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โครงการ Structural Determinants of Membrane Channel Formation And Receptor Recognition within the Vacuolating Cytotoxin VacA – a Model Virulence Factor from Human Pathogenic Helicobacter pylori

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ABSTRACT

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Project Title: STRUCTURAL DETERMINANTS OF MEMBRANE CHANNEL FORMATION AND RECEPTOR RECOGNITION WITHIN THE VACUOLATING CYTOTOXIN VacA – A MODEL VIRULENCE FACTOR FROM HUMAN PATHOGENIC *HELICOBACTER PYLORI*

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The vacuolating cytotoxin (VacA) from human-pathogenic *Helicobacter pylori* represents one of the most intensively characterized virulence factors of this bacterium. It is involved in the colonization of the gastric epithelium, the establishment of a prolonged inflammation and induces a multitude of cytopathic symptoms in the affected host. Within this project, it was proposed to establish molecular biological and biochemical work on VacA with a view to structure-mechanism relations. As practically no technical experience with VacA was available when the project started, the work was initiated by evaluating basic procedures for cloning, expression, purification and biochemical assays for VacA. In the first phase of this project, we have accomplished isolation, purification to homogeneity and an *in vitro* assay of apoptotic activity of a VacA toxin obtained from a Thai patient with gastrointestinal lymphoma. Sequencing revealed that the Thai isolate VacA is structurally similar to *H. pylori* s1/m2 type strains, whereas homology to the 60190 model strain was found to be lower. The purified VacA toxin exhibited significant apoptotic activity on two epithelial cell lines, T84 and MDCK, as revealed by DAPI staining, whereby the observed activity was significantly higher on MDCK cells. Preliminary experimental data have shown, however, that yield and purity of the recombinant VacA protein were insufficient for further biochemical and biophysical experiments, in particular for an intended x-ray crystallographic analysis of the pore-forming p33 domain. Therefore, further investigations on expression and purification of VacA led us to an intensive characterization of product toxicity within the recombinant *E. coli* host. We were able to demonstrate that biosynthesis of subdomains of VacA containing the p33 moiety was conducive to stalled growth of the host and low product yields. Currently we explore an improved expression system which uses a VacA-DHFR fusion protein that appears to be non-toxic for *E. coli* and offers substantial advantages for purification of recombinant VacA.

Keywords: *Helicobacter pylori*; VacA cytotoxin; sequence; apoptosis; assay; fusion protein.

1 INTRODUCTION

Presently *Helicobacter pylori* is considered as the most prevalent bacterial pathogen causing infection of humans (1). At least 50 % of the world populations are infected with *H. pylori* (2, 3). It is estimated that 90% of duodenal ulcers and up to 80% of gastric ulcers are caused by *H. pylori* (4). The rates of infection are substantially different for certain countries and suggest that dietary behaviors are directly related to the occurrence of infections. Less than 20% of all infected individuals develop gastric or peptic ulcer and only 1% of infected develop gastric cancer (5, 6). However, more than 80% of infected individuals remain asymptomatic for long periods of time and treatment is necessary only for patients with ulcer or ailing stomach. Diagnosis and treatment of infections caused by *H. pylori* now drain increasingly resources for public health care in developing countries (7). There is also an urgent need for the improvement of existing diagnostic and treatment procedures.

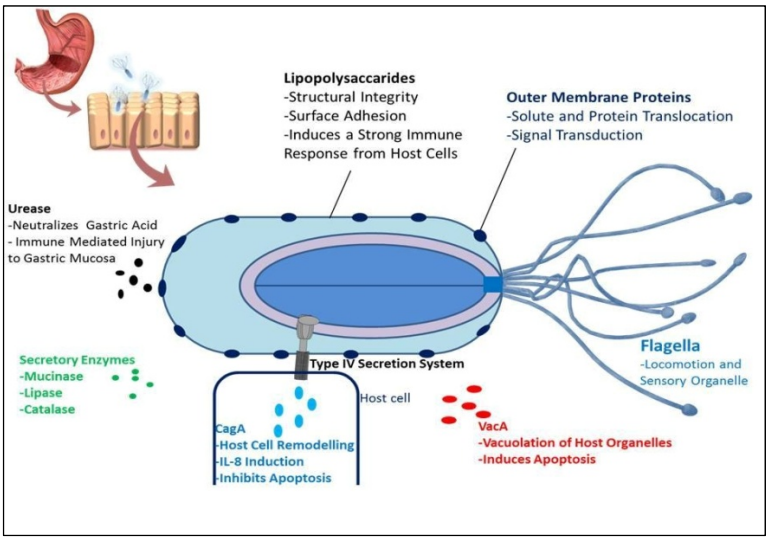

Figure 1. Virulence factors of *H. pylori*. The organism contains a multitude of virulence factors involved in host-pathogen interactions as well as components necessary for survival of the bacterium under highly acidic conditions in the stomach. The latter involves secreted enzymes such as lipase, catalase and urease, which is used to neutralize gastric acid in the stomach. A number of virulence factors encoded by the cytotoxin gene-associated pathogenicity island (cagPAI) and the VacA cytotoxin interact in a yet not completely resolved mechanism to maintain persistence of *H. pylori* in the stomach conducive to prolonged inflammation of the epithelial tissue.

Adhesion and colonization of the gastric mucosa by *H. pylori* is strain-dependent and related to the capability to produce a substantial number of virulence factors. Those include the cytotoxin-associated gene A (cagA) and the vacuolating cytotoxin A (VacA). CagA induces the release of cytokines such as interleukin IL-8 contributing to prolonged inflammation and subsequent ulceration of the epithelium (8, 9). CagA also appears to antagonize some activities of VacA by mediating opposing effects, especially on target cell apoptosis (10). Modulation of the host immune system has been reported a consequence of

infection with *H. pylori* (11). Several factors seem to influence long-term colonization including the host immune response, dietary intake, and probiotics (12, 13).

Vacuolating cytotoxin (VacA) is one of the major virulence factors produced by *H. pylori* and is found in all strains isolated so far (14). The VacA toxin activity appears to be directly related to incidence and/or severity of disease(s) caused by *H. pylori* infections (15). VacA is multifunctional toxin which seemingly is structurally and mechanistically unrelated to any other known bacterial toxin. The *vacA* gene is present as a single copy in the *H. pylori* genome and encodes a 140 kDa protoxin (in model strain 60190). The VacA protoxin is composed of a 33 residues N-terminal signal sequence, a 33 kDa C-terminal type V autotransporter domain and the 88 kDa mature toxin (16, 17. VacA is secreted as 88 kDa toxin and is cleaved by yet unidentified protease(s) which separate VacA into two domains: the 33 kDa N-terminal domain was reported to contain a pore-forming activity while the 55 kDa C-terminal domain is involved in receptor binding and toxin oligomerization (18). The two domains were designated p33 and p55, respectively. However, proteolytic separation of the two domains is not a prerequisite for VacA activity. VacA designated as p511 containing 100 residues of the p55 domain appears to represent the minimum sequence of VacA which is functionally active in vacuolation (19. This suggests that both domains are required for pore-forming activity. The architecture of the VacA toxin is shown in figure 2. Sequence analysis of VacA shows that 27 N-terminal amino acid residues contain a GXXXG motif necessary for VacA vacuolating activity and substitution of the glutamine residues in the GXXXG motif abolished VacA vacuolating activity (20). Recently, the crystal structure of the p55 domain has been solved (Figure 3) (21). No crystallographic structure is currently available for the p33 pore-forming domain. Homolgy comparisons suggest similarities to the mechanosensitive 'S channel' protein from *E. coli* (22). VacA forms water soluble hexameric or heptameric oligomers which dissociate under acidic or alkaline conditions. The oligomeric VacA was analyzed by electron microscopy which revealed a ring shaped "flower like structure" (23, 24). VacA forms anion-selective channels in the plasma membrane (25) and is therefore characterized as a pore-forming toxin. Secreted VacA contains soluble oligomeric structures when activated by host gastric acid (26). The monomeric active VacA can bind to host cell membranes either by sphingomyelin acting as a lipid-like receptor (27, 28) or by binding to several protein receptors such as receptor-like protein tyrosine phosphatases RPTPα and RPTPβ (31).

