to proinflammatory epithelial signaling (22). An OipA-positive status has been significantly associated with the presence of duodenal ulceration and gastric cancer, high *H. pylori* density and severe neutrophil infiltration (23). Sequencing of OipA in *H. pylori* strains revealed that the OipA genotype is linked to bacterial virulence determinants, such as functional vacA and most strongly, cagA genotypes (24).Member of the large Hop (*Helicobacter* outer membrane protein) family were

among the first characterized OMPs in *H. pylori.* Five members were purified and shown to have a highly conserved N-terminal motifs and demonstrated to function as protein (25). The *H. pylori* genome contains about 30 *hop* gene paralogous encoding (OMP). Study showed mutations in *hop* increased adherence of *H. pylori* to AGS gastric epithelial cells, CagA translocation into host cells and cellular alterations, demonstrating the importance of Hop for bacterial adherence. Other families of OMP, the Hof (*Helicobacter* OMP family) family of related proteins was identified from the *H. pylori* proteome based on the documentation of a 50 kDa heat-stable protein located in the outer membrane (26). The Hom, other smaller paralogus family of proteins. contains the C-terminal alternating hydrophobic motif and characteristic signal sequence typical of OMP (27).

**Table 1.1. Other potential virulence factors**

| | |
|---|---|
| *VacA* | Encodes a protein cytotoxin that induces vaculation in eukaryotic cells |
| *cagA* | Stimulate the production of interleukin-8; a part of it is also code for type IV secretion system |
| *babA* | Binds to lewis b antigen displayed on the surface of gastric epithelial cells |
| *iceA* | Up-regulated upon contact of *H. pylori* with the gastric epithelium |
| *oipA* | Induces IL-8 secretion by epithelial cells |
| *picB* | Induces IL-8 expression in gastric epithelial cells |
| *Urease* | Neutralizes acid |
| *Rocf* | Encode arginase that facilitate production of ammonia |
| *comB4* | Essential for colonization, as it encodes a putative ATPase which is a a part of DNA transformation associated type-IV system. |
| *Hop0169* | Essential for *H. pylori* stomach as it encodes for a collagenase |

## 1.3. Epidemiology

### 1.3.1. Prevalence of *H. pylori* infection

*H. pylori* is one of the most common bacterial infectious agents; it inhabits the stomachs of more than half of the world's population (28). The prevalence

of infection seems to mostly depend on the rate of acquisition, but also on the rate of loss of infection (29) and the length of the persistence period between acquisition and loss (30). Based on these factors, *H. pylori* prevalence differs from one country to another and may differ between different ethnic, social, or age groups within the same country (31). Globally, the prevalence of *H. pylori* infection in developing countries is markedly higher than that in developed countries (32). Moreover, the acquisition of *H. pylori* seems to occur at higher rates in developing countries (33).

### 1.3.2. Incidence of *H. pylori* infection

The geographic differences in *H. pylori* prevalence have been attributed to the differential rate of acquisition of the bacterium during the first years of life (28). In southern China, for example, the prevalence of *H. pylori* infection was shown to be significantly higher among Chinese subjects than that among Australians, a difference that was associated with the rate of acquisition of *H. pylori* under the age of ten years (34).

Acquisition of *H. pylori* is decreasing in developed countries at a faster rate than in developing countries, likely because of the faster improvement in hygiene practices in the developed world (35). Moreover, infection during childhood in developed countries is not frequent (36). In the United States, for example, the incidence of infection among children younger than five years is less than 5%, and only about 10% of the population is infected by adolescence (37). In contrast, the incidence of *H. pylori* infection in the developing world is higher and occurs at younger age (38). By five years of age, about 50% of children in developing countries are already infected and the infection rates in adults can reach 90% or higher (39).

Pounder and Ng classified the world into two groups according to the incidence of *H. pylori* infection (30). Group one consisted of countries where the majority of children become infected with *H. pylori* during childhood, while chronic infection continues during adult life. These are mostly developing countries, Algeria, Nepal, South Africa, Saudi Arabia, Thailand, and Vietnam. In group two, mostly comprising developed countries, only a minority of children becomes infected during childhood, but the prevalence of infection rises with age during adulthood. Examples of group

two countries are England, Finland, France, Japan, and the United States of America (30).

### 1.3.3. Incidence of *H. pylori* infection in Thailand

Thailand is a Southeast Asian country with a population of 64 million people and six geographic regions; the north (mountain and jungle community), the northeast (semiarid plateau community), the east (mountain and coastal community), the west (mountain and jungle community), the central (fertile plain community) and the south (peninsular community). Dyspepsia (feel a burning feeling in the upper abdomen) is a very common problem in this country and the surveys of community base data have shown that up to 66% of adults experience significant dyspeptic symptoms. Also dyspepsia is one of the most common reasons for a patient to visit a physician and accounts for between 3 and 4% of visits. Although more than half of these dyspeptic patients have functional dyspepsia, some have organic problems, the most severe being carcinoma of stomach. The discovery of *H. pylori* was a significant turning point in the understanding of the etiology of gastritis and its association with peptic ulcer diseases (41). This organism leads to gastritis and tissue atrophy, intestinal metaplasia with or without dysplasia and finally carcinoma (42). The whole nation incidence rate of *H. pylori* in Thailand was assessed by histopathological findings, geographic distribution of *H. pylori* in each region, type of chronic gastritis, atrophic change and intestinal metaplasia in each geographic region (41). In this study, of 3776 cases biopsies only 3066 data were enrolled in the typing of gastritis. The distribution of gastritis and types in each geographic region and for the whole country is shown in fig 1.1 (Gastritis typing is not available in biopsy materials from the north; the north-east and the west because the biopsy tissues were taken from only the antrum).

### 1.3.4. Treatment

The discovery of *H. pylori* as a causative agent of peptic ulcer disease has revolutionized the medical field's understanding of the treatment of this condition (1). Many patients still attribute symptoms of dyspepsia to an ulcer, and believe that ulcers are caused by diet, stress, and life style factors; however, it is now clear that eradication of *H. pylori* is central to the management of this illness. Primary care physicians are typically faced with patients who present with peptic ulcer disease.

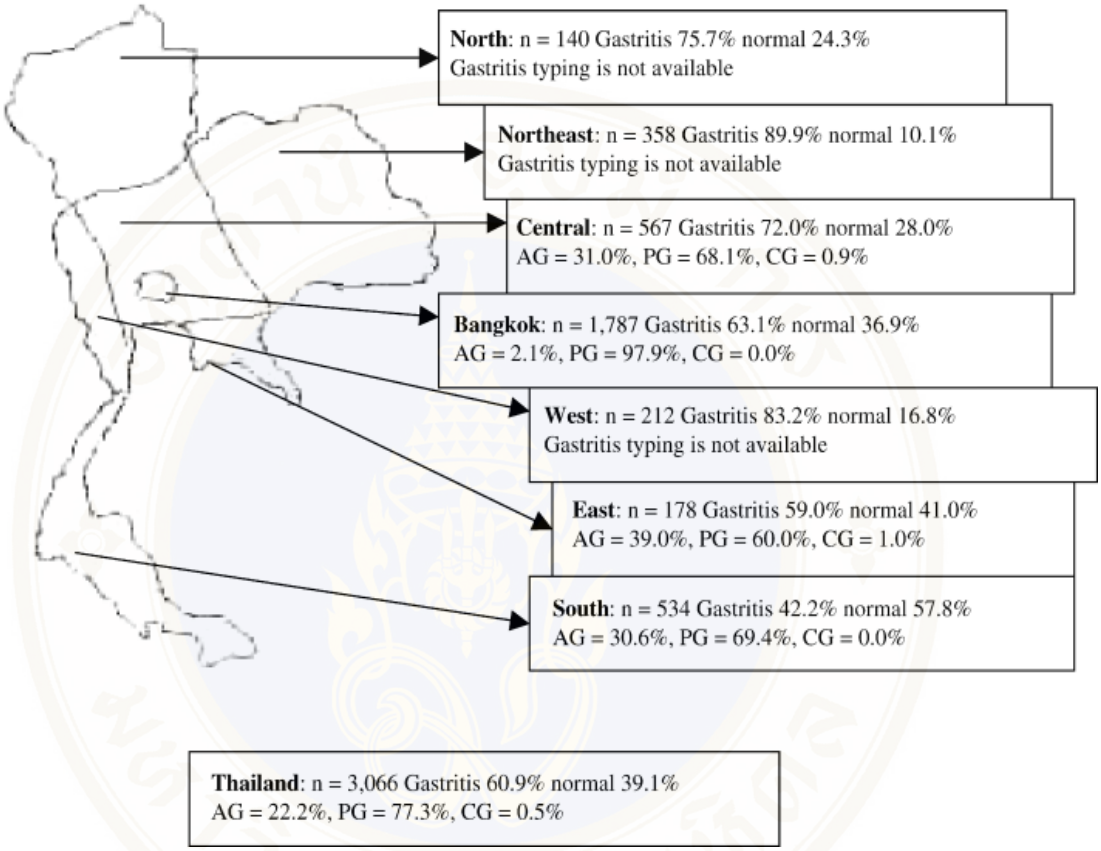



**Figure 1.1 Distribution of gastritis and type of gastritis in each geographic region of Thailand.**

AG = Antral gastritis

PG = Pan-gastritis

CG = Corporal gastritis

Patients undergo a noninvasive test for *H. pylori* infection and, if positive, are treated with eradication therapy. This strategy reduces the need for antisecretory medications as well as the number of endoscopies. The urea breath test or stool antigen test is recommended. Until recently, the recommended duration of therapy for *H. pylori* eradication was 10 to 14 days. Shorter courses of treatment (i.e., one to five days) have demonstrated eradication rates of 89 to 95 percent with the potential for greater patient compliance. A one-day treatment course consists of bismuth subsalicylate, amoxicillin, and metronidazole, all given four times with a one-time dose not to exceed 4.2 g, 2 g, and 1.5 g respectively.

## 1.4. The Vacuolating Cytotoxin A ( VacA)

### 1.4.1. VacA structure and function

*H. pylori* is believed to be a major causative agent of human peptic disorders including chronic gastritis and ulcers. The discovery in 1983 of the association between *Helicobacter pylori* and peptic ulceration (1) led to intense study of potential bacterial virulence factors. One such factor is the vacuolating cytotoxin (VacA). There has been a high level of interest in VacA study for many rational considerations, thus sequence divergence in vacA genes may explain the lack of functionally active cytotoxin production by some *H. pylori* isolates (44). VacA targets not only epithelial cells, but also cells of the immune system and induces immunosuppression (45). The vacA gene is present in basically all *H. pylori* strains as a sole chromosomal copy (44). A multifunctional toxin (VacA) that can have pleiotropic effects on mammalian cells and tissues is produced by the bacterium *H. pylori*. The actions of *H. pylori* VacA represent a paradigm for how bacterial secreted toxins contribute to colonization and virulence in multiple ways. The vacA gene is present in all strains and encodes 140-kDa protoxin (46). Among strains that were characterized, the genes encoding VacA vary in length from approximately 3860 to 3940 nucleotides (44). Transcriptional analysis of gastric biopsies specimens collected from *H. pylori*-infected human patients revealed that VacA was among the most highly expressed of the gene screened, including 18 putative virulence factors (47).

Higher levels of VacA transcripts were detected at one week post-infection, suggesting that there may be an important role of VacA during the early stages of colonization (47). The vacA gene of *H. pylori* strain 60190 encodes a 1287 amino acid protoxin (44). The VacA protoxin undergoes at least two proteolytic processing steps through maturation process to produce the active form of the toxin. The mature 90 kDa protein contains ≈821 amino acids (44). The first cleavage is at a 12 kDa (33 amino acids) amino terminal signal sequence while the toxin transports through the cytoplasm to the periplasmic space. After this step it is believed to be secreted across the outer membrane during further proteolytic processing with cleavage in a 33 kDa carboxy-terminal domain to yield the mature secreted toxin. VacA oligomer consists of 12 of 90-kDa subunits assembled into two interlocked six-membered arrays, overlap of which gives rise to a flower-like appearance. Support for this interpretation comes from electron microscope identification of small numbers of relatively "flat" oligomers composed of six teardrop-shaped subunits, interpreted to be halves of the complete flower. These flat forms in two different orientations corresponding to hexameric surfaces that are either exposed or sandwiched inside the dodecamer, respectively (48). Secreted VacA can be further processed into an N-terminal fragment of 33 kDa (p33) and a C-terminal fragment of 55 kDa (p55) as shown in (Figure 1.2); these proteolytic processesing is catalyzed by yet unidentified proteases. However, this cleavage does not seem to be necessary for VacA activity (49). The p33 domain exhibits pore-forming activity necessary for vacuole formation (50), whereas the p55 domain not only plays a role in binding to target cells but also in the formation of oligomeric structures and anionic membrane channels (74). The N-terminal of p55 subdomain is required for VacA-induced vacuolation, formation of VacA oligomeric structures, for host cell membrane depolarization, and necessary for formation of anionic membrane channels (51), besides to these information the crystal structure of p55, domain was determine. The p55 structure is predominantly a right-handed parallel helix (residues 355–735) but has a small globular domain at the C terminus (residues 736–811) with mixed secondary structure elements (Figure. 1.3).

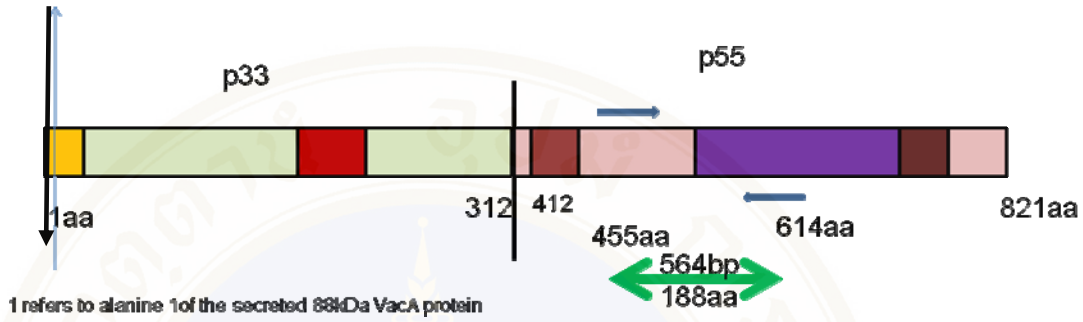



**Figure 1.2 VacA functional domains reproduced from Sewald *et al.*, 2008.** Mature secreted VacA comprises the p33 and p55 domains. The m-region contributes to cell type-specific receptor binding. Two domains in the N- and C-terminal part of p55 were highlighted, an oligomerization region and a conserved pocket, which is suggested to be involved in receptor binding. VacA forms hexamers with the pore-forming p33 domains (red) located in the centre of the oligomer. A hydrophobic region (orange) is located in the centre forming an anion-selective channel. The p55 domains (blue) are oriented outside the VacA oligomer and the conserved pocket in particular has a characteristic position for receptor binding.

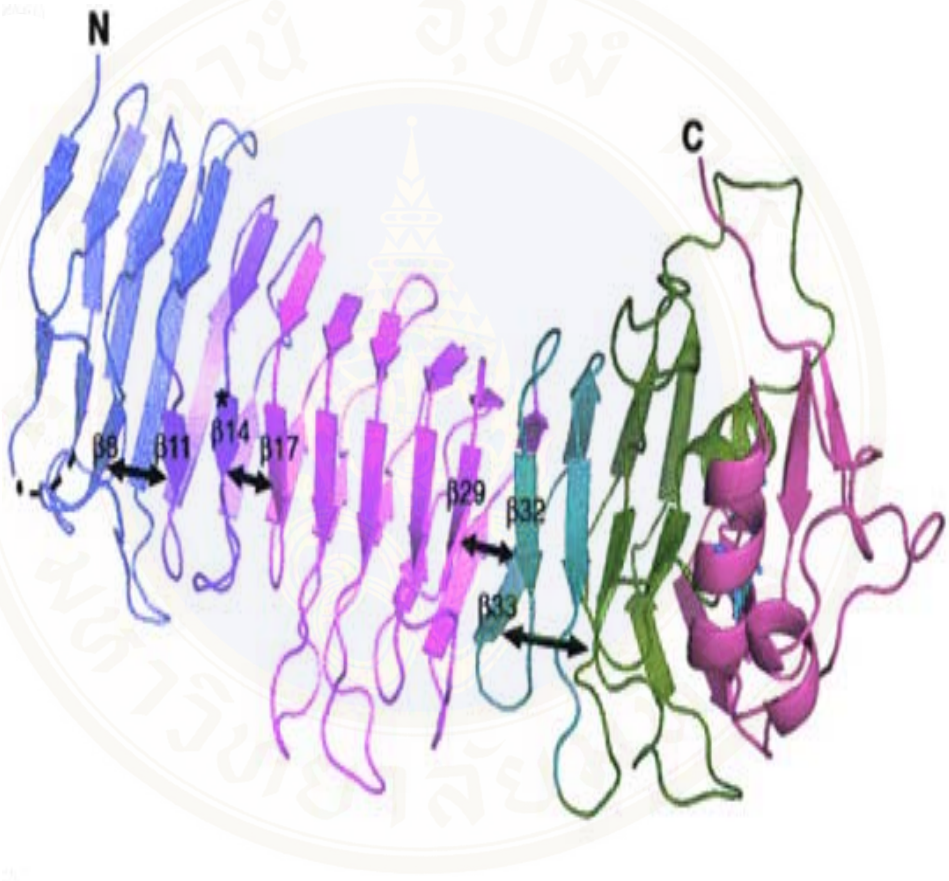



**Figure 1.3 The VacA p55 structure by Gangwer *et al.*, 2007**

The structure resembles a sock in which the C-terminal domain curves from the heel and extends to the tip of the foot which consists of a parallel β-helix with a carboxy-terminal globular domain, indicating how p55 monomers may assemble into oligomers capable of membrane pore formation (52). The vacuolating toxin VacA forms anion-selective channels in artificial planar lipid bilayers (53). VacA-dependent increase of current conduction both in artificial planar lipid bilayers and in the cellular system was effectively inhibited by the chloride channel blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and the pore formation by VacA accounts for plasma membrane permeabilization and is required for both cell vacuolation and increase of trans-epithelial conductivity. The secreted 88-kDa vacuolating cytotoxin (VacA) undergoes limited proteolytic cleavage to produce two fragments corresponding to two putative VacA domains (designated p33 and p55) and requires assembly of VacA monomers into oligomeric structures, formation of anion-selective membrane channels, and entry of VacA into host cells. Currently there is no evidence to show that the two fragments are required for VacA activity, but they are considered to represent two domains of VacA. Functional properties of recombinant VacA indicate that these two domains can interact with each other to form protein complexes. In comparison to the individual VacA domains, a mixture of the p33 and p55 proteins exhibited markedly enhanced binding to the plasma membrane of mammalian cells. Furthermore, internalization of the VacA domains was detected when cells were incubated with the p33/p55 mixture but not when the p33 and p55 proteins were tested independently. Incubation of cells with the p33/p55 mixture resulted in cell vacuolation, whereas the individual domains lacked detectable cytotoxic activity. Interestingly, sequential addition of p55 followed by p33 resulted in VacA internalization and reconstitute of cell vacuolation activity, whereas sequential addition in the reverse order was ineffective. These results indicate that both the p33 and p55 domains contribute to the binding and internalization of VacA and that both domains are required for vacuolating cytotoxic activity (54). Studies showed that the portion of the toxin molecule responsible for vacuolating activity (VacA-511) has about 422 amino acid residues at the N-terminal end of VacA which cover the p33 domain and about 100 amino acids of the p55 domain (55). The p33 domain appears to have a strongly hydrophobic region near the N-terminal and which is necessary for

membrane channel formation and VacA trafficking in host cells (50). Study hypothesized large portions of the p55 domain might not be essential for vacuolating toxin activity (51). Data show that a small alteration in only one of the two essential VacA domains is sufficient to inactivate toxin monomers (56). The amino-terminal sequence of the VacA protein is not homologous with the sequences of any other known bacterial toxin, but is partially homologous with internal sequences of numerous ion channel or transport proteins (57).

### 1.4.2. VacA genetic diversity

*H. pylori* strains vary considerably in their production of cytotoxin activity, and this is primarily due to variations in vacA gene structure which display differences between vacA from cytotoxic and non-cytotoxic strains. Some clinical isolates of *H. pylori* fail to express vacuolating cytotoxin, despite possessing a copy of the vacA gene on the chromosome, including internal duplication, large deletion, single base pair insertion, and non-sense mutations (58). The most variable region corresponds to ~800 base pair sequence located in the middle of the gene in the p58 domain ("mid region") which is designated 'm' for 'middle region'. The various m sequences have been grouped into two families of alleles, m1 and m2. Both types are pathogenic, but m2 have limited vacuolating activity on some specific cells (59). The analysis of several clinical isolates of *H. pylori* VacA revealed that vacoulating activity varies according to distinct families of *vacA* alleles. Considerable sequence variations are located at the s region which includes the amino terminal signal sequence and amino terminal sequence of the mature VacA toxin (60). Two main allelic families are recognized, designated s1 and s2 the s1 allelic type can be further divided into s1a, s1b and s1c subtypes. Certain types are associated with restricted geographical distribution. The variable regions are located near the 5′-end, s1 or s2, and in the mid-region, m1 or m2. All possible combinations of these regions (s1/m1, s1/m2, s2/m1 and s2/m2) have been reported in clinical isolates of *H. pylori* as seen in figure 1.2 (60, 54). The diversity in the middle region of VacA alleles comprises a sizable portion of the gene, and thus structural differences between type m1 and type m2 gene products could easily give rise to differences in cytotoxin phenotypes. However, the basis for the highly significant differences in phenotype between strains

with type s1 and type s2 signal sequences is less clear. One hypothesis is that strains with type s2 signal sequences export the VacA protoxin less efficiently across the cytoplasmic membrane. Alternatively, differences in the N-terminal residues of the mature secreted VacA products, arising from different signal sequence cleavage sites in type s1 and type s2 VacA proteins, may account for differences in protein function. The signal sequence cleavage site differs between the s1 and s2 VacA mature type. VacA s1 has a hydrophobic N terminus, but type s2 VacA has a short, predominantly hydrophilic, 12-amino-acid, N-terminal extension (60). Broth culture supernatants from *Helicobacter pylori* strains induce vacuolation and contain VacA s1 type in concentrations that are higher than those found in supernatants from *H. pylori* strains with VacA s2 type and s1, and s2 *H. pylori* strains typically differ not only in the VacA amino acid sequence but also in the level of VacA transcription (61). The mid region sequences between amino acids 501 and 647 (148 amino acid region) within the 58 kDa p55 domain of VacA govern the cell type specificity of m1 and m2 types (62). Possible roles and correlation of VacA in the pathogenesis of human gastrointestinal diseases and progression of disease in infected humans with *H. pylori* was examined in a hundred of studies. In a study on the clinical relevance of VacA genotypes, strong association between peptic ulcer disease and *vacA* type s1 strains corroborated the equally important finding that VacA type s2 strains are rarely associated with peptic ulceration (60). It is worth mentioning that (49) reported that s1 type VacA gene products are secreted at higher levels than s2 type VacA products. Other studies showed that VacA in gastric juice could be directly detected by a very sensitive method (bead-ELISA) are revealed that disease diversity was associated with not the allele type but the excreted amount of VacA. Therefore, the quality and the quantity of VacA are important factors in the pathogenesis of gastric disease (63). *H. pylori* strains with allelic form s1/m1 VacA are associated with an increased risk of gastric cancer and enhanced gastric epithelial cell damage compared with VacA s2/m2 alleles (64). The relationship between VacA genotypes and gastric cancer is consistent with the geographic distribution throughout the world. In regions such us Europe, North America, and South America, most patients infected with *H. pylori* strains harboring type s1 VacA alleles are at higher risk of developing peptic ulcer than those harboring strains with type s2 alleles (60, 64).