VacA binds to cells of the immune system by using lymphocyte function-associated antigen-1 (LFA-1) as its receptor on T-cells (29-31). In the present model of membrane channel formation which is supported by a number of ultrastructural studies, six N-terminal α-helices of VacA insert into the membrane and form a hexameric ring which constitutes the ion channel (32).

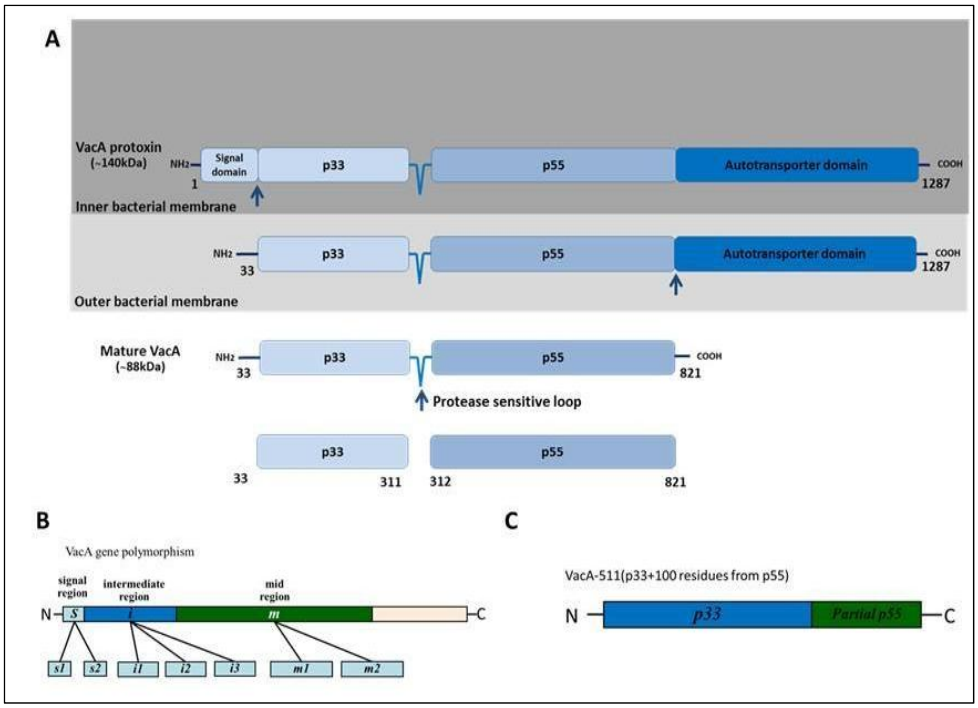

Figure 2. Architecture of the *vacA* gene from *H. pylori*. (**A**) VacA is produced as protoxin of approximately 140 kDa. The signal sequence is cleaved upon translocation in the periplasm and the C-terminal autotransporter domain is cleaved during transportation across the outer membrane. The mature 88 kDa protoxin is further cleaved by yet unidentified protease(s) into an N-terminal domain (amino acids 33 – 311) designated as p33, and a C-terminal p55 domain (residues 312 – 821). The p33 domain is involved in membrane insertion and the generation of ion channels whereas the p55 domain has a role in receptor binding and oligomerization. (**B**) Allelic diversity and polymorphisms within the *vacA* gene. The *vacA* gene contains three highly variable polymorphic regions which are the signal sequence region (s1, s2), the mid region (m1, m2) and the intermediate region (i1, i2). Strains of *H. pylori* with the s1m1 phenotype are more frequently associated with severe disease symptoms than strains carrying other combinations of these alleles. (**C**) The minimal structure of VacA necessary for vacuolation consists of the p33 domain and 100 additional residues of the p55 domain.

The region which is essentially required for oligomerization spans across the p33 and p55 domain interface (33). However, the precise structure of the VacA induced ion channel is not known in the absence of a 3-dimensional structure of the p33 domain. After entry into the target cells by receptor mediated endocytosis (34, 35), VacA induces the formation of large intracellular vacuoles.

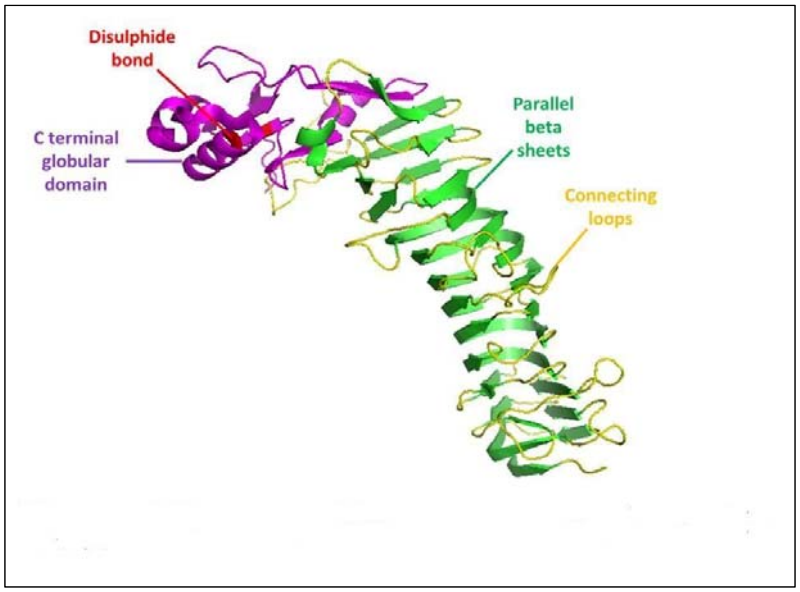

Figure 3. Crystallographic structure of VacA p55 domain. The p55 domain is composed mainly of beta sheets (green), connected by loops of varying size and shapes (yellow). The C-terminus of the p55 domain contains both alpha helices and beta sheets (violet) and a disulphide bond (red) is present in the globular domain (Gangwer et al., 2007).

VacA membrane channels lead to an influx of Cl^- ions into endosomal compartments which become highly acidic by subsequent activity of the vesicular ATPase resulting in an influx of weak bases such as NH_4^+ and water which leads to osmotic swelling of the vesicle. VacA interacts with mitochondrial membranes and induces apoptosis (36). VacA translocates to mitochondria by a yet unidentified mechanism and is imported into the inner mitochondrial membrane (37). It was proposed that VacA generates channels on mitochondria either on the outer membrane or the inner membrane and causes a depolarization of mitochondrial membrane potential releasing pro-apoptotic proteins (38). The N-terminal region of p33 was shown to be required for VacA translocation to the inner mitochondrial membrane (37).

The project proposed herein aims at initiating and expanding work on the detailed structural and functional characterization of the *H. pylori* VacA toxin. It is anticipated that the proposed investigations directly contribute to an improved understanding of VacA mechanism of action and therefore are useful for the development of vaccines as well as for the discovery of novel antimicrobial agents which target the VacA toxin. The investigations performed thus far were focusing on the following objectives: i) the isolation of a *vacA* gene from a Thai patient isolate and assay of apoptotic activity of the purified full-length toxin; ii) the analysis of a cytotoxic effect caused by VacA (or subdomains including p33) on recombinant *E. coli* host cells and iii) the methodological improvement and remedy of difficulties associated with low product yield by construction of a VacA-DHFR fusion protein.

2. MATERIALS AND METHODS

2.1 Cloning and Sequence Analysis of vacA Gene Isolated from a Thai Patient

H. pylori was cultured from a gastric biopsy sample from a Thai patient with diagnosed gastric lymphoma (62 years old male) obtained at Vichaiyut Hospital, Bangkok, Thailand, on the surfaces of horse blood agar plates (10% horse blood in Casman agar base [BBL Microbiology Systems, Cockeysville, Md.]), which were incubated in an atmosphere of 5% oxygen–10% carbon dioxide for 72 h at 37°C for up to 5 days (20-22). Chromosomal DNA was extracted and used as probe for PCR amplification of *vac*A. The following pair of primers derived from the sequence of *H. pylori* model strain 60190 was used:

SJ_*VacA*1F: 5`-CATGCCATGGCCTTTTTTACAACCGTGATCA-3` (underlined sequence represents the *Nco*I restriction site) and SJ_*VacA*821R: 5`-TGCACTGCAGAGCGT AGCTAGCGAAACGC-3` (underlined sequence represents the *Pst*I restriction site). Oligonucleotides were purchased from Proligo Singapore Pte Ltd.

PCR was performed with Pfu DNA polymerase (Fermentas Life Sciences, USA) using a thermal cycler GeneAmp PCR system Model 2400 (Perkin Elmer Cetus, USA), with pre-denaturing (98°C, 2 min), 25 cycles of denaturing (98°C, 0.1 min), annealing (65°C, 0.3 min), extension (72°C, 1.3 min), and final extension (72°C, 7 min). The resulting PCR products were subjected to agarose gel electrophoresis, and the 2.5 kb fragment representing the mature full-length VacA toxin was excised and purified by QIAquick® gel extraction kit (QIAGEN, Germany). The purified *vacA* PCR product and pTrcHisA (4.4 kb) vector (Invitrogen, USA) were digested with restriction enzymes *Nco*I and *Pst*I and purified by QIAquick purification kit. Insert DNA and pTrcHisA vector were combined at a 10:1 molar ratio in a ligation reaction containing 1× ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 25% (w/v) polyethylene glycol 8000) and five units of T4 DNA ligase (Gibco BRL, USA) in a final volume of 20 µl and incubated overnight at 14°C, resulting in pTrcHisA/VacA carrying a C-terminal $(His)_6$ purification tag and a translation initiation methionine residue at the N-terminus.

The correct sequence of the recombinant pTrcHisA/VacA$(His)_6$ construct was verified by restriction digestion and DNA sequencing analysis (Macrogen Inc., South Korea).

2.2 Expression and Purification of the Recombinant VacA Protein

Recombinant plasmid containing the VacA insert (pTrcHis2A/VacA) was transformed into *E.coli* TOP10 (GIBCO BRL, USA). To determine optimum conditions for the expression of theVacA protein, the construct was transformed into the *E. coli* and cells were incubated at 37°C in one liter LB medium containing 100 μg ml^{-1} ampicillin. At OD_{600} = 0.5, expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Protein biosynthesis was assayed at different IPTG concentrations (0.1-0.8 mM), incubation times (0 hr, 1 hr, 4 hr, 6 hr, overnight) and temperatures (18, 25, 30 and 37°C). Cells were harvested by centrifugation (6000 × *g*, 4°C, 10 minutes) and the pellet was resuspended in 30 ml lysis buffer (0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 0.25 mg ml^{-1} lysozyme, 10 mg ml^{-1} DNase, and 5 mM $MgCl_2$). Cells were lysed on ice by sonication using an Ultrasonic Processor XL (Misonix Inc. NY). The cell lysate was subjected to centrifugation (15000 × *g*, 4°C, 30 minutes), insoluble material was pelleted by centrifugation (15000 × *g*, 4°C, 20 minutes), and the soluble fraction was filtered through 0.22 micron pore-size filters (Pall Corporation, USA).

Histidine-tagged VacA protein was purified by immobilized metal ion affinity chromatography (IMAC), using nickel-sepharose HisTrap™ HP 5 ml columns (GE Healthcare, Sweden). The columns were pre-equilibrated with 5-10 column volumes sample buffer containing 100 mM Tris-HCl, pH 7.5, 300 mM NaCl, and the sample was loaded at a flow rate of 1 ml min^{-1}, using an FPLC pump (AKTA™ FPLC™ system, GE Healthcare). The column was washed with 10 column volumes of degased washing buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole) and the protein was eluted with elution buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 100 mM imidazole) at flow rate of 1.0 ml min^{-1}. Elution was monitored by absorbance at 280 nm using a UV detector (AKTA™ FPLC ™ system, GE Healthcare) and fractions of 2.0 ml were collected. From each fraction, 20 μl were loaded onto a 10% SDS-PAGE gel and electrophoresis was performed in Tris-glycine buffer (25 mM Tris-HCl, pH 8.3, 192 mM glycine and 0.1% SDS). Gels were stained in Coomassie-Blue (0.1% Coomassie-Brilliant-Blue R250, 50% methanol and 10% glacial acetic acid) with shaking at room temperature. Western blotting was performed using anti-VacA rabbit antiserum (Invitrogen, CA, USA) at 1: 2,000 dilution with alkaline phosphatase colour detection.

Fractions containing VacA were desalted by stepwise dialysis at 4°C by using SPECTRA/POR dialysis membranes (6–8 kDa MWCO) (Spectrum Medical Industries, Inc. MA, USA), against three batches of a 100-fold sample volume buffer A (100 mM Tris-HCl, pH 7.5, 200 mM NaCl), one batch of a 100-fold volume buffer B (100 mM Tris-HCl, pH 7.5, 100 M NaCl) and one batch of a 100-fold volume of buffer C (50 mM Tris-HCl, pH 7.5). Purified VacA was further concentrated to 1.0 mg ml^{-1} by centrifugal filter devices (Centricon 15 ml, 30 kDa MWCO, Millipore, USA) at 4°C. Protein concentrations were determined with a Bradford protein microassay (Bio-Rad, USA) using bovine serum albumin (Sigma Chemistry) as

calibration standard. Samples were used immediately or stored in 50 mM Tris-HCl, pH 7.5, 50% (v/v) glycerol, at -20°C.

2.3 Assay of VacA Apoptosis Activity

Human colonic adenocarcinoma (T84) and Madin-Darby canine kidney (MDCK) epithelial cells, originally purchased from the American Type Culture Collection (Manassas, Virginia, USA), 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^{-1} penicillin, 50 μg ml^{-1} streptomycin and 5% foetal bovine serum. The culture medium was replaced every other day. Monolayers were subcultured by trypsinization with 0.25% (w/v) trypsin and 5.3 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and plated on coverslips at a density of 10^5 cells ml^{-1} to study apoptosis caused by VacA. T84 and MDCK cells were seeded in 75 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2.

Apoptosis of the colonic epithelium was assessed using a nuclear stain, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, St.Louis, MO, USA). Cells were placed on coverslips at a density of 5×10^5 cells per well in DMEM medium and kept at the incubator. At the time of experiment, old medium was removed and cells were incubated with either 150 μg ml^{-1} VacA-containing medium or serum-free medium (as a control) for 24 h. Cells were washed in PBS (phosphate buffered saline) and fixed with 60 μl of 4% para-formaldehyde (PFA) for 8 minutes at 4°C followed by three times washing with 60 μl of 1× PBS. Cells were incubated with 60 μl of 0.1% Triton X-100 for 10 minutes and nonspecific binding sites were blocked by adding 2% skim milk powder for 1 h. Cells were washed in PBS three times, 10 minutes each. Finally, cells were stained with 50 μl of DAPI (1:2000 dilution in blocking buffer) for 15 minutes and mounted using 50% glycerol. The signals were visualized at wavelength 350/460 nm (excitation/emission) by using a fluorescence microscope (model IX71, Olympus, Japan). Binding of DAPI to dsDNA produced a ~20-fold fluorescence enhancement and a minimum of 100 cells was counted for each sample and the control by visual inspection of microscopic images. Results are represented as mean of three independent experiments and data are represented as mean ± standard deviation.