### 1.4.3. VacA multifunctional cell-modulating activities

#### 1.4.3.1. Membrane insertion and formation of channels

The effects of VacA on human cells have been experimentally investigated to understand how this toxin functions in gastroduodenal disease. VacA is inserts into host cells membrane and form pores across membrane and intoxicate a wide range of different cell types, like gastric epithelial cells and several immune type cells generating numerous cellular alterations. Native VacA can form low-conductance, voltage-dependent, anion-selective channels in planar lipid bilayers. The formation of membrane channels (pores) by VacA was investigated via the use of planar lipid bilayers, the height measurements of membrane associated VacA clearly indicate that the maximal height of the membrane-bound hexamers is markedly smaller than the total height of the soluble dodecamer. Association of VacA with membrane lipids at neutral pH was not observed, the acid activation of the monomers (pretreatment at low pH) appears to be a critical determinant for VacA binding to membrane and channel formation, conditions which also boost cell vacuolation. The formation of VacA channels requires the dissociation of the oligomer and the insertion of the released monomers into the membrane and disassembly into monomers in solution most likely precedes the formation of hexameric pores. Probably interaction of VacA monomers with membrane is followed by subsequent oligomerization and membrane entrance resulting in membrane channel formation. VacA induced cell vacuolation can be attributed to VacA channel formation in the membranes of the late endocytic compartment. A kinship between pore formation and the ability of VacA to induce intracellular vacuolation was suggested whose by monomers are formed from dissociation of inactive oligomers (48). Several amino acid residues close to the amino-terminal hydrophobic region of VacA contain three tandem GXXXG motifs which are essential for channel function (50). Mutagenesis of three nontoxic VacA proteins in the aminoterminal hydrophobic region abolishes membrane channel formation and vacuolating cytotoxin activity. Results obtained in a planar lipid bilayer assays were similar to those obtained in a HeLa cell depolarization assay. These data provide evidence that the amino-terminal hydrophobic region contains several amino

acid residues essential for membrane channel formation activity, and the membrane channel is required for the formation of intracellular vacuoles (50,55).

#### 1.4.3.2. Cellular vacuolization by VacA

Many cellular effects of VacA in various cellular locations are attributable to membrane channel formation but vacuolization is a unique and major detectable function of VacA which determines its name “vacuolating cytotoxin”. The membranes of these vacuoles contain the small GTP-binding protein Rab7, representing late endosome and lysosomal markers (66). Vacuolation depends not only on VacA, but also on the presence of permanent weak bases in the extracellular medium (57). Microinjection of VacA or the transfection of plasmids containing the VacA gene into HeLa cells resulted in the formation of intracellular vacuoles (67), providing evidence that VacA introduced into the cytosol acts on an intracellular target; potential targets include the vacuolar ATPase (V-ATPase), Rab7, and Rac1 (68). It is well established that VacA-induced vacuolation requires V-ATPase activity, and that its inhibitor, bafilomycin A1, reduced VacA-induced vacuolation in mammalian cells (69). Rab7 may be important for supporting membrane deposition and homotypic fusion between late endosomes. Dynamin, a high molecular weight GTP-binding protein that functions as a mechanochemical enzyme in vesicle formation, is involved in VacA-induced vacuolation (99). In addition, VacA-induced vacuolation was also inhibited in cells with transiently transfected dominant-negative mutant syntaxin 7 (70). Syntaxin 7 is an integral membrane protein present on both late endosomes and lysosomes. In addition, AGS (Human Stomach Adenocarcinoma cell line) cells expression of syntaxin 7 mRNA and protein is intensified by exposure to VacA, pointing to the participation of syntaxin 7 in VacA-induced vacuolation (70). At the final steps of vacuole formation by VacA, vesicle associated membrane protein 7 (VAMP7) is a partner of syntaxin 7 in the process of lysosome–endosome fusion (101). These results suggest that VacA-induced vacuolization is a result of a toxin-induced alteration of intracellular vesicle trafficking. Earlier studies have shown that any noticeable alterations in cellular morphology are not produce by purified VacA if the toxin is added to cells in the absence of previously treated with weak bases (57). Addition of VacA to cells under conditions that are not permissive for vacuole

formation (e.g., the absence of supplemental weak bases), may allow detecting subtle effects of the toxin on endocytic processes that are obscured by extensive changes in cellular architecture associated with VacA-induced vacuolation. The identification of any such effects would potentially be helpful for understanding the mechanism of VacA action. VacA on late endocytic membrane traffic are critical mechanistic steps in the process of VacA-induced cell vacuolation (74).

## 1.5. Role of VacA in gastroduodenal disease

Several experimental studies have examined and evaluated the potential roles of VacA in the gasroduodenal disease and vacA allelic type correlation with disease development. VacA toxin likes many, if not all bacterial toxin participate in some way in the ability of bacteria to colonize their hosts. Toxin producing bacteria strains of *H.pylori* that contain allelic forms of vacA are associated with an increased risk of symptoms of gastroduodenal disease compared with strains not containing other allelic forms of vacA. In particular strains that contain *H.pylori* harboring vacA allele s1 type are highly associated with an increased risk for the development of gastric ulcer disease and gastric cancer and theses data can be correlated with the failure of vacA s2 type to cause noticeable cytotoxicity *in vitro* (64,72). A new vacA polymorphic site, the intermediate (i) region which encodes part of the p33 domain of VacA, displays sequence variations at the nucleotide level. Two i-region types were identified; i1 and i2, and both were common among clinical isolates. Interestingly, only naturally occurring s1/m2 strains varied in i-type; s1/m1 and s2/m2 strains were exclusively i1 and i2, respectively. Vacuolation assays showed that i-type determined vacuolating activity among these s1/m2 strains, and exchange mutagenesis confirmed that the i-region itself was directly responsible for vacuolating activity. It was shown that gastric adenocarcinoma was strongly associated with i1-type strains not i2 strains in the Iranian population. Logistic regression analysis showed this association to be independent of and larger than, associations of vacA s- or m-type or *cag* status. The conclusion is that for gastric adenocarcinoma the vacA i-region is an important determinant of *H pylori* toxicity and the best independent marker of vacA-associated pathogenicity (73).

### 1.5.1. VacA binding to the cell surface

Many different cell lines were used to study vacA effects including gastric epithelial cells such as AGS and AZ-521, immune cells like T-cells, macrophages, and mast cells. Binding of VacA to a cell surface and entry into the cell is a crucial step in toxication pathway including vacuolation and mitochondrial perturbation .The binding of VacA to the surface of cells host contributes to a wide range of variable effects in intoxicated cells and are dependent on the ability of VacA to form membrane channels. Other effects take place through the channel independent pathway, thus VacA may act both at the cell surface and in at least one intracellular location (74). Cell modulating activity is markedly enhanced when water soluble oligimeric vacA was pre activated with acid or alkaline (67). Different VacA receptors have been described for the intoxication of different cell types (49).VacA binds to specific high – affinity cell surface receptors on target cells previosly identified as receptor-like protein tyrosine phosphatases RPTPα and RPTPβ. Chemical agents that promote the differentiation of HL-60 cells into macrophage- and monocytelike cells, but not granulocyte-like cells, enhance VacA sensitivity by increasing the expression of RPTPß (77). These receptors have been described as receptors for epithelial cells that co-immunoprecipitated with anti-vacA antibody (76). On the other hand, lymphocyte function-associated antigen-1 (LFA-1) was identified as a specific VacA receptor on T-cells (75) but not epithelial cells. Thus, VacA uses different receptors for intoxication or modulation of epithelial or immune cells. G401 cells, a human kidney tumor cell line, not expressing RPTPß, respond to VacA, and by co-immunoprecipitation p140 has been identified as receptor protein, p140 amino acid sequence was shown to be identical to those in RPTPα. Thus, two receptor tyrosine phosphatases, RPTPα and RPTPß, function as VacA receptors (76). Epithelial cell deficient in protein tyrosine phosphatase receptor type Z (also called RPTP-β, encoded by *Ptprz*) do not show mucosal damage by VacA, thus, supporting the idea that RPTP-β has a role in vacA induced toxicity binding. RPTP-β was reported to trigger a signaling pathway involving tyrosine phosphor-relation of Git1 (also called Cat-1). A substrate of RPTP-β, Git1 is a multidomain protein that is thought to function as an integrator of signaling pathways controlling vesicle trafficking, cell adhesion and cytoskeletal organization (78). Cell-specific binding has been attributed to differences

in the m1 and m2 alleles. Strains encoding s1/m1 *vacA* genes typically produce m1VacA with cytotoxic activity on human cervical carcinoma HeLa cells, whereas m2VacA, produced by strains with the s1/m2 vacA gene, induced vacuoles in primary cultured human gastric cell lines as well as non-gastric epithelial RK13 cells, but not in HeLa cells (65). Even in the absence of the 37 kDa subunit, P58 has a conformation capable of interacting with the cell and cell binding determinants of the m-region are found within p58 domain (79). The P58 subunit fails to form higher oligomers in the absence of the 37 kDa subunit and oligomerization and or hexameric structure of VacA is essential for endocytosis and a productive interaction with the cell. Immunoprecipitation experiments showed that, in AZ-521 cells, activated m2VacA, similar to m1VacA, binds to two receptor-like protein tyrosine phosphatases, RPTPα and RPTPß thus suggesting that activated m2VacA as well as m1VacA contribute to cell specific binding (80).

### 1.5.2. VacA-induced apoptotic cell death

Vacuolating cytotoxin purified from *H. pylori* causes mitochondrial damage, energy level decrease in human gastric cells and induces apoptotic cell death, and these effects of vacA on mitochondria have been detected after addition of VacA to the cell surface and also intracellular expression of VacA in transiently transfected cells (81). Purified activated VacA applied externally to cells reduces the membrane potential of mitochondria, resulting in cytochrome c release. The p37 domain genes transfected into Hep-2 cells localize specifically to the mitochondrial matrix, whereas the p58 domain remains in the cytosol. VacA accumulated into the mitochondria inner membrane, resulting in induction of cytochrome c release, activation of executioner caspase 3 and apoptotic cell death. Correspondingly intracellular expression of VacA in transiently transfected cells was reported to induce the release of cytochrome c from the intermembrane space of mitochondria, which suggests that VacA-induced apoptosis may occur via a mitochondria-dependent pathway (82). Study have demonstrated, that substantial fraction of full-length VacA added exogenously to cultured cells localizes to the mitochondria and modulates mitochondrial membrane permeability by a mechanism dependent on toxin channel activity ultimately resulting in cytochrome c release (83). On the other hand, bafilomycin A1 inhibited vacuole

formation but did not inhibit VacA-induced Bax activation and cell death. These data indicate that vacuole formation and Bax activation were independent effects of VacA; most VacA was localized to vacuoles, marked by Rab7-GFP, rather than mitochondria as analyzed by experiments using immunostaining and confocal microscopy. These results suggest that VacA may not directly induce cytochrome c release from mitochondria, and instead, suggest that apoptosis-related factors such as proapoptotic family proteins Bcl-2, Bax and Bak may be involved. In the VacA mediated process in which the Bax level was decreased, release cytochrome c indicates that VacA utilize both Bax and Bak to induce apoptosis (84). The time course of Bax activation in response to VacA paralleled that of cytochrome c release. In line with these findings activation of caspase 3 and cleavage of PARP (poly(ADP-ribose) polymerase) were observed. Thus, in response to VacA, Bax and Bak activation cause cytochrome c release from mitochondria and apoptotic cellular death. Pro-apoptotic Bcl-2 protein-mediated apoptosis execution was vacuolation-independent (84). The effects of VacA on late endosomal compartments and mitochondria are detectable within several hours of the addition of VacA to cells. By contrast, VacA causes several other cellular effects that can be detected after addition of VacA to a human gastric adenocarcinoma cell line (AZ-521). Two classes of mitogen-activated protein kinases (MAP KINASES) (p38 and ERK1/2) and the activating transcription factor 2 (ATF2) signalling pathway become activated. The inhibitor of p38 kinase activity (SB203580) did not block VacA-induced vacuolation or VacA-induced cytochrome *c* release, which indicates that VacA induced activation of the p38/ATF-2 signaling pathway is independent of the effects of VacA on late endocytic compartments and mitochondria (85). Thus, VacA may be liable for the triggering of several independent signaling pathways. The rapid cellular responses to VacA described above are likely to be consequences of VacA interaction with specific cell surface components, without a requirement for internalization of the toxin (54).

# CHAPTER II
# OBJECTIVES

*Helicobacter pylori* is the main cause of peptic ulceration and gastric adenocarcinoma. The vacuolating cytotoxin gene, *vacA*, is a major determinant of virulence. Three naturally polymorphic sites in *vacA*, the signal region, midregion and intermediate region are well-characterized determinants of toxicity and markers of pathogenesis. VacA, a large multifunctional protein of around 821 amino acids, displays with structural features and cell modulating activities that are distinct from those of any other known bacterial toxins. There is evidence that VacA contributes to the ability of *H. pylori* to colonize the stomach and contributes to the pathogenesis of gastro duodenal disease. The gene encoding VacA is characterized by a high degree of genetic variation specific allelic variants of VacA exhibit different levels of toxin activity. VacA is suggested to exert its cytotoxic activity after internalization by epithelial cells. Cell death can be executed via different mechanisms. One way is the apoptotic pathway. Apoptosis is characterized by morphological and biochemical changes such as membrane blebbing, chromatin condensation, and DNA fragmentation. In order to get insight into the comparative analysis of sequence diversity of *vacA* polymorphic sites and biological activities on eukaryotic cells between Thai isolate VacA toxin and compare it with model strains 60190. This study aimed of establishing molecular methods including PCR technique to construct a recombinant gene fragment encoding mature VacA, gel electrophoresis, SDS-PAGE, and Western blot analysis for cloning, sequencing, and expression of VacA in the *E.coli* system and purification by affinity chromatography. The later will provide sufficient amount and purity of protein for *in vitro* characterization of apoptotic activity (cell damage) of the toxin which is analyzed by biochemical method by which VacA induces apoptosis.

# CHAPTER III
# MATERIALS

## 3.1. Chemicals and reagents

All chemicals and reagents used were analytical grade purchased from a variety of suppliers

| **Chemicals** | **Company** |
|---|---|
| Ampicillin | Promega |
| Isopropyl-β-D-thiogalactopyranoside (IPTG) | Sigma |
| 1, 4-Dithiothreitol (DTT) | Sigma |
| Coomassie brilliant blue R-250 | Sigma |
| Deoxyribonucleotide triphosphates (dNTPs) | Promega |
| Standard DNA markers | Gibco BRL, Biolabs |
| SDS-PAGE molecular mass standard | Bio-Rad |
| Bradford protein assay reagent | Bio-Rad |
| Lambda DNA-*Bst*EII digested | Biolabs |
| Lambda DNA-*Hind*III digested | Biolabs |
| Standard protein marker | Bio-Rad |
| PROTEIN nitrocellulose transfer membrane | Bio-Rad |
| HisTrap chelating column | GE-Health Care |
| QIAquick® PCR purification kit | QIAGEN |
| QIAquick® gel extraction kit | QIAGEN |

## 3.2. Enzymes

| | |
|---|---|
| Phusion® DNA Polymerase | Biolabs, Inc. |
| T4 DNA ligase | Promega |
| Restriction endonucleases | Promega |

## 3.3. Antibodies

| | |
|---|---|
| Mouse anti-His antibody, | Invitrogen |
| Anti-*H. pylori* VacA protein (Toxin), | Austral Biologicals |
| Anti-rabbit IgG, alkaline phosphatase conjugate | Sigma Immune Chemicals |

## 3.4. Bacterial strains

TOP10 strain was provided to propagate vectors containing inserts in recombination deficient (*rec*A), endonuclease A-deficient (*end*A) *E. coli* strains.

Genotype: F-*mcr*A Δ (*mrr-hsd*RMS-*mcr*BC) Δ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139. (*ara-leu*) 7697 *gal*U *gal*K *rps*L *end*A1 *nup*G.

TOP10 contains:

*recA* for stable replication of high copy number plasmids

*endA* for improved yield and quality of miniprep DNA

*hsdRMS* to eliminate cleavage of recombinant plasmid by the endogenous *Eco*RI restriction system (Figure 2.1)

## 3.5. Culture media

**LB broth medium**

1% (w/v) tryptone

(w/v) 0.5% yeast extract,

(w/v) 0.5% NaCl

**LB Agar Plates**

1% (w/v) tryptone,

(w/v) 0.5% yeast extract,

(w/v) 0.5% NaCl

1.5 % (w/v) agar.

Both media were prepared as described in molecular cloning protocol (86).

**Cell culture media**

Dulbecco's modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM-Ham) (Sigma St. Louis MO, USA) supplemented with, 50 U/ml penicillin, 50 μg/ml streptomycin and 5% fetal bovine serum

## 3.6. Vector and Recombination plasmid

**pTrcHis2A plasmid** (Invitrogen) was used for expression the mature VacA gene of clinical Thai isolate of *H. pylori*. The pTrcHis2A vector contains the *trc* promoter, lacO operator, *Laq*I$^q$ repressor, gene 10 translational enhancer, and initiation ATG located at the N-terminal site. Multiple cloning site (MCS) Allows insertion of a gene for expression, polyhistidine (6x His) region fusion tag located at the C-terminal site allows detection of the fusion protein by the anti-*His antibody*, *rrn*B transcriptional termination sequence, ampicillin resistance gene ORF allows selection of the plasmid in *E. coli*, ribosome binding site (RBS) and a mini-cistron which contains a second ribosome binding site for efficient reinitiation of translation into the gene of interest (Figure 3.1).

**pTrcHis2A/VacA full length (figure 3.2)**

The recombinant plasmid containing the mature VacA gene amplified from a clinical Thai patient isolate (62 years old, male, at Vichaiyuth Hospital) digested by restriction enzymes *Nco*I and *Pst*I. The VacA gene was cloned in the pTricHis2A vector using the *Nco*I and *Pst*I cloning sites to construct the expression plasmid pTrcHis2A/VacA full length.

## 3.7. Synthetic oligonucleotides

All synthetic oligonucleotides used for PCR were purchased from Sigma-Aldrich Pte Ltd, Singapore. The sequence of oligonucleotides is shown below. The restriction enzyme recognition sites are underlined. The start codon is shown in bold. All primers were analyzed for melting temperature, % GC, and hairpin loops by primer designer at Invitrogen website (http://www.invitrogen.com).

Synthetic gene of vacA sequence of 60190 *H. pylori* strain bacteria in pLS vector synthesized from TOP Gene technologies, Incorporation for saving time and cost. The VacA sequence was cloned in the pLS vector (the company vector), that has chloramphenicol resistance gene as a biological marker.

### 3.7.1. PCR primers for amplification of full length mature VacA gene

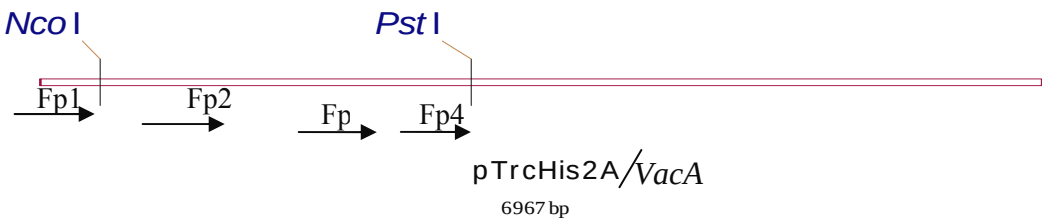
PCR primers used to amplify the vacA fragment encoding the mature vacA gene from *H. pylori* isolated DNA genome as a template. The sequence of *H. pylori* ATCC 49503 (strain 60190) (GenBank accession No.:U05676) with the VacA genotype s1/m1 was used as a template for primer design. Underlined nucleotides are restriction sites for *Nco*I and *Pst*I enzymes.