2.4 PCR Amplification of VacA Subdomains

The full-length VacA gene from *Helicobacter pylori* model strain 60190 was synthesized by TOP Gene Technologies, Canada. The company cloned a synthetic gene encompassing 2562 nucleotides into the pLS plasmid. The pLS-VacA was used as PCR

template for the amplification of VacA partial sequences. These encode the p33, p511, p55 sub-domains and a full-length VacA toxin which is commonly designated as p88. Specific primers were designed 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 and were purchased from Sigma-Aldrich Co., Ltd. The synthetic pLS-VacA (Top Gene Technologies, Canada) was used as a template. 50 µl of PCR reaction contained 100 ng template, 200 µM of dNTPs, 10 pmol of each primer, Phusion buffer and 1.5 units of Phusion polymerase enzyme (New England Biolabs, USA). PCR products were analyzed by 1% agarose gel electrophoresis. Subsequently, the PCR products were digested with compatible restriction endonucleases and were used for recombinant plasmid construction.

2.5 Cloning of VacA Domains

The full-length *vacA* gene from *Helicobacter pylori* model strain 60190 was cloned into pTrcHis2A plasmid vector as well as the p33, p511, p55 and p88 domains of VacA. The truncated domains were created for subsequent biochemical analysis of toxin activity. Each domain was cloned independently as described below.

The smallest domain is p33 that contains 1-311 amino acid residues of VacA. p33 is the N-terminal domain that is innately formed after proteolytic cleavage of full-length VacA. VacA ('p88') is cleaved by a yet unidentified protease(s) that separate VacA into two domains (59). The p33 PCR product harbors an *Nco*I restriction site at the 5' end and a *Sal*I site at the 3' end which were introduced by forward and reverse primers, respectively. PCR products were cut with *Nco*I and *Sal*I endonucleases. The plasmid pTrcHis2A was digested with *Nco*I and *Sal*I restriction enzymes and mixed with inserted fragments at 1:10 molar ratio. Ligations were performed at 16 °C overnight to obtain the pTrcHis2A/p33 recombinant plasmid. Ligation reactions were transformed into competent *E.coli* TOP10 cells. Transformants were screened for the recombinant plasmid by using the GeneJET Plasmid Miniprep Kit. Correct recombinant plasmids were verified by digestion analysis with *Nco*I, *Sal*I and *EcoR*I and DNA sequencing was performed by Macrogen (South Korea). The p33 sequence was compared to that of VacA strain 60190 (GenBank accession number U05676) and did not contain any mutation.

The p511 and the full-length p88 are two proteins of VacA that were reported to possess pore-forming and vacuolating activities (29). The p88 represents the full-length VacA toxin that is secreted by *H. pylori*. In contrast, p511 was made for analysis of VacA activity. The p511 domain was reported as comprising the minimal sequence which exhibits toxin activity that is not distinguishable from p88. The p511 protein contains 1-511 amino acid residues extended of 200 amino acid of p55 while p88 is composed of 821 amino acid residues. The p88 and p511 PCR products harbor an *Nco*I restriction site at the 5' end and a *Pst*I site at

the 3' end that were introduced by forward and reverse primers, respectively. Cloning strategies were identical to p33 as described above. Correct recombinant plasmids were verified by digestion analysis with *Nco*I, *Pst*I and *EcoR*I as shown in Figure 5.3 and 5.4 and DNA sequencing did not reveal any mutation.

The p55 domain is the product of proteolytic cleavage of the mature VacA toxin. The C-terminal domain p55 contains 312-821 amino acid residues and is presumed as the receptor binding domain. The p55 PCR product harbors the same restriction sites as described for p511 and p88. Cloning was performed in analogy to previously described procedures. Correct recombinant plasmids were verified by digestion analysis with *Nco*I, *Pst*I and *EcoR*I and DNA sequencing was performed by Macrogen (South Korea). The p55 sequence was compared and did not show any mutation.

The constructs of recombinant VacA were used to evaluate expression in *E.coli* strain TOP10 (Invitrogen, USA). The expression was under control of the inducible pTrc hybrid promoter in order to produce high yields of soluble target protein and/or inclusion bodies containing the target protein. *E.coli* TOP10 (Invitrogen, USA) containing the individual subclones (p88, p33, p55, p511) in the pTrcHis2A plasmid was cultivated in 10 ml LB broth containing 100 μg/ml of ampicillin at 37°C overnight. The culture was grown under vigorous shaking at 220 rpm. A milliliter of overnight culture was grown in 1000 ml LB broth containing 100 μg/ml of ampicillin at 37°C until OD_{600} was 0.5-0.6. Expression of the recombinant protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.1 mM and incubated at 37°C for 3 hours. Cells were harvested by centrifugation at 6,000 × *g* at 4°C for 20 minutes and stored at -20°C. The identities of the expressed proteins were determined by Western blot analysis.

Protein samples were separated on 12% SDS-PAGE and soaked in transfer buffer (Tris-Base 25 mM, pH 9.0, glycine 150 mM, 10% (v/v) ethanol) for 5 minutes. The nitrocellulose membrane and filter papers (eight pieces) were cut into the same size of the gel and were soaked in transfer buffer for 5 minutes. Protein samples were transferred to nitrocellulose membranes by semi-dry electrophoretic transfer (TE70 ECL Semi-dry Blotters) with constant current at 0.8 mA/cm^2 of gel at room temperature for 2 hours. After electro-blotting, the membrane was stained with Ponceau S staining for 2 minutes and destained by using 0.1 M NaOH. The membrane was soaked in blocking solution containing 5% (w/v) bovine serum albumin (BSA) in fresh PBS buffer (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), 0.1% (v/v) tween-20 at room temperature for 1 hour. The membrane was incubated with primary antibody solution (rabbit anti-VacA antibody, Austral Biological) at dilution 1：3,000 in blocking solution and incubated at room temperature for 1 hour. The membrane was washed three times for 10 minutes in PBS buffer, 0.1% tween-20 followed by incubated with the secondary antibody (anti-rabbit IgG horseradish peroxidase (HRP)-conjugated) at dilution 1：5,000 in blocking buffer at room temperature for 1 hour. Next,

the membrane was washed three times with PBS buffer, 0.1% tween-20 each 10 minutes. Signal was developed by using Super Signal® West Pico Chemiluminescent (Thermo Scientific, USA) as substrate. The developed membrane was exposed to autoradiography film for an appropriate period of time.

2.6 Purification of the Recombinant Proteins Representing Individual Domains of VacA

One gram cell pellet from *E. coli* grown under IPTG-induction was suspended in 1 ml binding buffer (20 mM Na_2HPO_4, 500 mM NaCl, pH 7.4, 10 mM imidazole). Sonication was performed by using Misonix XL2020 sonicator for 10 minutes. The supernatant was collected by centrifugation at 8,000 × *g*, 4°C for 30 minutes. The supernatant was filtered through 0.45 µm membrane filters.

The p55 6× His-tagged fusion protein was purified by a 2 step procedure. The first step of purification was Ni-column affinity chromatography by using a His-trap HP column (GE Healthcare). 5 ml column volume His-trap HP was washed with 25 ml MilliQ water. The column was pre-equilibrated with 5 column volumes of binding buffer. The supernatant was loaded into the column at a flow rate of 1 ml/min. Protein was eluted from the column with elution buffer (20 mM Na_2HPO_4, 500 mM NaCl, pH 7.4, 250 mM imidazole). Fractions of 500 µl were collected and analyzed by SDS-PAGE. The second step of purification was gel filtration chromatography by using a Sephadex 200 10/300GL column (GE Healthcare). 5 column volumes were washed with MilliQ water. A fraction from first step purification was loaded into the column at flow rate 0.5 ml/min. Fractions of 1 ml were collected and analyzed by SDS-PAGE. All purifications were performed on a HPLC system (Thermo Scientific, USA) and using a UV280 detector (Thermo Scientific, USA).

2.7 Cytotoxicity Analysis by Host Growth

Cytotoxicity analysis by recording bacterial growth curves was used to determine effects of cloned recombinant proteins on the cell growth of the *E. coli* expression host. *E.coli* clones were cultured in Luria broth containing 100 µg/ml of ampicillin with vigorously shaking at 220 rpm, 37 °C for overnight. The overnight culture was inoculated into pre-warmed Luria broth containing ampicillin (100 µg/ml) and further incubated with vigorously shaking at 220 rpm 37 °C. Optical density of the cell suspension was measured at 600 nm every hour from inoculation and following induction with 0.1 mM IPTG at OD_{600} equal to 0.6. *E. coli* harboring the pTrcHis2A plasmid was used as negative control.

3. RESULTS

3.1 VacA Isolated from a Thai Clinical Sample Induces Apoptosis in Epithelial Cell Lines

In the present study we constructed an expression system to obtain sequence information and to analyze the apoptotic activity of a *H. pylori* VacA toxin isolated from a Thai patient with gastric lymphoma ("Thai isolate"). We were seeking to understand how genetic differences and sequence polymorphisms between *m*1 and *m*2 types of *vacA* alleles are related to the Thai isolate VacA and how these could possibly influence the apoptotic activity of this protein.

The 2.5 kb *vacA* gene sequence encoding the mature VacA toxin was obtained by PCR amplification using *H. pylori* genomic DNA extracted from a patient isolate as template. VacA is the product of a single gene that encodes a 140 kDa precursor protein which upon proteolytical removal of the N-terminal signal sequence, yields the mature 88 kDa toxin containing alanine as N-terminal amino acid residue. A N-terminal methionine residue was included to ensure proper initiation of translation in the recombinant *E. coli* host and a C-terminal $(His)_6$ sequence was added for purification purposes. Recombinant plasmid DNA was transformed into *E. coli* TOP10, followed by rapid size screening and restriction digestion analysis.

The construct was analyzed by nucleotide sequencing and alignment with nucleotide sequences of *H. pylori* 60190 (*s*1/*m*1 allelic type) *vacA* sequence (GenBank accession no. U05676), and strain 95-54 (*s*1/*m*2 allelic type) (GenBank accession no. U95971). The sequence of the Thai strain isolate was deposited in GenBank at accession no. KC529337. Sequence analysis revealed that the sequence of the cloned Thai strain *vacA* gene encompasses 2577 bp encoding a mature VacA toxin of 859 amino acid residues. The sequence of the Thai isolate *vacA* gene is identical to a recently described strain isolated in China (CHN1811a; GenBank accession No. AF050326) and shows 89% homology to the toxigenic *H. pylori s*1/*m*2 strain 95-54, whereas homology to the *s*1/*m*1 model strain 60190 is only 83%. *H. pylori* strain 95-54 encodes an unusually large VacA toxin of 1323 amino acid residues and has been shown to possess an *s*1/*m*2 allelic phenotype.

Sequence comparison showed a well conserved p33 domain among the surveyed strains containing *m*1 and *m*2 alleles, and a diversification region which differentiates *m*1 and *m*2 allelic types. Residue D455 is located at the beginning of the mid – region in *H. pylori* 60190 and it was proposed that this area represents a receptor-binding site common to all *m*1 and *m*2 strains. The most notable difference between *H. pylori* strain 60190 and the Thai strain is the sizable insertion of 23 amino acid residues in the middle region making the Thai isolate an *m*2 type strain. The structural consequences of this insertion are not well understood at present. In particular, it is unclear whether the inserted sequence directly participates in receptor binding as both RPTPα and RPTPβ are recognized by the m2 type of *vacA* alleles.

Numerous studies have demonstrated a correlation between the allelic types of '*m*' and '*s*' regions and the occurrence of gastroduodenal diseases in humans, however, the pathophysiological mechanisms involved in these associations are characterized only to a limited extent at present. A study on the clinical relevance of VacA genotypes corroborated that VacA type *s*2 strains are rarely associated with peptic ulceration. As regions of diversity in *vacA* alleles comprise a sizable portion of the gene, structural differences between type *m*1 and type *m*2 gene products could give rise to differences in cytotoxin phenotypes affecting receptor recognition, internalization and vacuolation. Cell-specific binding has been attributed to differences in the *m*1 and *m*2 alleles. Strains encoding *s*1/*m*1 *vac*A genes typically produce VacA with cytotoxic activity on human cervical carcinoma HeLa cells, whereas *m*2-type VacA could induce vacuoles in primary cultured human gastric cell lines as well as non-gastric epithelial RK13 cells, but not in HeLa cells.

It is interesting to note that the third polymorphic determinant within the Thai isolate *vacA* sequence, the '*i*' region, shows marked differences to strain 95-54, whereas it shares greater homology to the other model strains. How the '*i*' region functions in the context of VacA allelic polymorphisms needs to be elucidated in future studies.

3.1.1 Expression and Purification of the Recombinant VacA Protein

The recombinant plasmid pTrcHis2A/VacA directed expression of the VacA toxin in *E.coli* TOP10 upon induction with IPTG. Optimization of expression conditions was attempted to achieve high levels of expression. Time-dependent expression of VacA was analyzed by SDS-PAGE of crude lysate and subsequent Western blotting with commercially available anti-VacA antiserum. It is interesting to note that growth of the recombinant stain carrying pTrHis2A/VacA ceased after addition of IPTG, and over a period of 14 hours, no further increase in bacterial growth rate was observed, thereby suggesting cytotoxic effects of the recombinant VacA on *E. coli* cell growth (data not shown). At lower temperatures (18-25°C), reduced amounts of recombinant VacA were detected after incubation for 14 hours and consequently, expression was performed at 37°C. Various amounts of IPTG appeared to have no effect on amounts of bioproduct formation and IPTG was used at a concentration of 0.1 mM for expression studies.

The recombinant VacA protein was purified to near-homogeneity in a single step procedure by metal chelate affinity chromatography (IMAC). At a concentration of 10 mM imidazole, most host proteins were eluted from the affinity matrix and 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 ≈88 kDa (Figure 4, panel A). Subsequent Western blotting with anti-VacA rabbit polyclonal antiserum produced a single protein band at ≈ 90 kDa (Figure 4, panel B). The purity of VacA

in the elution fraction was estimated to be >95%; however, the yield of the recombinant VacA protein was relatively low (< 1 mg l^{-1} of bacterial culture).