SJ_VacA1F 5`-CATGCCATGGCCTTTTTTACAACCGTGATCA-3`
TM = 79ºC *Nco*I
SJ_VacA821R 5`-TGCACTGCAGAGCGTAGCTAGCGAAACGC-3`
TM = 80 ºC *Pst*I

### 3.7.2 Sequencing primers

PCR primers used in the sequencing reaction are listed below

| | | |
|---|---|---|
| Fp1 | 5`-TTTATTATTAAAAATTAAAGAGGTATATAT-3` | TM= 54ºC |
| Fp2 | 5`-AACGCTGAAATTTCTCTTTATGATGGCGCC-3` | TM= 75ºC |
| Fp3 | 5`-AGAAGCAGGCGGTGCTGCTTTACCAGGCTC-3` | TM= 80ºC |
| Fp4 | 5`-TCTTTTTGTCCAAGATGGGCGTGTAGCGAC -3` | TM= 80ºC |



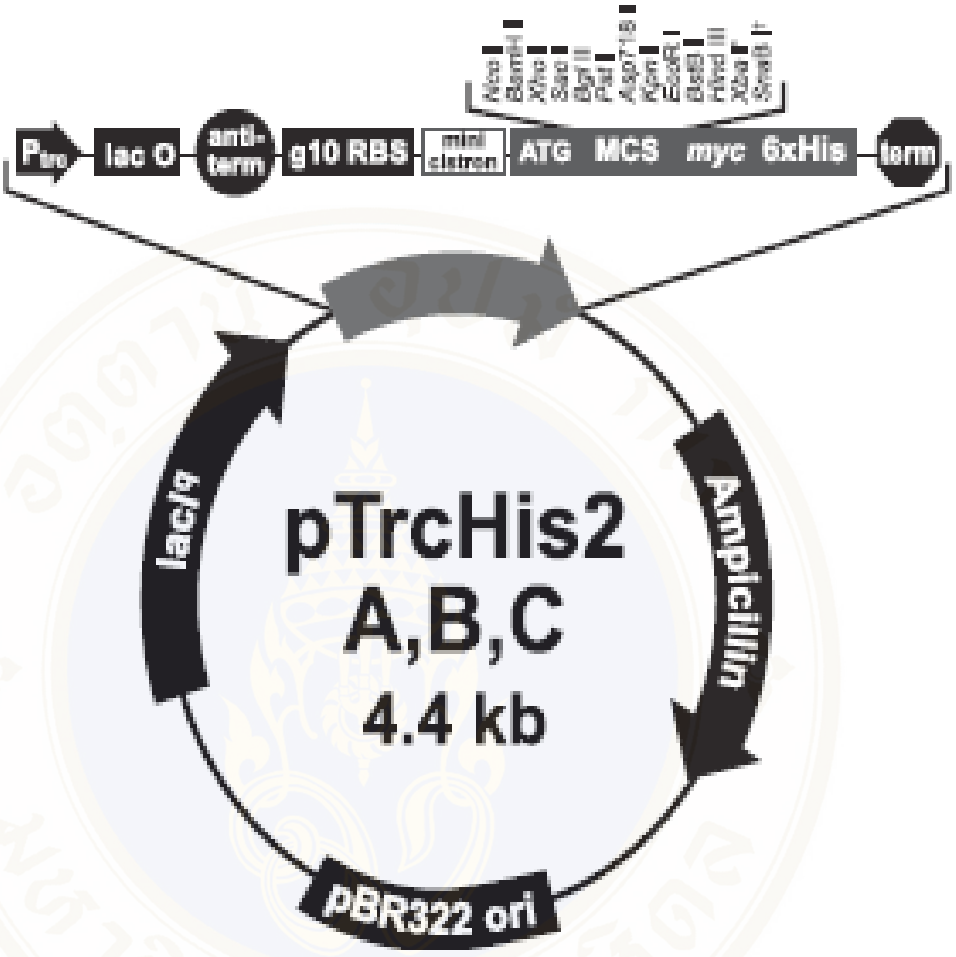



**Figure 3.1 Physical map of pTrcHis2A plasmid**

The figure shows the physical map of pTrcHis2A vector reproduced from the Invitrogen website for explanation of genetic symbols see text (http://www.invitrogen.com).

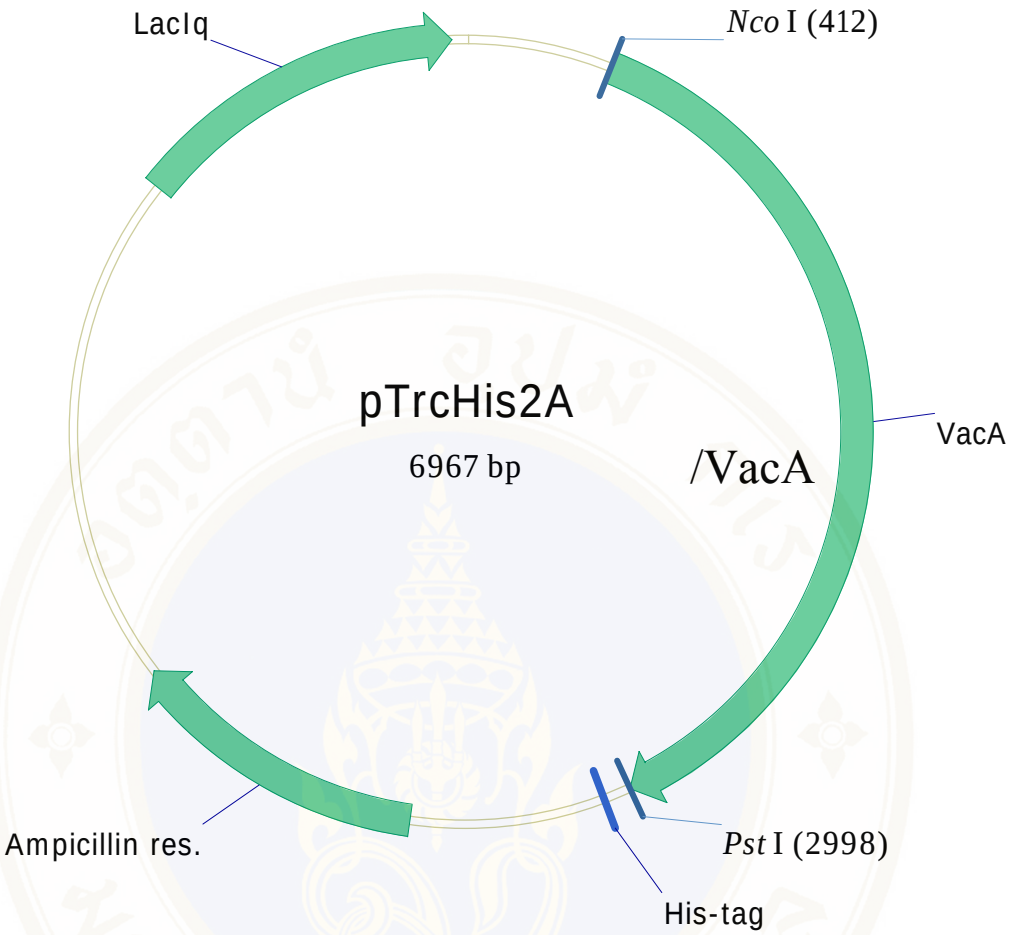



**Figure 3.2 Physical map of pTrcHis2A/VacA full length plasmid**

The figure shows the recombinant plasmid, pTrcHis2A/VacA, harboring 2577 bp of mature VacA gene from isolated DNA (*H. pylori)* of Thai patient. The plasmid backbone contains polyhistidine (6xHis) tag, Ampicillin resistance gene (Amp □) and repressor gene *LaqI*$^{q}$. The plasmid map was generated by Vector NTI program.

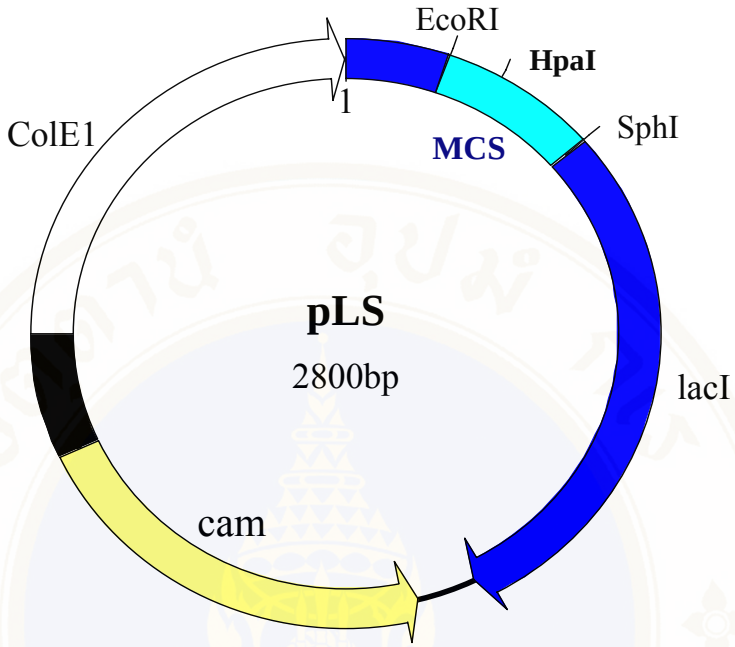



**Figure 3.3 Physical map of pLS full length plasmid**

The figure shows the full length plasmid that used for harboring the synthetic VacA gene. The synthetic fragment was cloned into the *Nco*I-*Hpa*I sites of the pLS vector. The synthetic gene and plasmid map were generated by TOP Gene Technologies, Inc. for explanation of genetic symbols and details see appendix.

# CHAPTER IV
# METHODS

## 4.1. Plasmid extraction

Methods were employed to obtain plasmid DNA contained in *E. coli*: alkaline lyses method and isolation of high copy plasmid DNA by commercial NucleoSpin®Plasmid kit.

### 4.1.1 Alkaline lysis method (86)

To grow bacterial culture as a starter, a single colony was inoculated in 3 ml of LB broth containing 100µg/ml ampicillin and incubated at 37ºC for 10-12 hours with 250 rpm shaking. One milliliter of the starter was inoculated in 100 ml of LB broth containing 100 µg/ml ampicillin to give 1% inoculation. The culture was incubated at 37ºC for 10-12 hours. Cells were harvested by centrifugation at 5,000 g for 10 min. Cell pellet was resuspended in 3.6 ml of ice-cold solution I (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl pH 8.0) containing 250 µg/µl lyzozyme. After mixing by vigorous vortexing, 8 ml of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added. The contents were gently and rapidly mixed by inverting the tubes five times and stored it on ice for 3 min then 4 ml of ice-cold solution III (3 M potassium, 5 M sodium acetate) was added to the mixture. The tube was gently and briefly vortexed for 15 seconds to mix the bacterial lysate and stored for 5 min. The supernatant was collected by centrifugation at 12,000 g, for 20 min. The supernatant was transferred to fresh tube and an equal volume of phenol: chloroform (1:1, v/v) was added. The mixture was mixed by vigorous vortexing and the aqueous phase was collected by centrifugation at 12,000 X g, for 20 min. the supernatant was mixed with an equal volume of chloroform in order to eliminate the phenol as much as possible. After vigorous vortexing, the aqueous phase was collected by centrifugation at 12,000 g, 4ºC for 20 min. The DNA was precipitated by addition of isopropanol to supernatant. The mixture was incubated at room temperature for 20 min and

centrifuged at 12,000 g for 30 min. The DNA pellet was washed twice with 70% ethanol. After air drying, the DNA pellet was resuspended in sterile distilled water.

#### 4.1.2. Isolation of high copy number plasmid DNA from *E. coli* by nucleoSpin®Plasmid kit

The procedure for the isolation of DNA plasmid from *E.coli* was based on manufacturer instructions. 1.5 ml of a saturated *E.coli* LB culture was pelleted in a standard benchtop microcentrifuge for 30 sec at 11,000 X g. The supernatant discarded and as much as possible liquid removed. 250 µl of buffer A1 were added and the cell pellet completely resuspended by vortexing. 250 µl of buffer A2 added, gently mixed by inverting the tube 6-8 times. Samples were incubated at room temperature until the lysate appeared clear. 300 µl of buffer A3 were added and, gently mixed by inverting the tube 6-8 times. Lysate clarified by centrifugation for 10 min at 11,000 g at room temperature. A NucleoSpin®Plasmid column was placed in a collection tube (2 ml) and 750 µl of the supernatant decanted from previous step were loaded onto the column. The column was centrifuged for 1min at 11,000 xg. The flow-through discarded and NucleoSpin®Plasmid column placed in the collection tube. The step was repeated to load the remaining lysate. 600µl of buffer A4 were added (supplemented with ethanol) and centrifuged for 1 min at 11,000 g. The flow-through was discarded and the NucleoSpin®Plasmid column was placed in the empty collection tube (2ml),and centrifuged for 2 min at 11,000 g and the collection tube discarded. The NucleoSpin®Plasmid column was placed in a 1.5 ml microcentrifuge tube and 50µl of buffer AE were added and incubated for 1min at room temperature and centrifuged for 1 min at 11,000 g.

## 4.2. Restriction endonuclease digestion

Restriction endonuclease analysis was used to analyze the authenticity of the plasmids. 2-5 units of appropriate enzymes were used to digest 1 µg of DNA under the conditions (optimum buffer, temperature and reaction duration) recommended by manufacturer. In general, 20 µl reactions composed of 1µg of DNA substrate, appropriate digestion buffer provided by the manufacturer and approximately 10 U of

restriction endonucleases. The digestion reaction was run on gel electrophoresis to visualize the reaction patterns.

## 4.3. Agarose gel electrophoresis

The separation of the DNA fragments depends on the concentration of the agarose gel and size of the DNA fragments. The agarose gel was prepared by dissolving the agarose powder in TAE buffer (40 mM Tris-acetate pH 8.3, 1 mM EDTA) at boiling temperature. After cooling down to 50-60 ºC, the gel mixture was then poured into the mold, allowed to solidify at room temperature. The DNA samples were mixed with 6 times gel loading dye (6X) (0.1% bromophenol blue, 40% ficoll 400 and 5 mM EDTA) to give a final concentration of one time gel loading dye (1X) before loading into slot of the gel which was submerged in TAE buffer. Electrophoresis was then performed at constant voltage (100 volts) for 1 hour. After electrophoresis was completed, the gel was stained in 2 µg/µl ethidium bromide solution for 5 minutes followed by destaining in sterile distilled water for 10 minutes. The DNA patterns were visualized under UV light. The amount of DNA was estimated by comparing the stained DNA bands with the standard DNA markers (λDNA digested with *Hin*dIII or *Bst*EII) of known concentrations under UV light and photographed.

## 4.4. Plasmid construction

### 4.4.1 PCR amplification of VacA

Based on the known sequence of vacA gene of *Helicobacter pylori* ATCC 49503 (strain 60190) (GenBank accession No.:U05676) with the VacA genotype s1/m1, specific primers for PCR amplification were designed and purchased from Sigma-Aldrich Co., Ltd (Singapore). The VacA fragment encoding the mature VacA gene from *Helicobacter pylori* isolated DNA genome from isolated Thai clinical patient used as a template. Many thanks for Dr. Wanpen and her student Muthanaporn for providing the chromosomal DNA sample and helpful comments on the laboratory

work direction. The plasmid was analyzed by restriction enzyme digestion. In 50µl of PCR reaction containing 118ng of template, 200 µM of dNTPs, 10 pmol of each primer 5x phusion buffer purchased (Fermantas company) and 1.5 units of polymerase phusion enzeme purchased from (Fermantas company). PCR profile used for amplification VacA fragment encoding mature VacA are represented in table1 below and the product of the amplification process was analyzed on 1% agarose gels.

**Table 4.1 PCR profile for amplification of VacA**

| Number of cycle | Period | Temperature (°C) | Time |
|---|---|---|---|
| 1 | Pre-denaturing | 98 | 2 min. |
| 25 | Denaturing | 98 | 10 sec. |
| | Annealing | 65 | 30 sec |
| | Extension | 72 | 90 sec |
| 1 | Final extension | 72 | 7 min |

**4.4.2 Purification of DNA from PCR and Enzymatic Reactions by QIAquick®PCR purification Kit**

The procedure for purification of DNA fragments was based on the manufacturer instruction. Five volumes of the P buffer were added to one volume of the sample (250 µl). The mixture was applied to a QIAquick column, centrifuged at 12,000 rpm for 30-60 sec and the flow-through was discarded. To the bound DNA, 0.75 ml of PE buffer was added and the column centrifuged at 12,000rpm for 20-60 sec. After discarding the flow through the column was centrifuged at 12000 rpm for additional 1 min to completely remove the residual ethanol from PE buffer. The QIAquick column was placed into anew 1.5 microcentrifuge tube and 30 µl sterile MilliQ water was added to the center of QIAmembrane to elude the bound DNA. The column was incubated at room temperature for 1 min and centrifuged at 12000rpm for 1 min.

### 4.4.3 Purification of DNA from TAE agarose gel

To prepare the PCR product or restriction endonuclease digestion product, about 250 µl of product were purified by QIAquick®PCR purification kit. 10 units of *Nco* I and *Pst* I (Promega) were used to digest 2.5 µg of PCR product. The digested product was analyzed on 1% agarose gel and purified by illustra GFX PCR DNA and GEL band purification Kit (GE Healthcare).

To prepare the vector, the pTrcHis2A vector (invitrogin) was extracted from *E.Coli* TOP 10 strain by nucleoSpin®Plasmid kit. Restriction analysis was performed to verify the restriction map. About 2 µg of pTrcHis2A vector was linearized at the concentration of 10 units of enzymes *Nco* I and *Pst* I (Promega) of 1 µg of DNA. The digested vector was analyzed on 1% agarose gel and purified by illustra GFX PCR DNA and GEL band purification Kit (GE Healthcare).

### 4.4.4 Preparation of competent cells by $CaCl_2$ method

A single colony of *E.coli* was inoculated into 3 ml of LB broth and incubated overnight at 37°C with vigorous shaking at 250 rpm. The overnight culture was diluted 1:50 in LB broth and incubated at 37°C with shaking until $OD_{600}$ reached 0.3-0.4. The culture was chilled on ice for 10 minutes prior to centrifugation at 4,000 rpm for 10min at 4°C.The supernatant was decanted and the pellet was resuspended in 10 ml of chilled 0.1 M $CaCl_2$ and stored on ice for 10min. After centrifugation at 4,000rpm for 10 min at 4°C, the pellet was resuspended in 10 ml of ice-cold 0.1 M $CaCl_2$ for additional time (5 min), placed on ice and centrifuged as described above. Glycerol was added to the cell suspension making a 30% final concentration and the suspension was stored on ice for 15 min. Aliquots of competent cells (100µl) were stored at -80°C until required.

### 4.4.5. DNA ligation and transformation

The ligation reaction was performed a 10:1 molar ratio of insert to vector. Ligation mixture contained 1X ligation buffer (Promega), 5 units of T4 DNA ligase enzyme and sterile MilliQ water in a final volume of 20 µl. The mixture was then

incubated at 14°C overnight. In order to calculate the appropriate amount of DNA inserts, the following equation was used.

$$\text{DNA inserts (ng)} = \frac{\text{amount of vector (ng) x insert size kb x (insert:vactor mass ratio)}}{\text{Vector (kb)}}$$

The reaction was transformed into competent *E.coli* cells strain TOP10. 10 µl of ligation reaction solution was mixed with 100 µl of competent cells. After incubation on ice for 35 min, cells were heat shocked at 42°C for 100 sec, followed by quick cooling on ice for 2 min. Then LB broth without antibiotic was added to give a final volume of 500 µl and cells were incubated at 37°C with shaking 250 rpm for 1 hour. Cells were resuspended in 200 µl LB media and plated on LB agar containing 100µg/ml ampicillin. Agar plates were incubated overnight at 37°C. Single colonies were selected and spotted on LB agar containing 100µg /ml ampicillin into 6 dots for each single colony. The master plates were incubated overnight at 37°C.

## 4.5. Isolation of recombinant clones

12 colonies were selected from master plate and inoculated into 3ml of LB broth and incubated overnight at 37°C with vigerous shaking at 250 rpm. The plasmid DNA of these colonies was extracted and band pattern analyzed by electrophoresis. The procedure for the isolation of DNA plasmid from *E.coli* was done by NucleoSpin®Plasmid kit based on manufacturer's instructions. Restriction enzyme digestion of plasmid DNA was used for verifying the clones with changed band pattern derived from the previous electrophoresis size screening. Plasmid was extracted from each clone by NucleoSpin®Plasmid kit and double digested with *Nco*I and *Pst*I enzymes that were compatible to the introduced restriction sites within the primers. The digested products were analyzed on 1% agarose gel.

## 4.6. DNA sequencing

DNA sequencing reactions were performed by Macrogen Incorporation at South Korea for 4 clones. The result of DNA sequencing was analyzed by alignment

with the sequence of VacA of *Helicobacter pylori* ATCC 49503 (strain 60190) (GenBank accession No.:U05676) with the VacA genotype s1/m1, which was used as template for primer design using Vector NTI program.

## 4.7. Recombinant protein expression

### 4.7.1 Optimum conditions for recombinant expression

Recombinant plasmid (pTrcHis2A/VacA) containing the vacA insert were transformed into *E.coli* TOP10 for protein expression. A single colony harboring the recombinant was inoculated into 3ml of LB broth containing 100 µg/ml ampicillin and the culture was incubated overnight at 37°C with 250 rpm shaking. The overnight culture was transferred to new flasks containing LB broth with 100µg/ml ampicillin to give 2% final concentration. The culture was incubated at 37ºC until $OD_{600}$ reached 0.5-0.6 then the flasks were incubated at various times (0 hr, 1 hr, 2 hr, 3 hr, 4 hr, 5 hr), temperature (25°C, 37°C) and IPTG was varied from 0.1 mM-0.8 mM.. Other flasks were incubated without IPTG with various times (0 hr, 1 hr, 4 hr, 6 hr, overnight) and the OD was measured accordingly. Cells were collected into one ml and mixed with SDS-PAGE dye buffer to perform the SDS-PAGE and Western blotting analysis

### 4.7.2 Protein (Toxin) expression

Recombinant plasmid (pTrcHis2A/VacA) containing the vacA insert was used for expression of C-terminal hexahistidin-tagged recombinant protein. Culture of *E.coli* Top10 transformed with the expression plasmid was grown in 800 ml LB broth with 100µg/ml ampicillin at 37ºC until $OD_{600}$ reached 0.5-0.6. The expression of the recombinant protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to give a final concentration of 0.1 mM and incubated for additional 3 hours at 37ºC. Cells were harvested by centrifugation at 6,000 rpm 4ºC for 10 minutes and stored at -20ºC until required.

## 4.8. Electrophoresis of Protein

### 4.8.1 Sample preparation

#### 4.8.1.1 *E. coli* crude lysate

After expression of the target protein, *E.coli* cell pellet (600 OD) was harvested by centrifugation at 6000 rpm for 30 sec. Cell pellet was resuspended by vortexing with sample buffer (50 mM Tris-HCl pH 6.8, 100 mM dithiotheritol, 2% (w/v) SDS, 10% glycerol, 0.1% (w/v) bromophenol blue) and the concentration was adjusted to 0.1 OD. The mixture was heated at 95ºC for 15 min followed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was loaded on SDS-PAGE and Western blot analyses were performed.