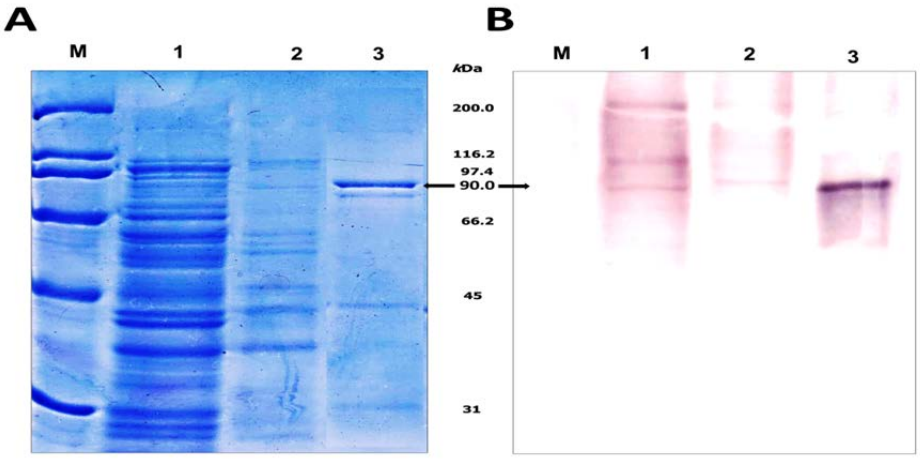

FIGURE 4: Purification of *H. pylori* VacA toxin by Ni^{2+} affinity chromatography. (**A**) SDS-PAGE (10% gel) analysis of *H. pylori* VacA toxin after IMAC purification. Lane M: broad range protein marker; lane 1, flow-through; lane 2, 10 mM imidazole wash; lane 3, 100 mM imidazole elution fraction of the proteins. (**B**) Corresponding Western blot of *H. pylori* VacA toxin after IMAC purification. Western blot profile of the gel as seen in (**A**) using anti-VacA antibody on 10% SDS-PAGE.Lane M: broad range protein marker; lane 1, flow-through; lane 2, 10 mM imidazole wash; lane 3, 100 mM imidazole elution fraction of the proteins.

3.1.2 Assay of VacA Apoptosis Activity

Apoptosis plays a major role in the pathogenic action of *H. pylori.* Previous studies have established that a correlation exists between the development of duodenal ulcer in *H. pylori* infection and the level of apoptosis in antral mucosal epithelium. Previous literature reports have also shown that the vacuolating toxin from *H. pylori* can increase the epithelial permeability of T84 and MDCK monolayers independent of its vacuolating activity and that *in vitro* infection of T84 intestinal epithelial cells with *H. pylori* can result in apoptosis. Moreover, earlier studies have shown that exposure to VacA induces the degradation of tight junctions in MDCK cells. The T84 and MDCK cell lines thus appear to represent interesting models to study the interaction of *H. pylori* with epithelial monolayers leading to apoptotic cell death.

Apoptotic activity of purified VacA was detected by incubation of T84 and MDCK cells for 24 hours in the presence of 150 µg ml^{-1} VacA protein and subsequent nuclear staining with DAPI analyzed by fluorescence microscopy. The nuclear (DAPI) staining revealed an increase in the number of nuclei which showed cytopathic symptoms typical of apoptosis such as chromatin condensation as well as DNA fragmentation (Figure 5).

Incubation of T84 cells with VacA 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 with a significance $p<0.05$.

VacA induced a marked apoptosis in MDCK cells with 25.4±3.06% increase when compared to 3.6±1.07% in control cells with P<0.001 significance. Similar to T84 cells, presence of VacA induced apoptotic symptoms in treated MDCK cells even at a higher degree. Figure 5 shows the percentage of T84 and MDCK cells exhibiting an apoptosis phenotype after incubation with VacA. Thus, MDCK cells appear to be significantly more sensitive to VacA than T84 cells (Figure 6).

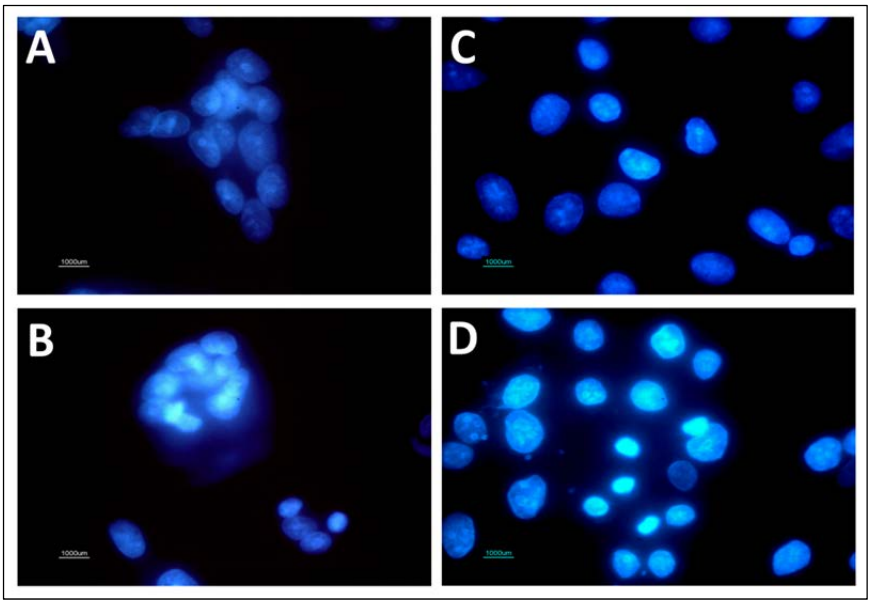

FIGURE 5: DAPI staining of intestinal epithelial cells (T84) and Madin-Darby Canine Kidney (MDCK) cells. (**A**) Control of untreated T84 cells showing normal nuclei. (**B**) T84 cells treated with VacA (150 µg ml^{-1}) cells showing chromatin condensation and DNA fragmentation. (**C**) Control of untreated MDCK cells. (**D**) MDCK cells treated with VacA (150 µg ml^{-1}), showing chromatin condensation and DNA fragmentation. DAPI was used at 1000-folddilution in buffer and the magnification of images is 1000×.

Differentiated T84 monolayers display high transepithelial resistance, a well-organized brush border, and the capacity to release IL-8 at the basal cell surface under adhesion with *H. pylori*. Previous studies demonstrated that stimulation of T84 monolayers with *H. pylori* soluble extracts has dramatic effects on epithelial physiological balance and integrity. Apical, but not basolateral, exposure of confluent monolayers of T84 cells to *H. pylori* extracts induces 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. It was previously proposed that during an infection with *H. pylori* the physiological gastric secretion in the antrum is impaired, eventually leading to the subsequent development of a duodenal ulcer. Although these findings suggest a correlation between the development of duodenal ulcer and the level of apoptosis in the antral mucosal epithelium, the precise molecular mechanism of the events leading to an accelerated disease development remain to be investigated.