#### 4.8.1.2 Soluble and insoluble fraction preparation

The pellet from 800 ml culture was resuspended by vortexing in 15 ml of buffer A (0.1 mM Tris-HCl pH 7.5, 0.3 M NaCl). Suspended cells were lysed by sonication (5 sec on, 5 sec off for 10 min) on ice using a Sonicator Ultrasonic processor XL (Misonix Inc. NY). The cell lysate was centrifuged at 10,000 g, 4ºC, for 30 min. After centrifugation, the insoluble fraction and the supernatant were collected and transferred to new tubes. The insoluble fraction was washed twice with buffer A containing 1% triton X-10 and with buffer A for an additional time. Inclusion bodies were solubilized in buffer B (0.1 mM Tris-HCl, pH 8, 0.3 M NaCl, 6 M urea) and clarified twice by centrifugation at 10,000 xg, 4ºC, for 30 min. The supernatant was discarded. The solubilized inclusion bodies were stored at -20ºC, for subsequent protein purification.

### 4.8.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (86)

The protein sample was prepared by mixing with 4x loading buffer (60 mM Tris-HCl, pH 7.5, 2% of SDS 10% glycerol, 0.025 % bromophenol blue, 100 m DTT) in 3:1 (v/v) ratio and boiled at 95ºC for 5 min. The heated sample was vigorously mixed and centrifuged at 10,000 rpm for 10 min to precipitate any

insoluble materials. Supernatant equivalent to 0.15 OD of the cell culture was loaded onto SDS-PAGE. Electrophoresis was performed according to the protocols for Mini-protein II electrophoresis (Bio-Rad) and Laemmli. The SDS-PAGE gel system is composed of separating and stacking gels. The separating gel is consist of 3% cross-linker (bis-acrylamid), 10% gel, 0.375 M Tris-HCl (pH 8.8) and 0.1% SDS. The stacking gel containing 3% cross-linker (bis-acrylamid), 5% gel, 0.125 M tris-HCl (pH 6.8) and 0.1% SDS. The gel was run in tris-glycine buffer (25 mM Tris, 192 mM glycin, 0.1% SDS) at constant voltage, at room temperature. After electrophoresis, the protein bands on the gel were visualized by 1 hour soaking in staining solution containing 50% methanol, 10% glacial acetic acid and 0.1 % Coomassie brilliant blue R-250 in water. The gel was then soaked in destaining solution (10% methanol, 10% glacial acetic) overnight to remove background staining. The protein molecular weight was estimated comparison with protein size marker.

## 4.9. Western blot analysis

Protein samples separated on 10% SDS-PAGE were soaked in cold 1x transfer buffer (Tris-Base 15.6 mM, glycin 120 mM) for 5 min. The nitrocellulose membrane (one piece) and filter paper (four pieces) were cut to the size of the SDS-PAGE gel and soaked in cold transfer buffer for 5 min. Protein samples were transferred to nitrocellulose membranes electrophoretically by submerged electrophoretic transfer (wet blot) for 16 hours at 4ºC and 30 V. After electro-blotting the membrane soaked in panceaue stain for 2 minutes to mark the marker protein and completely washed (destained) by distilled water. Then the membrane was soaked in blocking solution 5% (w/v) skimmed milk in fresh TBS buffer 7.5 pH (10 mM Tris-HCl, 50 m M NaCl) for 1 hr at room temperature. The membrane was washed for 5 min three times with TBS buffer containing 0.1 % tween 20. The membrane was incubated with primary antibody solution (anti-VacA antibody Austral biological, 1:2000 dilution in blocking solution) and mixed gently for 1 hour. The membrane was washed 10 min three times with (TBS buffer with 0.1% tween 20) followed by incubation with secondary antibody (anti-rabbit IgG alkaline phosphate conjugated) at 1:7000 dilution in blocking buffer for 1 hr. The membrane was washed two times with

(TBS buffer +0.1% Tween 20) each 5 minutes, then washed three times with 1x PBS buffer (120 mM NaCl, 16 mM $Na_2HPO_4 \cdot 2H_2O$, 4 mM $KH_2PO_4$, pH7.4). The signal was developed by incubating the membrane in developer solution (35 µl of 50 mg/ml BCIP in 100% (v/v) dimethyl formamide and 66µl of 50 mg/ml NBT in 70% (v/v) dimethyl formamide in 10 ml of buffer alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). After the color reaction was developed the membrane was washed and stored in a dark and dry place.

The same method was performed with primary antibody (anti-His antibody) at 1:10,000 and secondary antibody alkaline phosphatase conjugated anti-mouse IgG at 1:10,000 (Invitrogen).

## 4.10. Purification of the recombinant protein

The fusion protein was purified by using a His-trap® chelating column (GE Healthcare). The method is based on the affinity of the 6xHis affinity tag to Ni-NTI immobilized on a chromatography support matrix in the column. The 5ml His-trap® chelating column was washed with 25 ml of MilliQ water. The column was pre-equilibrated with 5-10 column volumes of buffer A containing 10mM imidazole. The supernatant was loaded onto the column at a flow rate of 1ml/min. The protein was eluted from the column with buffer A containing 100 mM imidazole. Fractions of 5 ml were collected and analyzed by SDS-PAGE and Western blotting.

## 4.11. Protein concentration determination

Protein concentrations were determined by using a Bio-Rad protein assay dye reagent based on the method described by Bradford (87). The standard protein calibration curve was constructed using bovine serum albumin (BSA) as a protein standard. The BSA standard was prepared dilutions in MilliQ water to yeild 0.02, 0,04, 0.08, 0.12, 0.16, and 0.20 mg/ml. A sample solution of 10 µl was mixed with 200 µl of dye reagent (Bradford reagent) on a 96-well flat bottom microtiter plate. The solution was gently mixed and incubated for 10 minutes at room temperature. Optical density of samples and standards were monitored at 595 nm using a Hitachi U-200

spectrophotometer. Protein concentrations of the samples were calculated from the standard curve.

## 4.12. Cell lines

Human adenocarcinoma (T84) and Madin-Darby canine kidney (MDCK) epithelial cells were originally purchased from the American Type Culture Collection (Manassa, Virginia, USA) and obtained from Professor Alan S. Verkman.

Cell culture T84 and MDCK cells were grown as monolayers in a 1:1 mixture of Dulbecco's modified Eagle's Medium/Nutrient Mixture F-12 Ham (DMEM-Ham) (Sigma St. Louis MO, USA) supplemented with, 50 U/ml penicillin, 50 μg/ml streptomycin and 5% fetal bovine serum. The culture medium was replaced every other day and after reaching confluent, monolayers were subcultured by trypsinization with 0.25% trypsin and 5.3 mmol/l EDTA in $Ca^{2+}$ and $Mg^{2+}$ free phosphate-buffered saline (PBS) and plated on coverslips at a density of $1x10^5$ cells/ml to study apoptosis caused by VacA . T84 and MDCK cells were seeded in 75 $cm^2$ flasks at 37ºC in a humidified atmosphere of 5% $CO_2$ and the following experimental protocol was followed.

## 4.13. DAPI staining

Apoptosis of the colonic epithelium was assessed using a nuclear stain 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, St.Louis, MO, USA). Briefly, cells were placed on coverslips at a density of 5 x $10^5$ cells/well in DMEM medium and kept at the incubator. At the time of experiment, old medium was removed and cells were incubated with either 50 μg/ml VacA containing medium or serum free medium (as a control) for 24 h. Next day, media was removed and cells were washed in PBS (phosphate buffered saline) then cells were fixed with 60 μL/well of 4% PFA (Para Formaldehyde) for 8 minutes at 4Cº followed by washing with 60 μL/well of 1x PBS, 3 times, 5 minutes per wash. Cells were permeabilized with 60μL of 0.1% Triton-X-100 for 10 minutes. After 3 times, 5 min, wash with PBS, nonspecific binding sites were blocked by 2% skim milk for 1h. Cells were washed in

PBS 3 times, 10 minutes each. Finally, cells were stained with 50 µL of DAPI (1:2000 dilutions in blocking buffer) for 15 minutes and mounted using 50% glycerol. The signals were visualized by a fluorescence microscope (model IX71, Olympus, Japan). The blue-fluorescent DAPI nucleic acid stain preferentially stains dsDNA. Binding of DAPI to dsDNA produces a ~20-fold fluorescence enhancement; DAPI exhibits wavelengths of 350 nm for fluorescence excitation and 460 nm for emission. In our study, cells nuclei were visualized with DAPI using standard fluorescence microscopy. The microscope was adjusted to be suitable for visualizing DAPI blue color, the settings included: Filters for excitation and emission of DAPI fluorescence (350 and 460 nm respectively), magnification lenses of 40 and 100x for capturing the pictures. Image J program used for adjusting pictures. Many thanks dedicated to Dr. Chartchai at faculty of science, physiology department payathai campus, and his student Mohammed Yousef for providing chemicals, equipments and very helpful comments.

# CHAPTER V
# RESULTS

## 5.1. Construction of pTrcHis2A/VacA expression plasmid

### 5.1.1 Amplification of the vacA gene

The recombinant plasmid pTrcHis2A/VacA was constructed by using *H. pylori* ATCC 49503 (strain 60190) (GenBank accession No.U05676) with the VacA genotype s1/m1 as a template for primer design. PCR primers used to amplify the VacA fragment encoding the mature vacA gene from *Helicobacter pylori* isolated genomic DNA from clinical Thai patient isolate (62 years old male at Vichaiyuth Hospital) as a template. Under the conditions used, the expected PCR products were produced, a 2.5 kb fragment of VacA (Figure 5.1).

### 5.1.2 Construction of synthetic vacA gene

Synthetic gene of vacA sequence of 60190 *Helicobacter pylori* strain bacteria in pLS vector synthesized from TOP Gene technologies, Incorporation for saving time and cost, and it was used as a template for primer design include nucleotides with restriction sites for *Nco*I and *Pst*I enzymes and methionine introduced as a starting codon in the expressing system. To clone the synthetic vacA in *E. coli* a vacA fragment encodes the mature VacA toxin (amino acids 1 to 821 amino acid residues not including the first amino acid added to construct pTrcHis2A/VacA). The synthetic fragment was cloned into the *Nco*I-*Hpa*I sites of the pLS vector (company vector). The correct synthetic gene sequence analysis was confirmed and indicated that its nucleotide sequence was identical to that of mature vacA from *H. pylori* 60190. This lead to suggest that VacA was not modified may posttranslationally in *E. coli*. For sequence alignment see appendix.

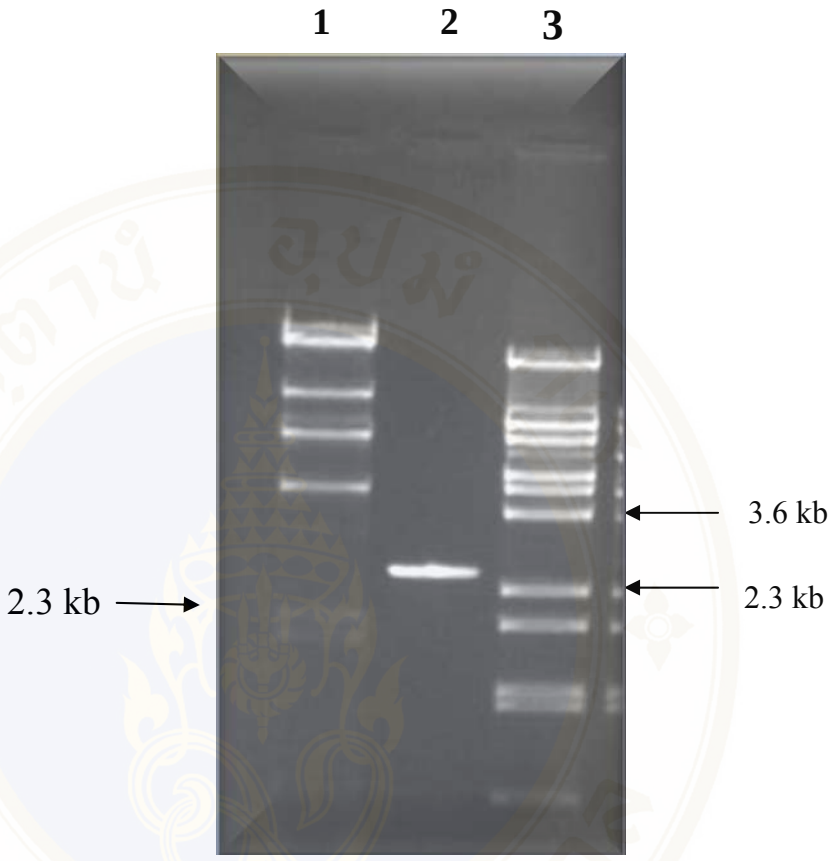



**Figure 5.1 Amplification of the VacA gene from a sample of chromosomal DNA obtained from Thai clinical isolate**

The shows 1% agarose gel electrophoresis (ethedium bromide stained) Lane 1 Lambda/*Hind*III DNA molecular marker, Lane 2 PCR product of VacA (single band) after purification with PCR Kit (expected size = 2.5 kb). Lane 3 Lambda/ *BstE*II Marker

### 5.1.3 Cloning of a VacA fragment encoding mature VacA

The vacA PCR products harboring *Nco*I and *Pst*I restriction sites at 5' and 3' ends introduced by forward and reverse primers respectively were cut with *Nco*I and *Pst*I restriction endonucleases. The plasmid pTrcHis2A (4.4 kb) was digested with *Nco*I and *Pst*I restriction enzymes and mixed with insert. Ligation reaction was performed to obtain the recombinant plasmid named pTrcHis2A/VacA (6.96 kb) as illustrated in (Figure 5.2-5.3). Ligation reactions contained 20 ng of vector, 120 ng of DNA insert (1:10 molar ratio) and were transformed into competent *E. coli* cells. Resulting transformants were screened for the presence of the recombinant plasmid by using nucleoSpin®Plasmid for kit plasmid extraction based on manufacturer's instructions. The correct recombinant plasmid was verified by restriction digestion analysis and DNA sequencing. The full length vacA gene fragment was sequenced by using a series of forward primers. DNA sequencing chromatogram encompassing the start and stop codons including the restriction sites are shown in (Figures 5.4A and 5.4B). The mature Thai isolate DNA sequence analysis of the plasmid encoding recombinant VacA indicates that the cloned vacA gene encode 2577 base pairs also is expected to encode 859 amino acids and the considerable sequence variation in the middle region (m2) (Figure 5.5). Interestingly, m2 VacA has an insertion of 23 residues at amino acid 475, consisting of an imperfect repeat of the upstream sequence (Figure 5.6). In addition it is unclear whether this region is directly involved in receptor binding because RPTPα, RPTPβ are both recognized by m2 VacA (80). The structural consequences of these extra 23 amino acid residues not clear yet and further investigations may provide new insight into structural properties of VacA that are required for other actions of this multifunctional toxin. Sequencing of the gene from strain 95-54 (Gene Bank accession no. U95971) revealed the presence of a 3,969-bp encoding a product of 1323 amino acids, analysis of the predicted amino acid sequence show that this protein consists of a combination of the s1 and m2 (60). Comparison of s1, m1 (60190), s1, m2 (95-54) and Thai clinical isolate VacA amino acid sequence shows >89% homology with VacA sequence from toxigenic strain 95-54 and less than 83% homology with the characterized VacA sequence from toxigenic model strain 60190 (Figure 5.7). Furthermore Thai isolate mature VacA was identical to Chinese strain CHN1811aVacA. See sequence detail for the strains at appendix.

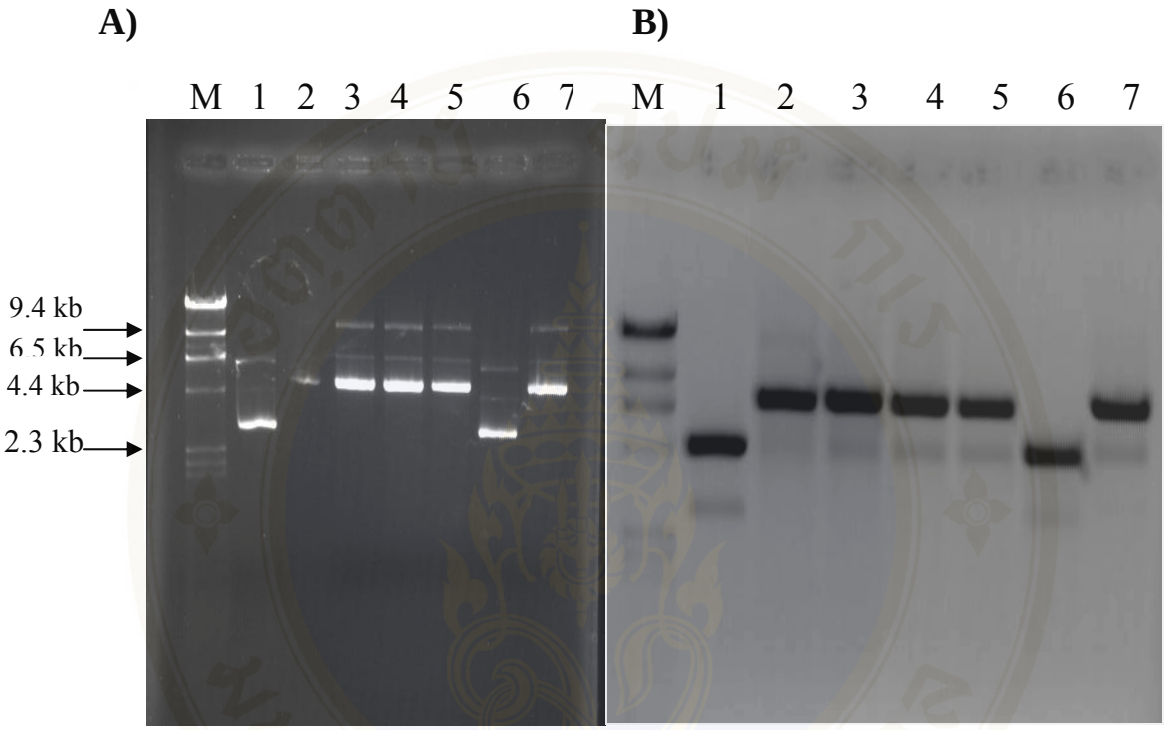



**Figure 5.2 Restriction endonuclease analysis of pTrcHis2A.The figure shows 1% agarose gel electrophoresis (ethidium bromide stained)**

A). Lane M: Lambda/HindIII digested DNA Marker

Lanes: 1, 2, 3, 4, 5, 6, 7 Extracted plasmid from transformed cells for 7 different colonies undigested.

B) Lane M: Lambda/HindIII digested DNA Marker

Lanes: 1, 2, 3, 4, 5, 6, 7 Extracted plasmid from same figure a transformed cells for 7 different colonies linearized by a single cut with *Pst*I enzyme. Transformants clones show a linear fragments size of 7kb corresponding to positive recombinant plasmid.

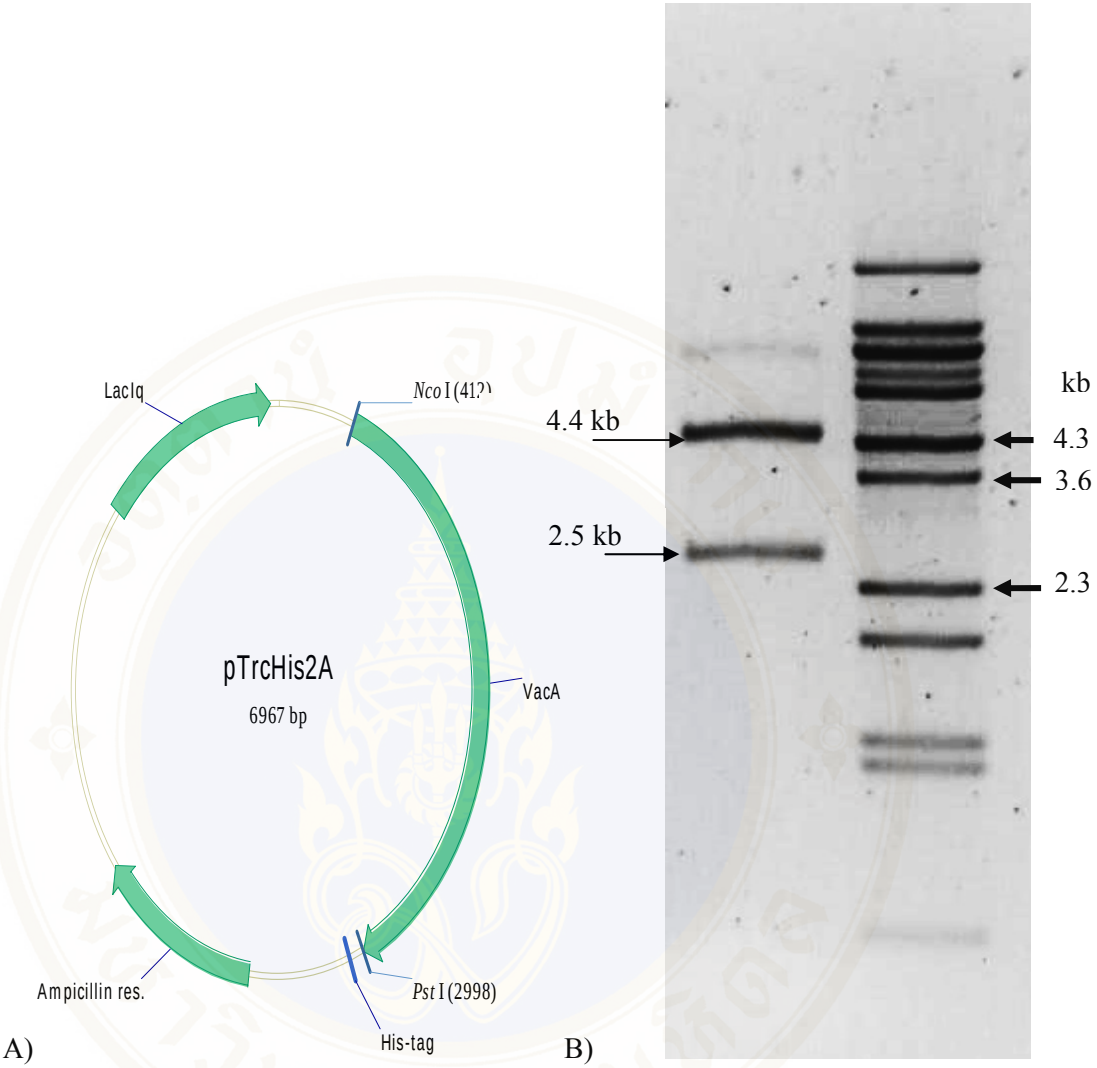



**Figure 5.3 Restriction endonuclease analysis of pTrcHis2A/VacA**

A restriction map of the pTrcHis2A/VacA shows the positions of NcoI and PstI restriction endonuclease recognition sites.

B) The figure shows 1% agarose gel electrophoresis (ethedium bromide stained ) of *Nco*I and *Pst*I double digested pattern of one sample recombinant plasmid. The upper fragment corresponds to the plasmid size and the lower band with 2586 bp band corresponds to the insert size.

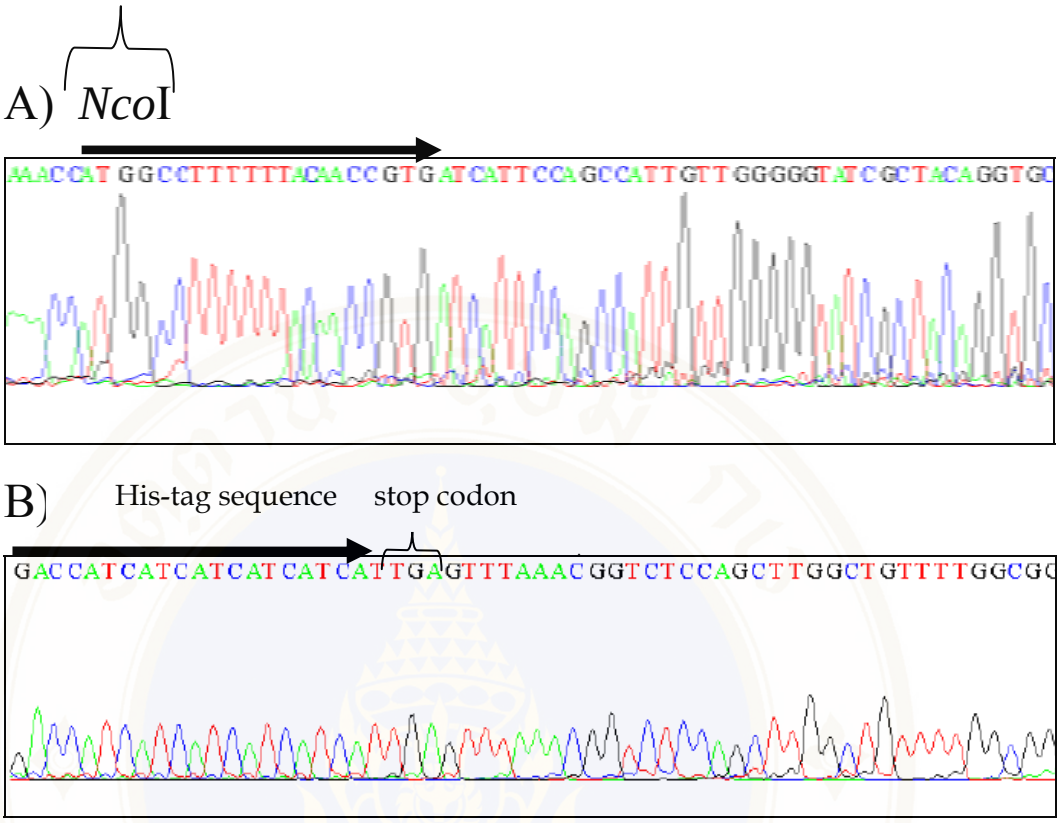



**Figure 5.4 DNA sequencing analysis of vacA gene**

A) DNA sequencing chromatogram of vacA, part of the sense sequence strand shows the *Nco*I restriction site and the ATG starting codon.The arrow shows the direction of the gene sequence.

B) DNA sequencing chromatogram of vacA, part of the sense sequence strand shows the His-tag sequence followed by stop codon. The arrow shows the direction of the gene sequence.

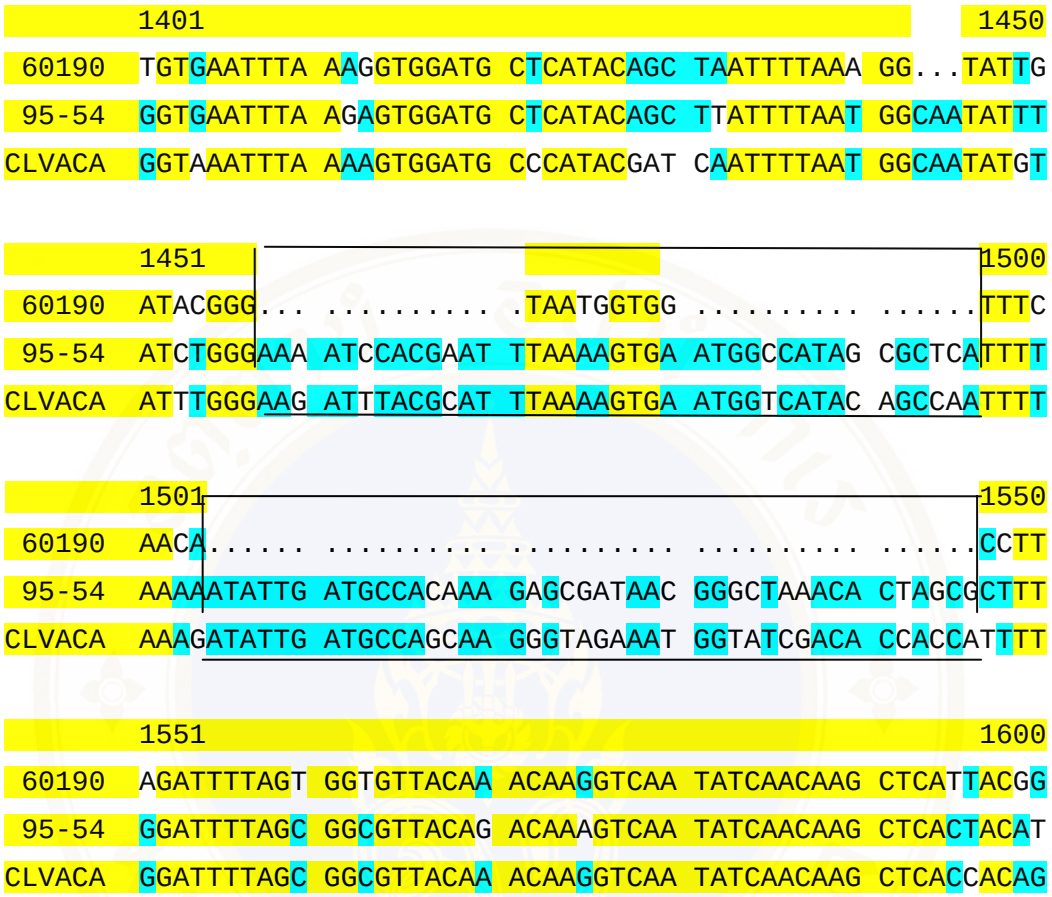



**Figure 5.5 Nucleotide sequence of recombinant VacA from Thai isolate aligned with that of model strain 60190 (S1/M1) and strain 95-54 (s1/m2).**

Identical nucleotide sequence shaded with yellow color, identical nucleotide sequence between two strains shaded with blue color. The white color region has nucleotide sequence consistently different between strains. The black box shows the nucleotide insertion in middle region of vacA gene.

```
          451                                                   500
60190aa   TKNGTATFNN DISLGRFVNL KVDAHTANFK G......... .........
95-54aa   TNNGTATFNN DIHLGKAVNL RVDAHTAYFN GNIYLGKSTN LKVNGHSAHF
  rVacA   TNNGTATFNN DIRLGKAVNL KVDAHTINFN GNMYLGRFTH LKVNGHTANF

          501                                                   550
60190aa   ......IDTG NGGFNTLDFS GVTNKVNINK LITASTNVAV KNFNINELIV
95-54aa   KNIDATKSDN GLNTSALDFS GVTDKVNINK LTTSATNVNI KNFDIKELVV
  rVacA   KDIDASKGRN GIDTTILDFS GVTNKVNINK LTTAATNAAI KNFDIKELVV

          551                                                   600
60190aa   KTNGVSVGEY THFSEDIGSQ SRINTVRLET GTRSIFSGGV KFKSGEKLVI
95-54aa   TTRVQSFGQY TIFGENIGDK SRIGVVSLQA GYSPAYSGGV TFKSGKKLVI
  rVacA   TTNVLSVGKY TDFTEDIGDQ SRIGIVSLQT GYGPAYSGGV TFKGGKKLVI

          601                                                   650
60190aa   DEFYYSPWNY FDARNIKNVE ITRKFASSTP ENPWGTSKLM FNNLTLGQNA
95-54aa   DEIYHAPWNY FDARNVTDVE INKRILFGSP GNIAGKTGLM FNNLTLNSNA
  rVacA   DEIYHAPWNY FDARNVTDVE INKRILFGAP GNIAGKTGLM FNNLTLNSNA

          651                                                   700
60190aa   VMDYSQFSNL TIQGDFINNQ GTINYLVRGG KVATLNVGNA AAMMFNNDID
95-54aa   SMDYGKDLDL TIQGHFTNNQ GTMNLFVQDG RVATLNAGHQ ASMIFNNLVD
  rVacA   SMDYGKDLDL TIQGHFTNNQ GTMNLFVQDG RVATLNAGHQ ASMIFNNLVD

          701                                                   750
60190aa   SATGFYKPLI KINSAQDLIK NTEHVLLKAK IIGYGNVSTG .....TNGIS
95-54aa   SATGFYKPLI KINNAQNLTK NKEHVLVRAR NIDYNLVGVQ GASYDNISAS
  rVacA   SATGFYKPLI KVNNAQNLTK NKEHVLVKAR NIDYNLVGVQ GASYDNISAS
```

**Figure 5.6 Alignment of amino acid sequence of recombinant VacA from Thai isolate aligned with the sequence of 60190 strain (m1) and 95-96 (m2).**

Identical amino acids residues are shaded with yellow. Identical amino acids residues between two strains shaded with blue color. The white color region has amino acids residues consistently different between strains. The black box shows the amino acids residues insertion in middle region of vacA gene. The amino acids residues are fairly conserved when exclusively m1 or exclusively m2 sequence are analyzed. However, when m1 and rVacA (m2) sequence are analyzed together residues are highly variable.

```
                201                                                 250
   821aa   (201) AEISLYDGATLNLASNSVKLNGNVWMGRLQYVGAYLAPSYSTINTSKVTG
   S72494  (201) AEISLYDGATLNLASSSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG
AF191639   (201) AEISLYDGATLNLASNSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG
  95-54aa  (201) AEISLYDGATLNLASNSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG
   rVacA   (201) AEISLYDGATLNLASNSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG

                251                                                 300
   821aa   (251) EVNFNHLTVGDHNAAQAGIIASNKTHIGTLDLWQSAGLNIIAPPEGGYKD
   S72494  (251) EVNFNHLTVGDKNAAQAGIIANKKTNIGTLDLWQSAGLNIIAPPEGGYKD
AF191639   (251) EVNFNHLTVGDHNAAQAGIIASKKTYIGTLDLWQSAGLNIIAPPEGGYKD
  95-54aa  (251) EVDFNHLTVGDHNAAQAGIIASNKTHIGTLDLWQSAGLNIIAPPEGGYKD
   rVacA   (251) EVDFNHLTVGDHNAAQAGIIASKKTYIGTLDLWQSAGLNIIAPPEGGYKD

                301                                                 350
   821aa   (301) KPNNTPSQSGAKNDK--------QESSQNNSNTQVINPPNSTQKTEVQPT
   S72494  (301) KPNNTPSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSAQKTEVQPT
AF191639   (301) KPNNTNSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSGQKTEIQPT
  95-54aa  (301) KPKDKPSNTTQNNANNNQQNSAQN-----NSNTQVINPPNSAQKTEIQPT
   rVacA   (301) KPNNTNSQSGAKNDKNESAKNDKQ-----DSNTQVINPPNSGQKTEIQPT

                351                                                 400
   821aa   (343) QVIDGPFAGGKDTVVNIDRINTKADGTIKVGGFKASLTTNAAHLNIGKGG
   S72494  (351) QVIDGPFAGGKDTVVNINRINTNADGTIRVGGFKASLTTNAAHLHIGKGG
AF191639   (351) QVIDGPFAGAKDTVVNINRINTNADGTIKVGGYTASLTTNAANLNIGKGG
  95-54aa  (346) QVIDGPFAGGKDTVVNINRINTNADGTIRAGGYKASLTTNAAHLYIGKGG
   rVacA   (346) QVIDGPFAGAKDTVVNINRINTNADGTIKVGGYTASLTTNAADLNIGKGG

                401                                                 450
   821aa   (393) VNLSNQASGRTLLVENLTGNITVDGPLRVNNQVGGYALAGSSANFEFKAG
   S72494  (401) VNLSNQASGRSLIVENLTGNITVDGPLRVNNQVGGYALAGSSANFEFKAG
AF191639   (401) VNLSNQASGRSLLVENLTGNITVDGALMVNNQVGGYALAGSSANFEFKAG
  95-54aa  (396) VNLSNQASGRSLLVENLTGNIAVEGTLRVNNQVGGSAVAGSSANFEFKAG
   rVacA   (396) INLSNQASGRSLLVENLTGNITVDGALMVNKEAGGAALPGSSANFEFKAG

                451                                                 500
   821aa   (443) VDTKNGTATFNN---------------------DISLGRFVNLKVDAHTA
   S72494  (451) TDTKNGTATFNN---------------------DISLGRFVNLKVDAHTA
AF191639   (451) VDTKNGTIAFN----------------------NNISLGRFVNLKASAHTV
  95-54aa  (446) TDTNNGTATFNNDIHLGKAVNLRVDAHTAYFNGNIYLGKSTNLKVNGHSA
   rVacA   (446) VDTNNGTATFNNDIRLGKAVNLKVDAHTINFNGNMYLGRFTHLKVNGHTA

                501                                                 550
   821aa   (472) NFKGIDTGNG----GFNTLDFSGVTNKVNINKLITASTNVAVKNFNINEL
   S72494  (480) NFKGIDTGNG----GFNTLDFSGVTDKVNINKLITASTNVAVKNFNINEL
AF191639   (480) NFKDIDTGNG----GFNTLDFSGVTNKVNINKLITASTNVAVKNFNINEL
  95-54aa  (496) HFKNIDATKSDNGLNTSALDFSGVTDKVNINKLTTSATNVNIKNFDIKEL
   rVacA   (496) NFKDIDASKGRNGIDTTILDFSGVTNKVNINKLTTAATNAAIKNFDIKEL

                551                                                 600
```

**Figure 5.7 Alignment of amino acid sequence of recombinant VacA from Thai isolate with several strains.** Identical amino acids residues are shaded with yellow**.** Identical amino acids residues between two strains shaded with blue color. The white color region has amino acids residues consistently different between strains. We show here strictly conserved VacA domain among several surveyed strains m1and m2 sequences and diversification region which differentiate m1 and m2.

## 5.2. Optimization of expression condition for the recombinant VacA protein

The recombinant plasmid pTrcHis2A/VacA directed expression in *E. coli* TOP10 by induction with IPTG (method 7). Cells corresponding to 1 $OD_{600}$ were collected and the pellet was suspended in 4x gel sample buffer and then the resuspended buffer loaded on the gel. The identification of the expressed proteins was done by Western blot analysis (method 9). Proteins from *E. coli* cell lysate were probed with anti-His antibody which recognizes the C-terminal (His)6-tag and anti-VacA (polyclonal) that raised against the purified VacA antigen. The high number of expression systems shows that there is not one that works for all cases. Optimization of expression conditions was attempted to produce high levels of expression. Time and IPTG conc. dependant expression of VacA was analyzed by SDS-PAGE of crude lysate (Figure 5.8) and subsequent Western blotting (Figure 5.9-5.10). It is interesting to note that growth of recombinant stain having pTrHis2A/VacA ceased after addition of IPTG and over period of 14 hrs, no further increase in growth rate was observed. At lower temperature 25ºC reduced amounts of recombinant VacA were detected after incubation for 14 hrs. Various amounts of IPTG appeared to have no effect on amounts of bioproduct formation and consequently, IPTG was used at concentration of mM for an expression studies.

## 5.3. Purification of VacA protein by immobilized metal affinity chromatography (IMAC).

Expression of soluble VacA fused to a (His)6-tag at the carboxyl-terminus was induced by 0.1mM IPTG at 37ºC for 3 hours *in E. coli* TOP10. The recombinant VacA was purified by affinity chromatography on a nickel chelating resin. As shown in, single band of apparent molecular weight of 90 kDa was recognized in immunoblots with polyclonal anti-rVacA antibody. The 90 kDa protein was present in most likely represents full length form of recombinant VacA. The protein was eluted from the column in a buffer containing 100 mM imidazole as a single peak level of > 95% purified recombinant VacA about 0.2 mg/ml protein concentration (Figures 5.11, 5.12 and 5.13)

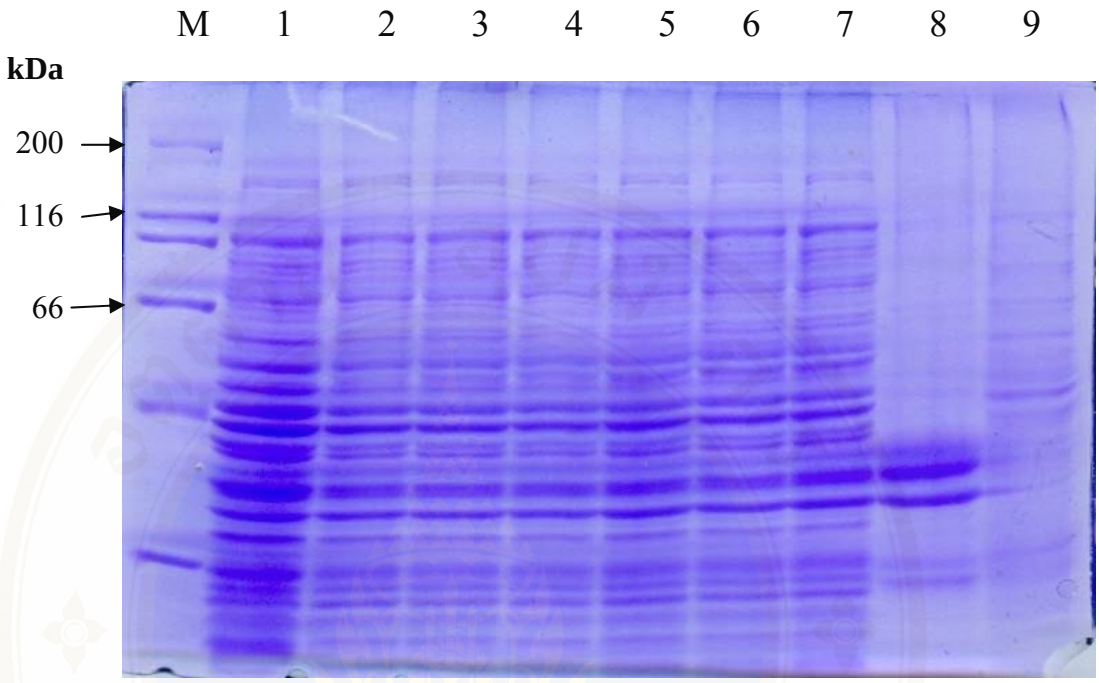



**Figure 5.8 SDS-PAGE analysis of temperature and time dependant of rVacA**

The figure shows the PAGE gel analysis of crude lysate of *E.coli* TOP10 expressing rVacA protein on SDS-PAGE gel (Coomassie staining) using 10% SDS-PAGE at 37°C and 25°C.

Lane M: Broad range Marker (Biorad)

Lane 1: pTrcHis2A plasmid used as a negative control

Lane 2: 0 mM IPTG at 37°C

Lane 3: 1hr at 37°C

Lane 4: 2hr at 37°C

Lane 5: 3hr at 37°C

Lane 6: 4 hr at 37°C

Lane 7: 5hr at 37°C

Lane 8: supernatant portion (soluble fraction) incubated at 25°C

Lane 9: pellet (insoluble fraction) incubated at 25°C


**Figure 5.9 Western blot analysis of rVacA expressed at different time**

The figure shows the Western blot profile using (anti-rVacA antibody) of crude lysates of recombinant *E. coli* culture expressing VacA protein. Cells were incubated at 37ºC and samples were collected at intervals of 0, 1, 2, 3, 4 and 5 hrs. Molecular mass standards are indicated on the left. Lane N: pTrcHis2A (-ve control), Lane 0: 0 hr (no IPTG added) Lanes 1, 2, 3, 4 and 5 (1, 2, 3, 4 and 5 hours after addition of IPTG respectively).

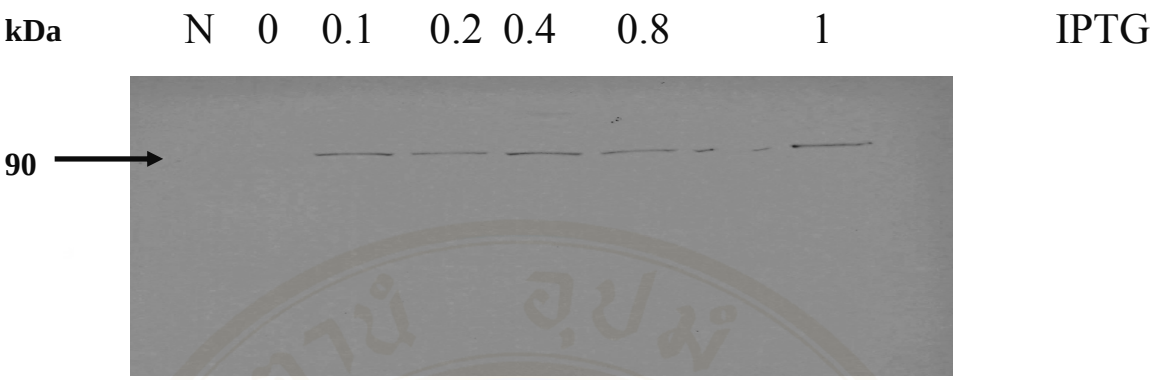



**Figure 5.10 Western blot analysis of rVacA expressed at different IPTG concentration**

The figure shows the Western blot profile using (anti-His antibody) of crude lysates of recombinant *E. coli* culture expressing VacA protein. Cells were incubated at 37ºC and collected at 3 hrs intervals. Lane N: pTrcHis2A plasmid.