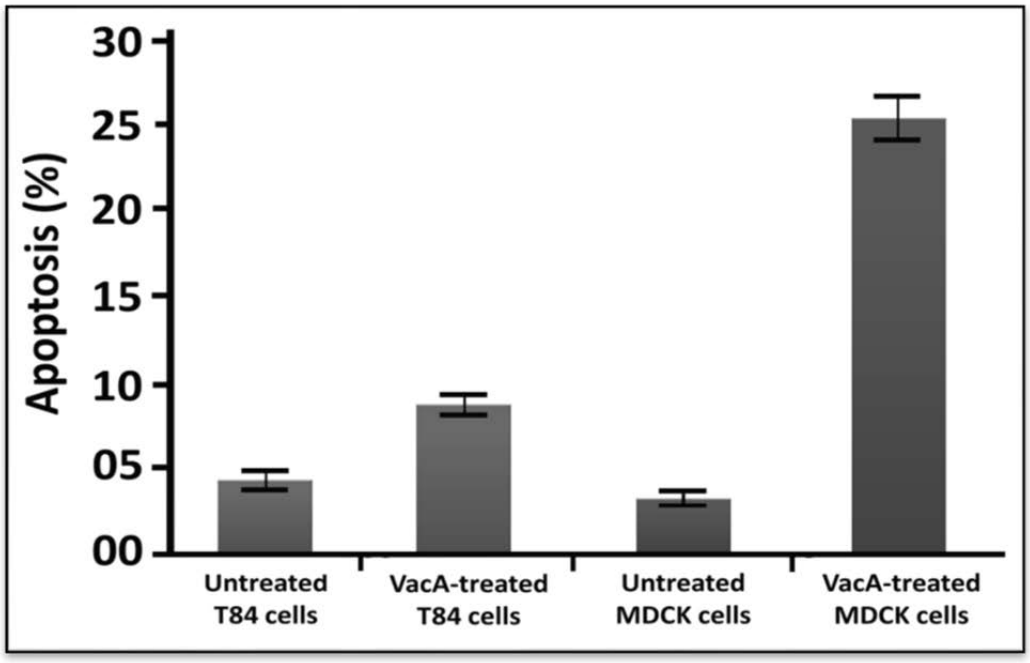

FIGURE 6: Percentage of apoptotic cells as observed after DAPI staining in T84 and MDCK cells. A mean of six pictures from each sample with at least 200 cells was counted for apoptosis-positive cells and the mean percentage of apoptotic cells wascompared with the untreated control. Error bars represent SEM.

In conclusion, we have successfully started the construction of a prokaryotic expression system for *vacA* gene from a clinical isolate and expressed and purified the active toxin. This is the first reported analysis of a VacA toxin from a patient sample obtained in Thailand. The purified VacA toxin was able to induce apoptosis in 2 epithelial cell lines. This study could serve as an entry point to future investigations on genetic diversity within the *vacA* gene and their role for the differential pathogenicity of different strains of *H. pylori.*

3.2 Expression Studies and Analysis of Cytotoxicity

Growth curves were recorded for recombinant *E. coli* cells containing the various types of VacA derivatives and the full-length p88 toxin. A rapid stall of cell growth following addition of IPTG was observed in case of the p33, p511 and p88 proteins, whereas clones expressing the p55 domain appeared to be less affected. Interestingly, all three cytotoxic domains contain the p33 N-terminal domain. The N-terminal region of p33 contains the GXXXG motif and mutations of the glycine residues abolish vacuolating activity. The GXXXG motif is probably required for helix dimerization during the process of membrane insertion and/or pore formation. Therefore, it seems tempting to speculate that cytotoxicity is probably caused by VacA pore-forming activity at the *E.coli* cell membrane. The gene sequences encoding p33, p55, p511 and the full-length p88 VacA toxin were designed to allow expression under control of the pTrc promoter with the aim to produce high yields of either soluble protein or in the form of inclusion bodies. The p55 domain showed relatively highest

levels of expression as soluble protein. In contrast, the p33, p511 and p88 did exhibit low levels of protein expression. The low expression yields of p33 were realized earlier in the literature and possible product toxicity of p33 was suspected as reason for the impaired cell growth. Cytotoxicity of p33 could represent a distinct feature of this single domain. Reduced growth rates for clones containing the pore-forming p33 domain were demonstrated after induction of expression. Clones with stalled growth were unsuitable for the efficient purification of the target proteins what forced us to consider alternative expression systems for the p33 domain. The difficulties associated with inefficient expression of the p33 domain could also provide a possible explanation for the delay in acquisition of a crystallographic structure of this protein.

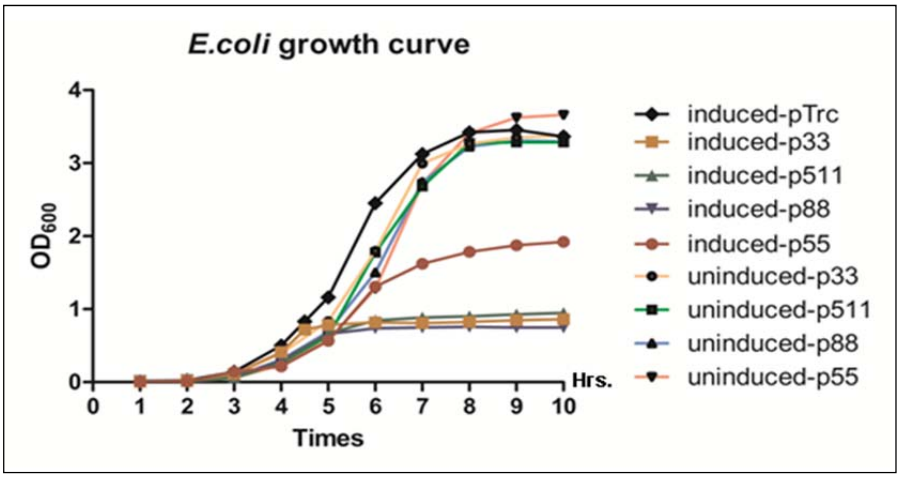

Figure 7: The *E.coli* growth curves under expression of VacA Shown are *E.coli* growth curves under induction with 0.1 mM IPTG and a no induction - control.

We attempted to express these proteins under control of the T7 promoter by subcloning of p33, p511 and p88 into the pPETBlue-2 plasmid vector. The pPETblue-2/VacA recombinant plasmids were expressed in Tuner™ (DE3) pLacI competent cells (Novagen, Germany) for up to three hours as suggested to achieving high yields in short time. Not surprisingly, the analysis of expression did not result in significantly higher levels of protein expression when compared to the pTrc expression system. Therefore, we concluded that protein yield did not depend on the type of promoter used. Recent work in our laboratory has shown that a full-length VacA expressed as an N-terminal fusion protein with mouse DHFR can be obtained at high yields thus suggesting that conformational features of the N-terminal VacA sequence are mainly responsible for adverse effects on host cell growth (*see below*). We may speculate that the N-terminal p33 domain of VacA is capable of disturbing the membrane integrity of *E. coli*, conceivably by the formation of ion pores in the cell membrane. It is well established that the action of VacA is relatively indiscriminative to the cell type and that a variety of biological membranes is susceptible to pore formation by VacA. To the best of our knowledge, VacA would be the first example of a channel-forming toxin which provokes a marked effect on a prokaryotic host cell. However, this model does not yet have a sound

explanation on how the toxin produced inside the cell would be interacting with the membrane in the absence of receptor–binding interactions.

3.2.1 Purification of VacA Domains

For all cloned VacA toxin derivatives, we attempted to establish a straightforward protocol for purification which was based on Ni-column affinity chromatography (IMAC) followed by gel-filtration chromatography. The procedure, however, was found to be efficient only for the isolated p55 domain. The yield of purified p55 was unexpectedly high and estimated to approximately 10 mg/l cells culture, whereas other the domains could not be efficiently purified to near-homogeneity due to low level expression resulting in a high undesired background of contaminating proteins. The purification of N-terminal tagged p55 by Ni-affinity column chromatography, ion exchange and gel filtration chromatography was useful to produce sufficient amounts for subsequent crystallization. The p55 we were using was conjugated with a C-terminal 6×His tag to prevent possible functional disturbances particularly in the putative oligomerization region at the N-terminus. The p33 domain was reported earlier not to yield a soluble protein upon expression. Inclusion bodies produced by this method would require lengthy procedures to prove functional activity after refolding.