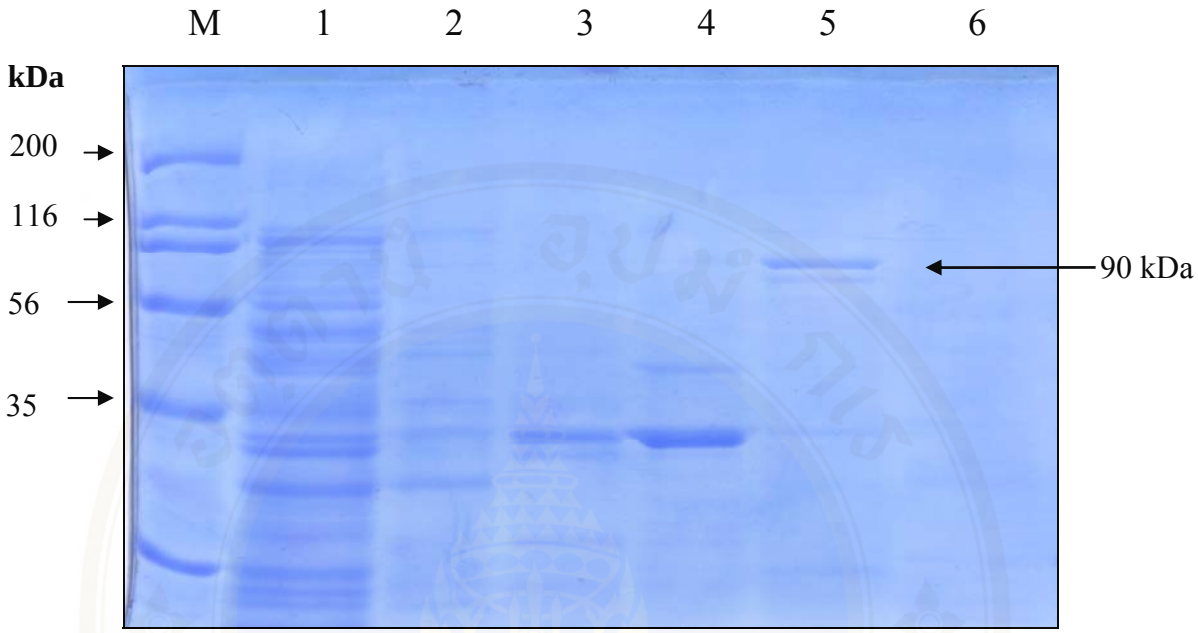



**Figure 5.11 Purification profile of VacA protein IMA chromatography**

Expression of soluble rVacA fused to (His)6-tag in *E.coli*: SDS-PAGE analysis of elution fractions from IMAC chromatography of rVacA has a molecular weight of ~90kDa the arrowhead indicates the position of 90 kDa of rVacA.

Lane M: Molecular weight marker

Lane 1: Unbound fraction (flow through)

Lane 2: elution fraction of 10mM Imidazole

Lane3: elution fraction of gradient Imidazole

Lane 4: elution fraction of gradient Imidazole

Lane 5: elution fraction of 100mM Imidazole

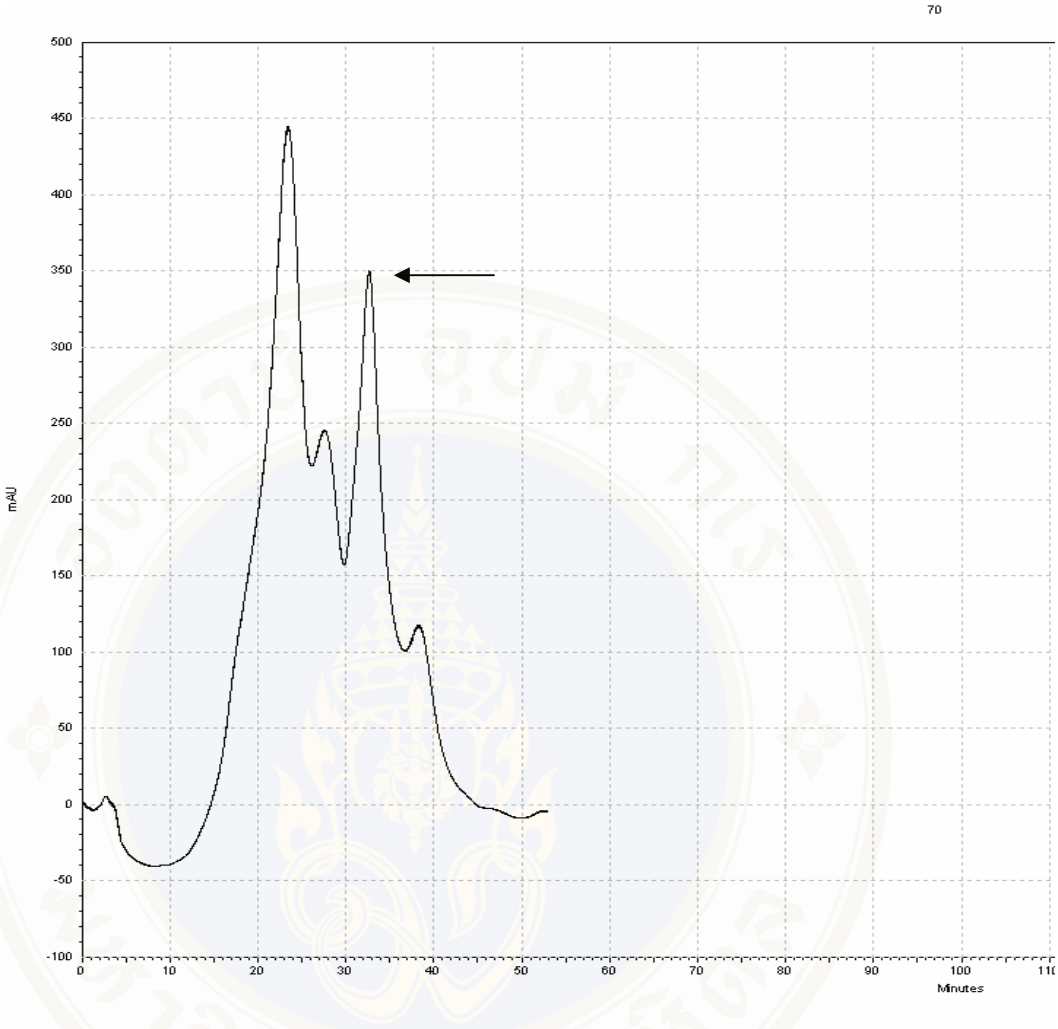



**Figure 5.12 Chromatogram of the elution fractions of rVacA protein**

The chromatographic elution profile from His-trap column shows absorbance at 280 nm (mAU) and elution volume (ml). SDS-PAGE gel of the selected peak fraction (arrow) containing the 90 kDa purified VacA protein

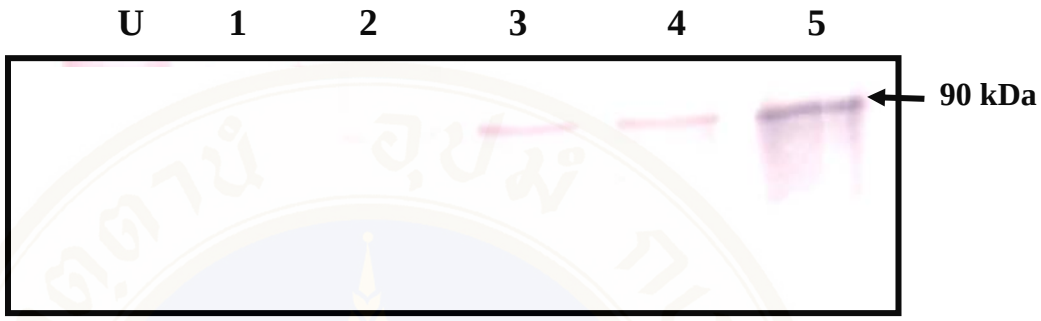



**Figure 5.13 Detection of rVacA with anti-rVacA antibody using immunoblot**

The rVacA s1/m2 appeared at 90 kDa of the purification fraction obtained after IMAC. The presence of rVacA in the supernatants was confirmed by Western blotting with anti-VacA antibodies

Lane U: Unbound fraction (flow through)

Lane 1: The elute fraction with 10mM Imidazole

lane 2: The elute fraction with gradient Imidazole

Lane3: The elute fraction with gradient Imidazole

Lane 4: The elute fraction with gradient Imidazole

Lane 5: The elute fraction with 100mM Imidazole

## 5.4. Induction of apoptosis by VacA in intestinal epithelial cells

We aimed to detect the activity of the Thai strain purified VacA protein on human colonic adenocarcinoma (T84) cells. T84 cells were plated on cover slips and divided into two groups; the treated group received 50 µg/ml purified VacA protein and an untreated group that kept as a control. Apoptosis was determined by DAPI staining, and apoptosis induction in T84 cells was observed after 24h of VacA incubation.

The summary of the results as shown in Table 3 and obtained from 6 pictures taken from both control and VacA treated T84 cells. From this table it is clear that, incubation of VacA to T84 cells for 24h resulted in an increase in apoptosis of T84 cells with 8.25 ± 1.6% in VacA treated cells (mean ± standard deviation) compared with 4.22 ± 1.36% in the control cells, and with a $p<0.05$ significance.

The nuclear staining, DAPI staining, revealed a slightly increase in the number of nuclei which showed chromatin condensation and DNA fragmentation, a typical signs for apoptosis, in VacA treated T84 cells compared with an increased number of normal nuclei in untreated cells (Figure 5.14 A). Figure 5.14 B, shows the percent of T84 cells which underwent apoptosis in control or VacA treated cells. From this figure we conclude that, Thai strain purified VacA has a weak activity upon intestinal epithelial cells which confirmed by the slight apoptosis induced by this protein upon T84 cells.

## 5.5. Induction of apoptosis by VacA in kidney epithelial cells

To further confirm the apoptosis-induced activity of VacA on epithelial cells, Madin-Darby Canine Kidney (MDCK) cells were used in our study. MDCK cells were found to be a *suitable in vitro* model for studying the activity of VacA. VacA was shown to induce initial loosing of the tight junctions of MDCK cells this notion suggests that purified VacA have receptors on MDCK cells through which this protein can induce its action. We studied the apoptosis induced by VacA in MDCK cells and Table 3 shows the summary of 6 pictures taken after incubation of MDCK cells with the purified protein for 24h.

VacA induced a marked apoptosis in MDCK with 25.4±3.06% increase in cells underwent apoptosis compared with 3.6±1.07% in normal control cells and with P<0.001 significance. (Figure 5.15A) shows normal and VacA treated MDCK cells stained with the nuclear staining DAPI and detected by fluorescence microscopy. VacA induced more chromatin condensation and DNA fragmentation in VacA treated cells. (Figure 5.15B) shows the percentage of MDCK cells that have apoptosis in normal and after VacA treatment.

Taken together, these results suggest that our purified VacA protein has lower toxic activity on intestinal cells (Figure 5.16) which eventually is due to the increase in nucleotides at the m region of the mature VacA protein (64, 65, and 80).

**Table 5.1 Data obtained after staining with DAPI of both T84 and MDCK Cells.**

Results were taken for six pictures of all VacA treated cells and compared with the normal control. Data were calculated as mean ± standard deviation

| N | T84 Cells | | MDCK cells | |
|---|---|---|---|---|
| | Control | VacA | Control | VacA |
| **1** | 3.0 | 6.6 | 3.2 | 25.6 |
| **2** | 4.5 | 8.4 | 2.8 | 20.8 |
| **3** | 2.8 | 11.1 | 3.6 | 28.0 |
| **4** | 3.6 | 9.0 | 2.4 | 26.0 |
| **5** | 5.0 | 7.4 | 4.6 | 29.0 |
| **6** | 6.4 | 7.0 | 5.2 | 23.0 |
| **Mean** | 4.21 | 8.25 | 3.6 | 25.4 |
| **Std. Deviation** | 1.36 | 1.65 | 1.07 | 3.06 |
| **Std. Error** | 0.56 | 0.67 | 0.44 | 1.2 |
| **P value (two tailed)** | P<0.05 | | P<0.001 | |

A) B)

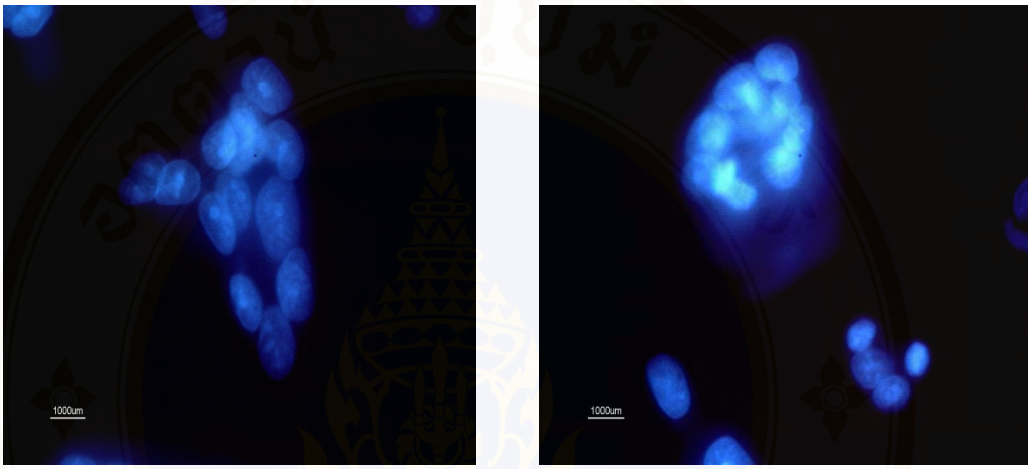



**Figure 5.14 DAPI staining of Intestinal Epithelial Cells (T84 Cells)**.

(A) normal T84 cells showing normal nuclei.

(B) VacA (50 µg/ml) treated T84 cells showing chromatin condensation and DNA fragmentation. DAPI was used at a dilution of (1:1000) and the magnification of pictures is 100X.

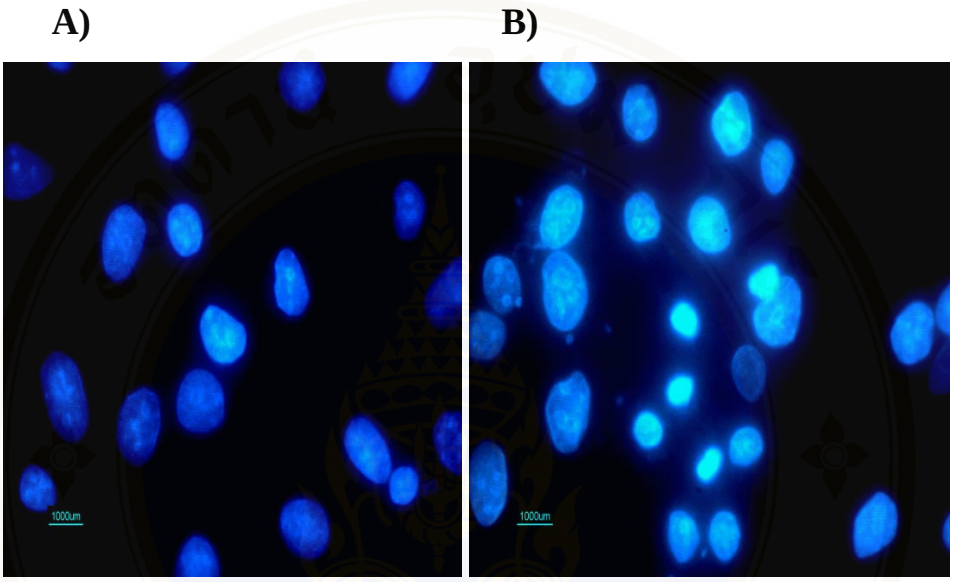



**Figure 5.15 DAPI staining of Kidney Epithelial Cells (MDCK Cells)**.

(A) Normal MDCK cells showing normal nuclei.

(B) VacA (50 µg/ml) treated MDCK cells showing signs of apoptosis such as chromatin condensation and DNA fragmentation. DAPI was used at a dilution of (1:1000) and the magnification of pictures is 100X

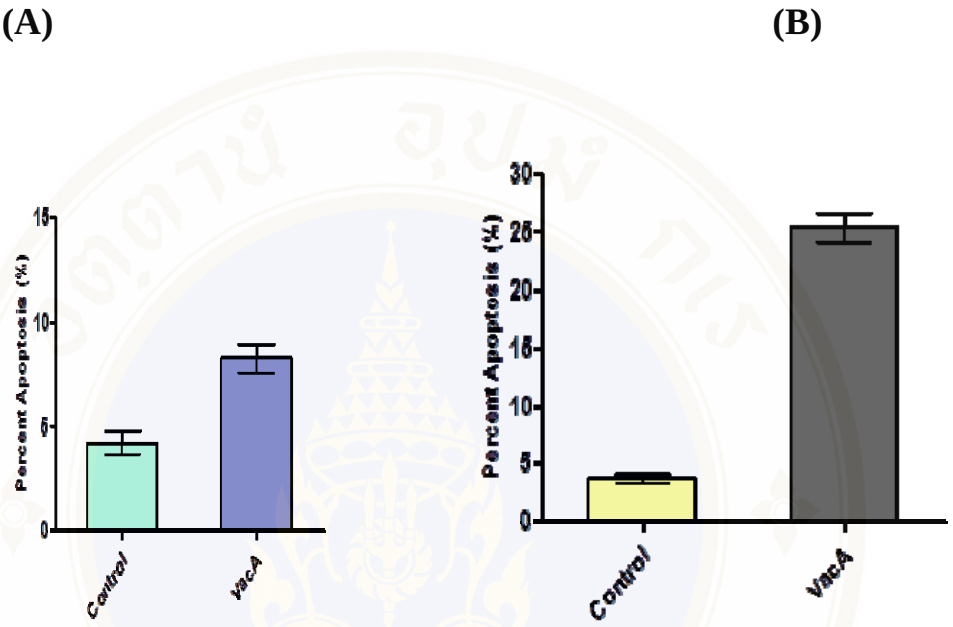



**Figure 5.16 Summary of percent of apoptosis after DAPI staining in T84 (A) and MDCK (B) cells**. A mean of six pictures from each cell line was calculated and the percentage of apoptotic cells in VacA treated cells were blotted against normal untreated cells.

# CHAPTER VI
# DISCUSSION

## 6.1. Construction of pTrcHis2A/VacA plasmid

The aim of the present study was to construct an expression system to analyze the biological activity of the *Helicobacter pylori* VacA toxin. We have successfully started construction of a prokaryotic expression system for vacA gene from a clinical Thai isolate and expressed and purified the active toxin. This is the first reported and analysis of VacA toxin from a patient sample obtained in Thailand. We were seeking to understand how genetic differences and sequence polymorphisms between m1 and m2 of *vacA* alleles are related to the Thai isolate vacA. *H. pylori* VacA is the product of a single gene that encodes a 140 kDa precursor protein; this precursor protein is proteolytically processed to yield the mature 88 kDa toxin (44). Upon removal of the amino-terminal signal sequence, the mature VacA toxin starts with an alanine as the amino-terminal amino acid. To express the mature VacA in *E coli*, a methionine must be present at the amino terminus in order to support initiation of translation. Previous studies had shown that several mutations near the VacA amino terminus result in an ablation of functional activity of the VacA toxin (55,102). Therefore conjugation with N-terminal (His)6-tag at this site could potentially abolish toxin activity. Based on these considerations, addition of methionine and or addition of extra amino acids to the N-terminus were avoided by introducing a *Nco*I site in the oligonucleotide primer SJ_VacA1F. The PCR products (2.5 kb) from Thai clinical isolate of *H. pylori* corresponds to the mature portion of the VacA toxin and were ligated to the pTrcHis2A vector (4.4 kb) upstream of the C-terminal (His) 6-tag. The expression plasmid pTrcHis2A/VacA (6.9 kb) was generated for expression and purification of the target protein. Construct we obtained was analyzed by nucleotide sequencing and alignment with nucleotide sequences of *H. pylori* 60190 vacA sequence (GenBank accession No.:U05676) and strain 95-54 (s1/m2) (GenBank accession No.U95971) and other strains. The results showed strictly conserved VacA

domain p33 among several surveyed strains allelic types m1and m2 and a diversification region which differentiate m1 and m2.

The amino acids residues are >85% conserved when exclusively m1 or m2 sequences are analyzed. However, when m1 and rVacA (m2) sequence are analyzed together residues are highly variable. The amino acid residue of mid region m1 for *H. pylori* vacA 60190 corresponding to D455 represents the start of the midregion that defines m1 and m2 strains. It is suggested that this area may represent a receptor-binding site which is shared by m1 and m2 forms of the toxin (52). The biggest difference between the model strain 60190 and the Thai strain is the sizable insertion of 23 amino acid residues in the middle region. The position of these residues in the context of allelic variants m2 strains is impossible to predict (52). This sequence difference between the model strain and the Thai strain gene could eventually result in differences in cytotoxin phenotypes.

## 6.2. Expression of recombinant VacA toxin

Optimization of expression conditions was attempted to produce high levels of expression, it is interestingly to note that growth of recombinant strain having pTrHis2A/VacA ceased after addition of 0.1 mM IPTG and over a period of 14 hrs, no further increase in bacterial growth rate was observed. It is worth mentioning that *Helicobacter pylori* produces a number of proteins associated with the outer membrane, including adhesins and the vacuolating cytotoxin and the functional expression of such proteins is reportedly deleterious to *Escherichia coli*, the host bacterium used for gene cloning. Therefore, a general method was previously developed for the functional expression of such genes by using a shuttle vector in *H. pylori* and *E. coli* (103). Western blot analysis of rVacA expressed in *E. coli* with an anti-His antibody and an anti-rVacA rabbit polyclonal antibody identified a single band protein which migrates at an apparent molecular weight of approximately 90 kDa. The 90 kDa band is considered to represent the mature form of VacA. This observation corresponds to the predicted molecular weight ≈ 95 kDa in the presence of (His) 6-tag and the extra amino acids at the C-terminus. Western blotting showed that rVacA was expressed both as an inclusion body and as soluble form the amount of

protein present in the soluble fraction appeared to be higher. Therefore protein purification was attempted from the soluble fraction thus avoiding refolding of insoluble proteins from inclusion bodies. The results obtained also subject to further degradation or premature termination of translation suggests that the expressed protein is stable or not.

## 6.3. Purification of recombinant VacA

Expression of a soluble recombinant VacA from *H. pylori* patient isolate from Thailand fused to a (His)6 tag at the carboxy terminal was achieved in *E. coli* TOP10. The recombinant VacA was purified by affinity chromatography by using nickel chelating chromatography using imidazol at a concentration of 10 mM, most host proteins were eluted from the affinity matrix. The (His)6 tag recombinant VacA was eluted from the column with 100 mM imidazole as a single peak. Elution fractions analyzed on SDS-PAGE revealed a major protein band at 90 kDa and another minor protein band at lower molecular weight ≈88 kDa. Subsequent Western blotting with anti-rVacA rabbit polyclonal antibody showed a single protein band at ≈ 90 kDa. The purity of (His)6 tag recombinant VacA in the elution fraction was estimated to >95% and approximately 0.2 mg/ml were obtained after affinity chromatography purification of soluble fraction.

## 6.4. Induction of apoptosis by VacA in intestinal epithelial cells

The loss of epithelial homeostasis and integrity are likely to be the first steps in the cascade of events that leads to *H. pylori*-associated gastrointestinal pathogenesis. The enterocytes perform the barrier function by maintaining their polarity and tightly associating with each other through lateral adherents and tight junctions (90). These cells also maintain a stable connection to basement membranes through adhesive interactions between the cells and extracellular matrix proteins (91).

It has been previously shown that sonicated bacterial extracts from *H. pylori* can alter the paracellular barrier function (92, 93) that the bacterial vacuolating toxin can selectively increase the permeability of this cell line. These data indicate that

*H. pylori* epithelial pathogenesis may be mediated not only by direct interaction with the organism, but also by released bacterial elements such as lipopolysaccharides (LPS) or secreted proteins. Therefore alterations of the epithelial physiology could contribute to the onset of *H. pylori*-associated disease.

It has been shown previously that *H. pylori* could adhere to epithelial cell membranes by different forms of adhesion (94), but the consequences of such an interaction on the turnover and the onset of apoptosis of epithelial cells have been poorly investigated. Previous studies have shown a correlation between the development of duodenal ulcer in *H. pylori* infection and the level of apoptosis in the antral mucosal epithelium (95), but the molecular events mediating enhanced epithelial cell apoptosis associated with duodenal ulcer diseases remain under investigation. To date, there is no gastric epithelial cell model able to grow as a polarized monolayer. In contrast, differentiated T84 monolayers display high transepithelial resistance (96), a well-organized brush border, and the capacity to release IL-8 at the basal cell surface under adhesion with *H. pylori* (97). Moreover, it is well known that freshly isolated human gastric epithelial cells do not proliferate in primary cultures and do not form uniform confluent monolayers.

The T84 cell line thus appears as an interesting model to study the interaction of *H. pylori* with an epithelial monolayer. Previous studies by (104) showed that, stimulation of T84 monolayers with *H. pylori* soluble extracts has dramatic effects on the epithelial physiological balance and integrity. Apical, but not basolateral, exposure of confluent monolayers of T84 cells to *H. pylori* extracts induced a rapid decrease in TER as well as the formation of domes. Domes are fluid-filled blister-like areas which form due to separation of the monolayer from the substrate, while the cells remain attached to each other (104). More specifically, (93) have shown that the vacuolating toxin from *H. pylori* can increase the epithelial permeability of T84 and MDCK monolayers, independently of its vacuolating activity (107).

Apoptosis plays a major role in the pathogenic action of *H. pylori* (105). Previous studies have already established that a correlation exists between the development of duodenal ulcer in *H. pylori* infection and the level of apoptosis in antral mucosal epithelium (94). One hypothesis could be that during *H. pylori*

infection in the gastric antrum, the physiological mechanism of both gastrin and gastric secretion is impaired, leading to the subsequent development of a duodenal ulcer (95). Study provided evidence that in vitro *H. pylori* infection of T84 intestinal epithelial cells induced apoptosis (106).

We investigated the apoptotic activity of a Thai isolate VacA protein on two types of epithelial cell lines, T84 and MDCK cells, using the nuclear staining DAPI. Our results showed that, Thai isolate VacA is at least one of the *H. pylori* virulence factors capable of inducing apoptosis in both intestinal and kidney epithelial cells. As was shown by DAPI staining that the supernatant of the cytotoxic Thai isolate *H. pylori* strain induces apoptosis in cells and its effects upon kidney epithelial cells was greater than that upon intestinal epithelial. Taken together, the work presented in this thesis represents an entry point to the further molecular biological characterization of the VacA toxin isolated from a Thai clinical sample.

Future studies focus on the genetic mechanism and diversity contributing to differential pathogenesis of VacA and structural determination of receptor interaction, pore formation cell vacuolation.

# CHAPTER VII
# CONCLUSION

## 7.1. Construction of pTrcHis2A/VacA expression plasmid

The recombinant plasmid pTrcHis2A/VacA was constructed by using *Helicobacter Pylori* ATCC 49503 (strain 60190) (GenBank accession No.:U05676) with the VacA genotype s1/m1 as a template for primer design. The vacA fragment encoding the mature vacA gene from *Helicobacter pylori* isolated from a Thai clinical isolate was used as a template. VacA from Thai clinically isolate gene was successfully amplified and cloned by PCR. The vacA gene is present as a single band. Correct insert orientation was analyzed with restriction endonuclease mapping. The VacA gene of four identical clones was sequenced and no polymorphism introduced by PCR amplification detected. Alignment gives 89% homology to VacA genotype s1/m2 (95-54).The homology to model strain 60190 genotype s1/m1 was lower than the expected (83%). Interestingly it was identical to Chinese strain CHN1811a VacA.

## 7.2. Expression conditions for recombinant VacA

The recombinant plasmid pTrcHis2A/VacA directed expression of VacA in *E. coli* TOP10. Western blot analysis showed that the recombinant protein had an apparent molecular weight of approximately 90 kDa corresponding to the predicted molecular weight of VacA and no degradation product was observed. An optimization of expression conditions was attempted to produce high levels of protein the bacterial cultures is grow normally but the growth rate of recombinant culture after induction almost the same for 14 hours. It appears that VacA has deleterious effect on growth of *E. coli*. At 25ºC, decreased amount of product was observed.

## 7.3. Purification of recombinant VacA protein

The recombinant VacA toxin was expressed as a soluble protein in *E. coli.* Immobilized metal chelate affinity chromatography was used for purification utilizing the hexahistidine tag. A major band of > 95% pure protein was obtained and the yield was estimated to 0.2 mg/ml of bacterial culture.

## 7.4. Apoptotic activity assay of recombinant VacA protein

VacA is a potent toxin that considered being an important determinant of *Helicobacter pylori* virulence, and therefore, it is important to have an in-depth understanding of VacA biological activity. The multitude of allelic variation and other bacterial toxin makes extremely difficult to test the contribution of each individual factor. However sequence analysis indicated the presence of substantial differences between strains and that sequence divergence between VacA may explain the loss of biological activity in strains.

The purified recombinant VacA protein was added at 50 µg/ml to human colonic adenocarcinoma (T84) and Madin-Darby canine kidney (MDCK) cells and apoptosis was observed by fluorescence microscopy of DAPI stained cells showing chromatin condensation and DNA fragmentation. MDCK cells appear to be significantly more sensitive to VacA than T84.

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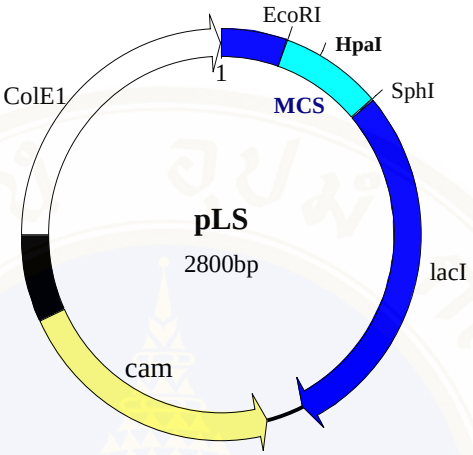
# Appendix

**Appendix 1: Physical map of pLS (company vector). The synthetic fragment was cloned into the *Nco*I-*Hpa*I sites of the pLS vector**




T7 primer

*EcoRI* *AgeI* *AatII*

CTGGCGGAAT TAGTAATACG ACTCACTATA GGGAATTCCT GTG AAA CCG GTA ACG TTA TAC GAC GTC GCA

GACCGCCTTA ATCATTATGC TGAGTGATAT CCCTTAAGGA CAC TTT GGC CAT TGC AAT ATG CTG CAG CGT

*PmlI* *HpaI* *Nhe* *PmlI* *AfeI*

GAG TAC GCC GGT GTC TCT TAT CAG ACC GTT TCA CGT GTG GTT AAC CAG GCT AGC CAC GTG AGC

CTC ATG CGG CCA CAG AGA ATA GTC TGG CAA AGT GCA CAC CAA TTG GTC CGA TCG GTG CAC TCG

*SmaI* *NcoI* *SacI*

GCT AAA ACC CGG GAA AAA GTG GAA GCG GCC ATG GCG GAG CTC AAT TAC ATT CCC AAC CGC GTG

CGA TTT TGG GCC CTT TTT CAC CTT CGC CGG TAC CGC CTC GAG TTA ATG TAA GGG TTG GCG CAC

*MfeI* *NaeI* *HindIII* *MscI* *XhoI* *SphI*

GCA CAA CAA TTG GCC GGC AAA CAA AGC TTG CTG ATT GGC GTG GCC ACC TCG AGT CTG GCC CTG

CGT GTT GTT AAC CGG CCG TTT GTT TCG AAC GAC TAA CCG CAC CGG TGG AGC TCA GAC CGG GAC

***R2 primer***

CAT GCA CCG TCG CAA ATT GTC GC

GTA CGT GGC AGC GTT TAA CAG CG

***R1 primer***

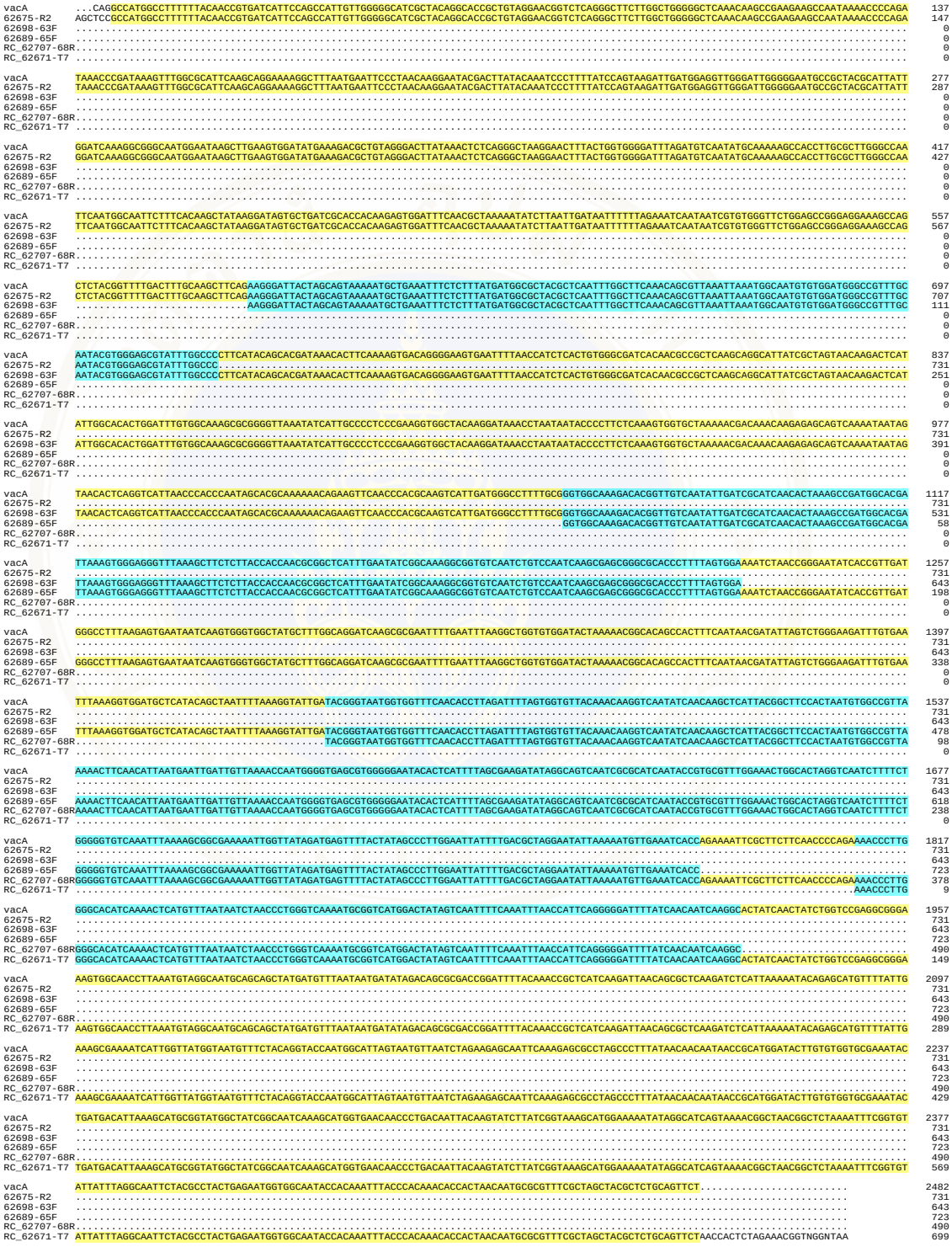
**Appendix 2: Nucleotide coding sequence of pLS (company vector) pLS sequence:**

CCAGCTTACTTCCCATCGAGCTGTTGACAATTAATCATCGGACTCGTATAATGTGTGGCACACAG
GAAAAAGGGCGTTACCCAATTCAATCGAATATCATGCACATCCCCCATTCTCTAGCTGGCGGAATTAGT
AATACGACTCACTATAGGGAATTCCTGTGAAACCGGTAACGTTATACGACGTCGCAGAGTACGCCGGTG
TCTCTTATCAGACCGTTTCACGTGTGGTTAACCAGGCTAGCCACGTGAGCGCTAAAACCCGGGAAAAAG
TGGAAGCGGCCATGGCGGAGCTCAATTACATTCCCAACCGCGTGGCACAACAATTGGCCGGCAAACAAA
GCTTGCTGATTGGCGTGGCCACCTCGAGTCTGGCCCTGCATGCACCGTCGCAAATTGTCGCGGCGATTA
AATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTTGTGTCGATGGTAGAACGAAGCGGCGTCGAAGCCT
GTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTATCCGCTGTATG
ACCAGGATGCGATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACC
AGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTACGCGACTGGGCGTGGAGCATCTGGTCG
CATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGG
CTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGGGAAGGCGACTGGAGTG
CCAAGTCCGGTTTTCAACAAACCATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTG
CCAACGATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGCGGATA
TCTCGGTAGTGGGTTACGACGATACCGAAGACAGCTCATGTTATATCCCGCCGCTAACCACCATCAAAC
AGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGA
AGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCGCCCTGGCTCCCAATACGCAAACCG
CCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGC
AGTGAGCTTCCTTTGCTTTGCATACCGGATTTTCTTTTTAGCTCGCCCCGCCCTGCCACTCATCGCAGT
ACTGTTGTAATTCATTAAGCATTCTGCCGACATGGAAGCCATCACAAACGGCATGATGAACCTGAATCG
CCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAATATTTGCCCATCGTGAAAACGGGGGCGAAGAAGT
TGTCCATATTCGCCACGTTTAAATCAAAACTGGTGAAACTCACCCAGGGATTGGCTGAGACGAAAAACA
TATTCTCAATAAACCCTTTAGGGAAATAGGCCAGGTTTTCACCGTAACACGCCACATCTTGCGAATATA
TGTGTAGAAACTGCCGGAAATCGTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAGTTTGCTCAT
GGAAAACGGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATACGAA
ATTCCGGATGAGCATTCATCAGGCGGGCAAGAATGTGAATAAAGGCCGGATAAAACTTGTGCTTATTTT
TCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCAACTG

continued

ACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTT
TTTTCTCCATTTTATTTTCCTCCTCTCATAGCTGTTTCCTGTGTGAAATTGTTATTCGCTCACAATTCC
ACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATT
AATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCACCGTAGAAAAGA
TCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGC
TACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCA
GAGCGCAGATACCAAATACTGTCCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAG
CACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTC
TTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGT
GCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAGAAA
GCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTGTCAGGTAAGCGGCAGGGTCGGAACAGGAGAGC
GCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGAC
TTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCT
TTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTC

# Appendix 3: Synthetic gene coding sequence confirmation by alignment with 5 primers



**Appendix 4: Alignment of amino acid sequence of recombinant VacA from Thai isolate with the sequence of 60190 strain (m1) and 95-96 strain (m2).**

Identical amino acids residues are shaded . The break points between m1 like and m2 like regions and extra amino acid residues that introduced in m2 region of recombinant VacA shown.

```
          1                                                  50
60190aa   AFFTTVIIPA IVGGIATGTA VGTVSGLLGW GLKQAEEANK TPDKPDKVWR
95-54aa   AFFTTVIIPA IVGGIATGAA VGTVSGLLSW GLKQAEEANK TPDKPDKVWR
  rVacA   AFFTTVIIPA IVGGIATGAA VGTVSGLLGW GLKQAEEANK TPDKPDKVWR
          51                                                 100
60190aa   IQAGKGFNEF PNKEYDLYKS LLSSKIDGGW DWGNAATHYW IKGGQWNKLE
95-54aa   IQAGRGFNNF PNKEYDLYRS LLSSKIDGGW DWGNAARHYW VKGGQWNKLE
  rVacA   IQAGRGFNEF PNKEYDLYKS LLSSKIDGGW DWGNAARHYW VKGGQWNKLE

          101                                                150
60190aa   VDMKDAVGTY KLSGLRNFTG GDLDVNMQKA TLRLGQFNGN SFTSYKDSAD
95-54aa   VDMKDAVGTY TLSGLRNFTG GDLDVNMQKA TLRLGQFNGN SFTSYKDSAD
  rVacA   VDMKDAVGTY KLSGLRNYTG GDLDVNMQKA TLRLGQFNGN SFTSFKDSAD

          151                                                200
60190aa   RTTRVDFNAK NILIDNFLEI NNRVGSGAGR KASSTVLTLQ ASEGITSSKN
95-54aa   RTTRVDFNAK NILIDNFLEI NNRVGSGAGR KASSTVLTLQ ASEKITSREN
  rVacA   RTTRVDFNAK NISIDNFLEI NNRVGSGAGR KASSTVLTLQ ASEGITSGKN

          201                                                250
60190aa   AEISLYDGAT LNLASNSVKL NGNVWMGRLQ YVGAYLAPSY STINTSKVTG
95-54aa   AEISLYDGAT LNLASNSVKL MGNVWMGRLQ YVGAYLAPSY STINTSKVTG
  rVacA   AEISLYDGAT LNLASNSVKL MGNVWMGRLQ YVGAYLAPSY STINTSKVTG

          251                                                300
60190aa   EVNFNHLTVG DHNAAQAGII ASNKTHIGTL DLWQSAGLNI IAPPEGGYKD
95-54aa   EVDFNHLTVG DHNAAQAGII ASNKTHIGTL DLWQSAGLNI IAPPEGGYKD
  rVacA   EVDFNHLTVG DHNAAQAGII ASKKTYIGTL DLWQSAGLNI IAPPEGGYKD

          301                                                350
60190aa   KPNNTPS... QSGAKNDKQE SSQNN...SN TQVINPPNST QKTEVQPTQV
95-54aa   KPKDKPSNTT QNNANNNQQN SAQNN...SN TQVINPPNSA QKTEIQPTQV
  rVacA   KPNNTNS... QSGAKNDKNE SAKNDKQDSN TQVINPPNSG QKTEIQPTQV

          351                                                400
60190aa   IDGPFAGGKD TVVNIDRINT KADGTIKVGG FKASLTTNAA HLNIGKGGVN
95-54aa   IDGPFAGGKD TVVNINRINT NADGTIRAGG YKASLTTNAA HLYIGKGGVN
  rVacA   IDGPFAGAKD TVVNINRINT NADGTIKVGG YTASLTTNAA DLNIGKGGIN

          401                                                450
60190aa   LSNQASGRTL LVENLTGNIT VDGPLRVNNQ VGGYALAGSS ANFEFKAGVD
95-54aa   LSNQASGRSL LVENLTGNIA VEGTLRVNNQ VGGSAVAGSS ANFEFKAGTD
  rVacA   LSNQASGRSL LVENLTGNIT VDGALMVNKE AGGAALPGSS ANFEFKAGVD

          451                                                500
60190aa   TKNGTATFNN DISLGRFVNL KVDAHTANFK G......... ..........
95-54aa   TNNGTATFNN DIHLGKAVNL RVDAHTAYFN GNIYLGKSTN LKVNGHSAHF
  rVacA   TNNGTATFNN DIRLGKAVNL KVDAHTINFN GNMYLGRFTH LKVNGHTANF
```

Continued

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          501                                                        550
60190aa   ......IDTG NGGFNTLDFS GVTNKVNINK LITASTNVAV KNFNINELIV
95-54aa   KNIDATKSDN GLNTSALDFS GVTDKVNINK LTTSATNVNI KNFDIKELVV
  rVacA   KDIDASKGRN GIDTTILDFS GVTNKVNINK LTTAATNAAI KNFDIKELVV

          551                                                        600
60190aa   KTNGVSVGEY THFSEDIGSQ SRINTVRLET GTRSIFSGGV KFKSGEKLVI
95-54aa   TTRVQSFGQY TIFGENIGDK SRIGVVSLQA GYSPAYSGGV TFKSGKKLVI
  rVacA   TTNVLSVGKY TDFTEDIGDQ SRIGIVSLQT GYGPAYSGGV TFKGGKKLVI

          601                                                        650
60190aa   DEFYYSPWNY FDARNIKNVE ITRKFASSTP ENPWGTSKLM FNNLTLGQNA
95-54aa   DEIYHAPWNY FDARNVTDVE INKRILFGSP GNIAGKTGLM FNNLTLNSNA
  rVacA   DEIYHAPWNY FDARNVTDVE INKRILFGAP GNIAGKTGLM FNNLTLNSNA

          651                                                        700
60190aa   VMDYSQFSNL TIQGDFINNQ GTINYLVRGG KVATLNVGNA AAMMFNNDID
95-54aa   SMDYGKDLDL TIQGHFTNNQ GTMNLFVQDG RVATLNAGHQ ASMIFNNLVD
  rVacA   SMDYGKDLDL TIQGHFTNNQ GTMNLFVQDG RVATLNAGHQ ASMIFNNLVD

          701                                                        750
60190aa   SATGFYKPLI KINSAQDLIK NTEHVLLKAK IIGYGNVSTG .....TNGIS
95-54aa   SATGFYKPLI KINNAQNLTK NKEHVLVRAR NIDYNLVGVQ GASYDNISAS
  rVacA   SATGFYKPLI KVNNAQNLTK NKEHVLVKAR NIDYNLVGVQ GASYDNISAS

          751                                                        800
60190aa   NVNLEEQFKE RLALYNNNNR MDTCVVR..N TDDIKACGMA IGNQSMVNNP
95-54aa   NTNLQEQFKE RLALYNNNNR MDICVVRKNN LNDIKACGMA IGNQSMVNNP
  rVacA   NTNLQEQFKE RLALYNNNNR MDICVVRKNN TDDIKACGMA IGNQSMVNNP

          801                                                        850
60190aa   DNYKYLIGKA WKNIGISKTA NGSKISVYYL GNSTPTENGG NTTNLPTNTT
95-54aa   ESYKYLEGKA WKNTGINKTA NNTTIAVNLG NNSTPTENGG NTTDLPTNTT
  rVacA   ENYKYLEGKA WKNTGINKTA NNTTIAVNLG NNSAPTENGG NTTNLPTNAT

          851                                                        900
60190aa   NNARFASYAL IKNAPFAHS. ATPNLVAINQ HDFGTIESVF ELANRSKDID
95-54aa   NNARFASYAL IKNAPFAHYN ATPNLVAINQ HDFGTIESVF ELANRSSDID
 rVacA    NNARFARFAS YA
```

**Appendix 5: Alignment of amino acid sequence of recombinant VacA from Thai isolate with several strains.**

Identical amino acids residues are shaded with yellow. Identical amino acids residues between two strains shaded with blue color. The white color region has amino acid residues consistently different between strains. We show here strictly conserved VacA domain among several surveyed strains m1 and m2 sequences and diversification region which differentiate m1 and m2.

```
                1                                                  50
821AA    (1) AFFTTVIIPAIVGGIATGTAVGTVSGLLGWGLKQAEEANKTPDKPDKVWR
S72494   (1) AFFTTVIIPAIVGGIATGTAVGTVSGLLSWGLKQAEEANKTPDKPDKVWR
AF191639(1) AFFTTVIIPAIVGGIASGAAVGTVSGLLGWGLKQAEEANKTPDKPDKVWR
95-54AA  (1) AFFTTVIIPAIVGGIATGAAVGTVSGLLSWGLKQAEEANKTPDKPDKVWR
RVACA    (1) AFFTTVIIPAIVGGIATGAAVGTVSGLLGWGLKQAEEANKTPDKPDKVWR
               51                                                 100
821aa   (51) IQAGKGFNEFPNKEYDLYKSLLSSKIDGGWDWGNAATHYWIKGGQWNKLE
S72494  (51) IQAGKGFNEFPNKEYDLYRSLLSSKIDGGWDWGNAARHYWVKGGQQNKLE
AF191639(51) IQAGRGFNEFPNKEYDLYKSLLSSKIDGGWDWGNAARHYWVKGGQWNKLE
95-54aa(51) IQAGRGFNNFPNKEYDLYRSLLSSKIDGGWDWGNAARHYWVKGGQWNKLE
rVacA   (51) IQAGRGFNEFPNKEYDLYKSLLSSKIDGGWDWGNAARHYWVKGGQWNKLE
              101                                                150
 821aa(101) VDMKDAVGTYKLSGLRNFTGGDLDVNMQKATLRLGQFNGNSFTSYKDSAD
 S72494(101) VDMKDAVGTYTLSGLRNFTGGDLDVNMQKATLRLGQFNGNSFTSYKDSAD
AF191639101) VDMKDAVGTYKLSGLINYTGGDLDVNMQKATLRLGQFNGNSFTSFKDSAD
95-54aa(101) VDMKDAVGTYTLSGLRNFTGGDLDVNMQKATLRLGQFNGNSFTSYKDSAD
 rVacA(101) VDMKDAVGTYKLSGLRNYTGGDLDVNMQKATLRLGQFNGNSFTSFKDSAD
              151                                                200
 821aa(151) RTTRVDFNAKNILIDNFLEINNRVGSGAGRKASSTVLTLQASEGITSSKN
 S72494(151) RTTRVDFNAKNISIDNFVEINNRVGSGAGRKASSTVLTLQASEGITSDKN
 F191639151) RTTRVDFNAKNISIDNFLEINNRVGSGAGRKASSTVLTLQASEGITSSKN
 95-4aa(151) RTTRVDFNAKNILIDNFLEINNRVGSGAGRKASSTVLTLQASEKITSREN
 rVacA(151)  RTTRVDFNAKNISIDNFLEINNRVGSGAGRKASSTVLTLQASEGITSGKN
               201                                               250
 821aa(201)  AEISLYDGATLNLASNSVKLNGNVWMGRLQYVGAYLAPSYSTINTSKVTG
S72494(201)  AEISLYDGATLNLASSSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG
AF1916 (201) AEISLYDGATLNLASNSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG
95-54a(201)  AEISLYDGATLNLASNSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG
rVacA(201)   AEISLYDGATLNLASNSVKLMGNVWMGRLQYVGAYLAPSYSTINTSKVTG
```

```
           251                                                      300
821aa(251) EVNFNHLTVGDHNAAQAGIIASNKTHIGTLDLWQSAGLNIIAPPEGGYKD
S72494(251)EVNFNHLTVGDKNAAQAGIIANKKTNIGTLDLWQSAGLNIIAPPEGGYKD
AF91639(251EVNFNHLTVGDHNAAQAGIIASKKTYIGTLDLWQSAGLNIIAPPEGGYKD
95-54(251) EVDFNHLTVGDHNAAQAGIIASNKTHIGTLDLWQSAGLNIIAPPEGGYKD
VacA (251) EVDFNHLTVGDHNAAQAGIIASKKTYIGTLDLWQSAGLNIIAPPEGGYKD
           301                                                  350
821aa(301) KPNNTPSQSGAKNDK--------QESSQNNSNTQVINPPNSTQKTEVQPT
S72494(301)PNNTPSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSAQKTEVQPT
AF1916(301)KPNNTNSQSGAKNDKNESAKNDKQESSQNNSNTQVINPPNSGQKTEIQPT
95-54a(301 KPKDKPSNTTQNNANNNQQNSAQN-----NSNTQVINPPNSAQKTEIQPT
rVacA(301) KPNNTNSQSGAKNDKNESAKNDKQ-----DSNTQVINPPNSGQKTEIQPT
           351                                                  400
821aa (343)QVIDGPFAGGKDTVVNIDRINTKADGTIKVGGFKASLTTNAAHLNIGKGG
S72494(351)QVIDGPFAGGKDTVVNINRINTNADGTIRVGGFKASLTTNAAHLHIGKGG
AF1919(351)QVIDGPFAGAKDTVVNINRINTNADGTIKVGGYTASLTTNAANLNIGKGG
95-54a(346)QVIDGPFAGGKDTVVNINRINTNADGTIRAGGYKASLTTNAAHLYIGKGG
rVacA (346)QVIDGPFAGAKDTVVNINRINTNADGTIKVGGYTASLTTNAADLNIGKGG
           401                                                  450
821aa(393) VNLSNQASGRTLLVENLTGNITVDGPLRVNNQVGGYALAGSSANFEFKAG
S72494(401)VNLSNQASGRSLIVENLTGNITVDGPLRVNNQVGGYALAGSSANFEFKAG
AF19169401)VNLSNQASGRSLLVENLTGNITVDGALMVNNQVGGYALAGSSANFEFKAG
95-54a(396)VNLSNQASGRSLLVENLTGNIAVEGTLRVNNQVGGSAVAGSSANFEFKAG
rVacA(396) INLSNQASGRSLLVENLTGNITVDGALMVNKEAGGAALPGSSANFEFKAG
           451                                                  500
821aa 443) VDTKNGTATFNN----------------------DISLGRFVNLKVDAHTA
S72494451) TDTKNGTATFNN----------------------DISLGRFVNLKVDAHTA
AF1916(451)VDTKNGTIAFN-----------------------NNISLGRFVNLKASAHTV
95-54a(446)TDTNNGTATFNNDIHLGKAVNLRVDAHTAYFNGNIYLGKSTNLKVNGHSA
rVacA(446) VDTNNGTATFNNDIRLGKAVNLKVDAHTINFNGNMYLGRFTHLKVNGHTA
           501                                                  550
821aa (472)NFKGIDTGNG----GFNTLDFSGVTNKVNINKLITASTNVAVKNFNINEL
S72494(480)NFKGIDTGNG----GFNTLDFSGVTDKVNINKLITASTNVAVKNFNINEL
AF1916(480)NFKDIDTGNG----GFNTLDFSGVTNKVNINKLITASTNVAVKNFNINEL
95-54a(496)HFKNIDATKSDNGLNTSALDFSGVTDKVNINKLTTSATNVNIKNFDIKEL
rVacA 496) NFKDIDASKGRNGIDTTILDFSGVTNKVNINKLTTAATNAAIKNFDIKEL
           551                                                  600
821aa(518) IVKTNGVSVGEYTHFSEDIGSQSRINTVRLETGTRSIFSGGVKFKSGEKL
S7249(526) IVKTNGISVGEYTHFSEDIGSQSRINTVRLETGTRSLFSGGVKFKGGEKL
AF193(526) LVKTNGISVGEYTNFSEDIGNQSRINTVRLETGTRSIYSGGVKFKGGEKL
95-54a(546)VVTTRVQSFGQYTIFGENIGDKSRIGVVSLQAGYSPAYSGGVTFKSGKKL
rVacA (546)VVTTNVLSVGKYTDFTEDIGDQSRIGIVSLQTGYGPAYSGGVTFKGGKKL
Conssus551)IVKTNGISVGEYT FSEDIG QSRINTVRLETGTRSIYSGGVKFKGGEKL
           601                                                  650
821aa(568) VIDEFYYSPWNYFDARNIKNVEITRKFASSTPENPWGTSKLMFNNLTLGQ
S72494576) VIDEFYYSPWNYFDARNIKNVEITNKLAFGPQGSPWGTSKLMFNNLTLGQ
AF1939(576)VINDFYYAPWNYFDARNIKNVEITNKLAFGPQGSPWGTSKLMFNNLTLNS
95-54a(596)VIDEIYHAPWNYFDARNVTDVEINKRILFGSPGNIAGKTGLMFNNLTLNS
rVacA(596) VIDEIYHAPWNYFDARNVTDVEINKRILFGAPGNIAGKTGLMFNNLTLNS
```

Continued

```
          651                                                   700
821aa(618)NAVMDYSQFSNLTIQGDFINNQGTINYLVRGGKVATLNVGNAAAMMFNND
S7244(626)NAVMDYSQFSNLTIQGDFINNQGTINYLVRGGKVATLSVGNAAAMMFNND
AF1916626)NASMDYGKDLDLTIQGHFTNNQGTMNLFVQDGRVATLNAGHQASMIFNNL
95-5a(646)NASMDYGKDLDLTIQGHFTNNQGTMNLFVQDGRVATLNAGHQASMIFNNL
rVacA(646)NASMDYGKDLDLTIQGHFTNNQGTMNLFVQDGRVATLNAGHQASMIFNNL
          701                                                   750
821aa(668)IDSATGFYKPLIKINSAQDLIKNTEHVLLKAKIIGYGNVSTG-----TNG
S7249(676)IDSATGFYKPLIKINSAQDLIKNTEHVLLKAKIIGYGNVSTG-----TNG
AF939(676)VDSATGFYKPLIKINNAQNLTKNKEHVLVKARNIDYNLVGVQGASYDNIS
95-5a(696)VDSATGFYKPLIKINNAQNLTKNKEHVLVRARNIDYNLVGVQGASYDNIS
rVacA(696)VDSATGFYKPLIKVNNAQNLTKNKEHVLVKARNIDYNLVGVQGASYDNIS
          751                                                   800
821aa(713)ISNVNLEEQFKERLALYNNNNRMDTCVVR--NTDDIKACGMAIGNQSMVN
S724(721) ISNVNLEEQFKERLALYNNNNRMDTCVVR--NTDDIKACGMAIGDQSMVN
AF116(726)ASNTNLQEQFKERLALYNNNNRMDICVVRKDNLNDIKACGMAIGDQAMVN
95-5a(746)ASNTNLQEQFKERLALYNNNNRMDICVVRKNNLNDIKACGMAIGNQSMVN
rVacA746) ASNTNLQEQFKERLALYNNNNRMDICVVRKNNTDDIKACGMAIGNQSMVN
```

## Appendix 6: Nucleotide sequence of the vacA gene strain 60190 in GenBank database GenBank accession no. U05676

```
LOCUS       HPU05676              4738 bp    DNA     linear   BCT 15-APR-1994
DEFINITION  Helicobacter pylori 60190 cysteinyl-tRNA synthetase homolog gene,
            partial cds, and vacuolating cytotoxin (vacA) gene, complete cds.
ACCESSION   U05676
VERSION     U05676.1  GI:471727
KEYWORDS    .
SOURCE      Helicobacter pylori
  ORGANISM  Helicobacter pylori
            Bacteria; Proteobacteria; Epsilonproteobacteria;
Campylobacterales;
            Helicobacteraceae; Helicobacter.
REFERENCE   1  (bases 1 to 4738)
  AUTHORS   Cover,T.L., Tummuru,M.K., Cao,P., Thompson,S.A. and Blaser,M.J.
  TITLE     Divergence of genetic sequences for the vacuolating cytotoxin
among
            Helicobacter pylori strains
  JOURNAL   J. Biol. Chem. 269 (14), 10566-10573 (1994)
   PUBMED   8144644
REFERENCE   2  (bases 1 to 4738)
  AUTHORS   Cover,T.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-JAN-1994) Timothy L. Cover, Division of Infectious
            Disease, Vanderbilt University School of Medicine, A3310 Medical
            Center North, Nashville, TN 37232, USA
FEATURES             Location/Qualifiers
     source          1..4738
                     /organism="Helicobacter pylori"
                     /mol_type="unassigned DNA"
                     /strain="60190 (ATCC 49503)"
                     /db_xref="taxon:210"
                     /clone_lib="lambda ZAP II"
     CDS             <1..567
                     /note="putative"
                     /codon_start=1
                     /transl_table=11
                     /product="cysteinyl-tRNA synthetase homolog"
                     /protein_id="AAA17656.1"
                     /db_xref="GI:471728"

/translation="FFIKDALKNYDGEILRNYLLGVHYRSVLNFNEEDLLVSKKRLDK

IYRLKQRVLGNLGGINPNFKKEILECMQDDLNVSKALSVLESMLSSTNEKLDQNPKNK

ALKGEILANLKFVEELLGIGFKDPSAYFQLGVSESEKQEIENKIEERKRAKEQKDFLK
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     RBS             788..792
                     /note="putative"
     gene            797..4660
                     /gene="vacA"
     CDS             797..4660
                     /gene="vacA"
                     /codon_start=1
                     /transl_table=11
                     /product="vacuolating cytotoxin"
                     /protein_id="AAA17657.1"
                     /db_xref="GI:471729"

/translation="MEIQQTHRKINRPLVSLALVGALVSITPQQSHAAFFTTVIIPAI

VGGIATGTAVGTVSGLLGWGLKQAEEANKTPDKPDKVWRIQAGKGFNEFPNKEYDLYK
```

SLLSSKIDGGWDWGNAATHYWIKGGQWNKLEVDMKDAVGTYKLSGLRNFTGGDLDVNM

QKATLRLGQFNGNSFTSYKDSADRTTRVDFNAKNILIDNFLEINNRVGSGAGRKASST

VLTLQASEGITSSKNAEISLYDGATLNLASNSVKLNGNVWMGRLQYVGAYLAPSYSTI

NTSKVTGEVNFNHLTVGDHNAAQAGIIASNKTHIGTLDLWQSAGLNIIAPPEGGYKDK

PNNTPSQSGAKNDKQESSQNNSNTQVINPPNSTQKTEVQPTQVIDGPFAGGKDTVVNI

DRINTKADGTIKVGGFKASLTTNAAHLNIGKGGVNLSNQASGRTLLVENLTGNITVDG

PLRVNNQVGGYALAGSSANFEFKAGVDTKNGTATFNNDISLGRFVNLKVDAHTANFKG

IDTGNGGFNTLDFSGVTNKVNINKLITASTNVAVKNFNINELIVKTNGVSVGEYTHFS

EDIGSQSRINTVRLETGTRSIFSGGVKFKSGEKLVIDEFYYSPWNYFDARNIKNVEIT

RKFASSTPENPWGTSKLMFNNLTLGQNAVMDYSQFSNLTIQGDFINNQGTINYLVRGG

KVATLNVGNAAAMMFNNDIDSATGFYKPLIKINSAQDLIKNTEHVLLKAKIIGYGNVS

TGTNGISNVNLEEQFKERLALYNNNNRMDTCVVRNTDDIKACGMAIGNQSMVNNPDNY

KYLIGKAWKNIGISKTANGSKISVYYLGNSTPTENGGNTTNLPTNTTNNARFASYALI

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**Appendix 7: Nucleotide sequence of the vacA gene strain 95-54 in GenBank database GenBank accession no. U95971**

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08-JUL-1998
DEFINITION  Helicobacter pylori 95-54 (J128) inactive cytotoxin
(vacA) gene,
complete cds.
ACCESSION   U95971
VERSION     U95971.1  GI:3294545
KEYWORDS    .
SOURCE      Helicobacter pylori
ORGANISM  Helicobacter pylori
Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales;
Helicobacteraceae; Helicobacter.
REFERENCE   1  (bases 1 to 4243)
AUTHORS   Pagliaccia,C., de Bernard,M., Lupetti,P., Ji,X.,
Burroni,D.,
Cover,T.L., Papini,E., Rappuoli,R., Telford,J.L. and Reyrat,J.M.
TITLE     The m2 form of the Helicobacter pylori cytotoxin has cell
type-specific vacuolating activity
JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 95 (17), 10212-10217 (1998)
PUBMED   9707626
REFERENCE   2  (bases 1 to 4243)
AUTHORS   Pagliaccia,C., Cover,T., Rappuoli,R., Telford,J.L. and
Reyrat,J.M.
TITLE     Direct Submission
JOURNAL   Submitted (01-APR-1997) Biol Mol, IRIS Chiron-Biocine, via
Fiorentina 1, Siena 53 100, Italy
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**Appendix 8: Nucleotide and deduced amino acid sequence of the recombinant *vacA* gene from a clinical Thai isolate.**

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CTGGGGGCGT TACTTTTAAA GGTGGTAAAA AACTGGTTAT AGATGAAATT
TACCATGCCC CTTGGAATTA TTTTGACGCT AGGAATGTTA CCGATGTTGA
AATCAACAAG AGGATTCTTT TTGGAGCCCC AGGAAACATT GCCGGCAAAA
CAGGGCTTAT GTTTAACAAC CTAACCCTAA ATAGTAATGC GAGCATGGAT
TATGGTAAAG ATTTAGACTT AACCATTCAA GGGCATTTCA CTAACAATCA
GGGCACGATG AATCTTTTTG TCCAAGATGG GCGTGTAGCG ACCTTAAATG
CAGGCCATCA AGCAAGCATG ATATTTAATA ATTTAGTGGA TAGCGCGACC
GGATTTTACA AACCGCTCAT TAAGGTTAAT AACGCTCAAA ACCTCACTAA
AAATAAAGAA CATGTGTTAG TGAAAGCGCG AAACATTGAT TATAATTTAG
TGGGAGTGCA AGGTGCTAGT TATGACAATA TTTCTGCAAG CAACACCAAT
CTGCAAGAGC AATTCAAAGA GCGCCTAGCC CTTTATAACA ACAATAACCG
CATGGATATT TGTGTGGTGC GAAAAAATAA TACCGATGAC ATTAAAGCAT
GCGGGATGGC TATCGGCAAT CAAAGCATGG TGAATAACCC TGAAAATTAC
AAGTATCTTG AAGGTAAGGC ATGGAAAAAT ACAGGGATTA ATAAAACGGC
TAACAACACC ACAATCGCTG TTAATTTAGG CAACAATTCT GCACCTACTG
AGAATGGTGG CAATACCACA AATTTACCCA CAAACGCCAC TAACAATGCG
CGTTTTGCGC GTTTCGCTAG CTACGCT

**Translation**

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  1    AFFTTVIIPA IVGGIATGAA VGTVSGLLGW GLKQAEEANK TPDKPDKVWR
 51    IQAGRGFNEF PNKEYDLYKS LLSSKIDGGW DWGNAARHYW VKGGQWNKLE
101    VDMKDAVGTY KLSGLRNYTG GDLDVNMQKA TLRLGQFNGN SFTSFKDSAD
151    RTTRVDFNAK NISIDNFLEI NNRVGSGAGR KASSTVLTLQ ASEGITSGKN
201    AEISLYDGAT LNLASNSVKL MGNVWMGRLQ YVGAYLAPSY STINTSKVTG
251    EVDFNHLTVG DHNAAQAGII ASKKTYIGTL DLWQSAGLNI IAPPEGGYKD
301    KPNNTNSQSG AKNDKNESAK NDKQDSNTQV INPPNSGQKT EIQPTQVIDG
351    PFAGAKDTVV NINRINTNAD GTIKVGGYTA SLTTNAADLN IGKGGINLSN
401    QASGRSLLVE NLTGNITVDG ALMVNKEAGG AALPGSSANF EFKAGVDTNN
451    GTATFNNDIR LGKAVNLKVD AHTINFNGNM YLGRFTHLKV NGHTANFKDI
501    DASKGRNGID TTILDFSGVT NKVNINKLTT AATNAAIKNF DIKELVVTTN
551    VLSVGKYTDF TEDIGDQSRI GIVSLQTGYG PAYSGGVTFK GGKKLVIDEI
601    YHAPWNYFDA RNVTDVEINK RILFGAPGNI AGKTGLMFNN LTLNSNASMD
651    YGKDLDLTIQ GHFTNNQGTM NLFVQDGRVA TLNAGHQASM IFNNLVDSAT
701    GFYKPLIKVN NAQNLTKNKE HVLVKARNID YNLVGVQGAS YDNISASNTN
751    LQEQFKERLA LYNNNNRMDI CVVRKNNTDD IKACGMAIGN QSMVNNPENY
801    KYLEGKAWKN TGINKTANNT TIAVNLGNNS APTENGGNTT NLPTNATNNA
851    RFARFASYA
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