3.3 Construction and Assay of a VacA -DHFR Fusion Protein

The sequence of VacA Thai clinical isolate (s1/m2 strain) (GenBank accession number KC529337) was subjected to codon optimization and the gene was custom synthesized as fusion protein with DHFR by TOP GENE©, Canada. In the project previously described, our laboratory cloned and expressed VacA(thai) fused to the 6×His tag at the N-terminus, however, achieved only a relatively low expression. Moreover, we have observed that host cells expressing recombinant VacA proteins containing the p33 portion exhibited permanently decreased and stalled growth upon induction of expression. The VacA gene was fused to DHFR at the N-terminus for further detection and purification. DHFR offers advantages not only in terms of efficiency of expressing the fusion protein as a soluble form but also in stabilizing unstable polypeptides and facilitating purification of the expressed protein by means of methotrexate-bound affinity chromatography and by making use of enzyme activity. The restriction sites *Nco*I and *Psi*I were placed on each side of a DHFR-VacA fusion protein for subcloning into the pTrc His2A vector (Invitrogen). To efficiently isolate the VacA full-length (p88) after purification of the fusion protein, a thrombin cleavage site was placed between VacA and DHFR. The fusion protein was then subcloned into the pTrcHis2A vector.

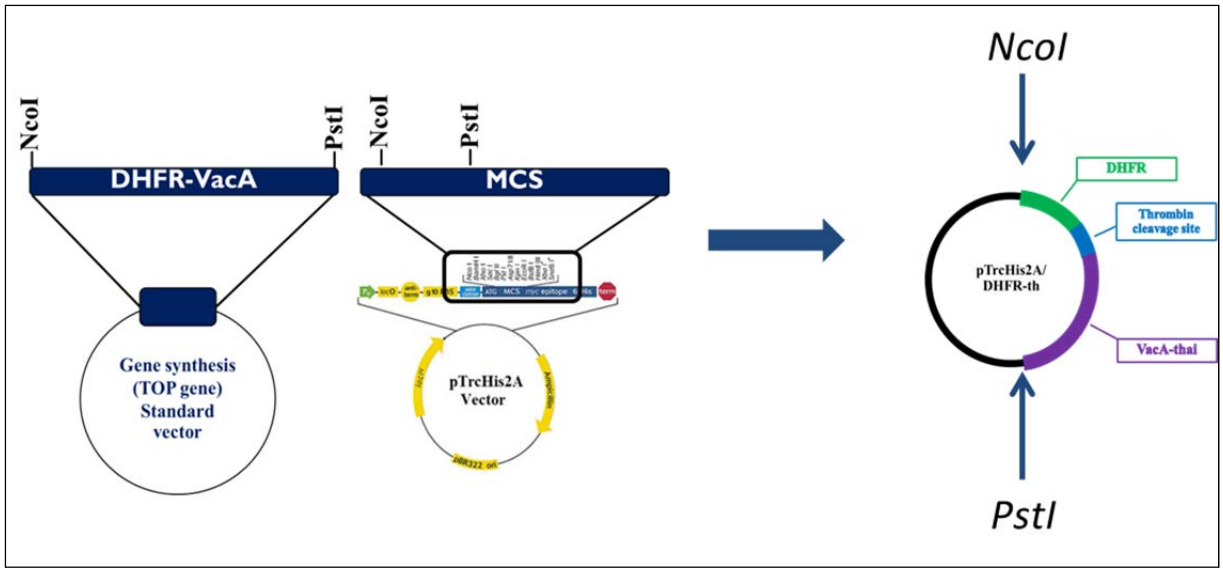

Figure 8: The recombinant protein DHFR-VacA was synthesized byTOP GENE® Canada. The restriction sites used for cloning were *Nco*I and *Pst*I. The gene was then subcloned into pTrcHis2A vector (Invitrogen).

The recombinant DHFR-VacA protein was expressed upon induction of biosynthesis with IPTG, and several parameters (temperature, induction time, IPTG-concentration) were optimized to achieve the maximum expression of the protein in the cell lysate soluble fraction. The expression profile of DHFR-VacA can be seen in figure 9. Expression induction with 0.1 mM IPTG at OD 0.5-0.6 in SOB media for 1 hr at 37 ˚C gives the highest quantity of soluble fusion protein.

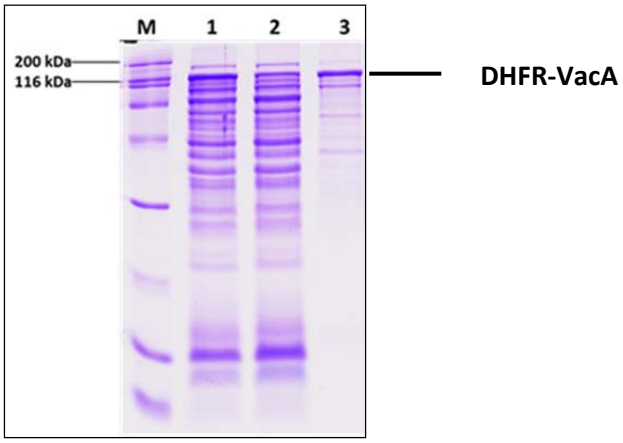

Figure 9: Comassie blue-stained 10% SDS-PAGE gel of pTrc-VacA-DHFR (3 fractions): lane 1, molecular weight markers; lane 2, total protein lysate; lane 2, soluble fraction; lane 3, inclusion bodies.

Antisera with specificity towards VacA, DHFR and the His-tag were used to evaluate the identity of expressed protein (figure 10).

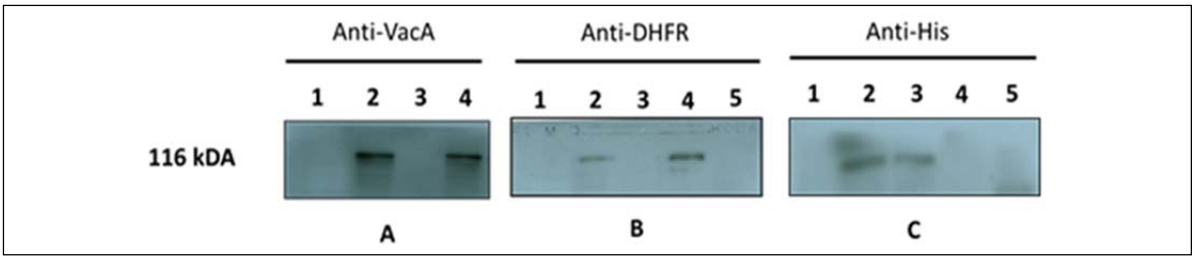

Figure 10: Western Blot analysis of pTrc-VacA-DHFR protein using different antibodies: **A**. Anti-VacA; exposure time 5 seconds; lane 1, markers; lane 2, cell lysate; lane 3, soluble fraction; lane 4, inclusion bodies. **B.** Anti-DHFR; exposure time 5 seconds; lane 1, markers; lane 2, cell lysate; lane 3, soluble fraction; lane 4, inclusion bodies; lane 5- pTrcHis2A vector (control). **C.** Anti-His; exposure time 5 seconds; lane 1, markers; lane 2, cell lysate; lane 3, inclusion bodies; lane 4, soluble fraction; lane 5- pTrcHis2A vector (control).

The expression of tagged VacA as DHFR fusion protein was markedly enhanced when compared to previous constructs (data not shown). In particular, it appeared that the cytotoxic effects resulting in decelerated host cell growth were not observed during expression of the VacA-DHFR fusion protein. Therefore, it is conceivable that the N-terminal modification at the p33 domain would be conducive to conformational rearrangements abolishing product toxicity to the host cell. It is also possible that the optimization of codon usage contributed to the increased yields of the fusion protein during expression.

To closer analyze the contribution of the N-terminal DHFR fusion to product yield, we have designed specific primers to delete the DHFR tag from the N-terminus of p33. Expression profiles were then compared between N-terminal tagged VacA and N-terminal untagged VacA. The 6×His tag remained present in all constructs tested. Expression of VacA in the recombinant clones was markedly reduced upon removal of the N-terminal DHFR tag which shows the importance of this N-terminal tag in stabilizing and enhancing target protein expression. The soluble form of the VacA-DHFR fusion protein was used for purification by HPLC.

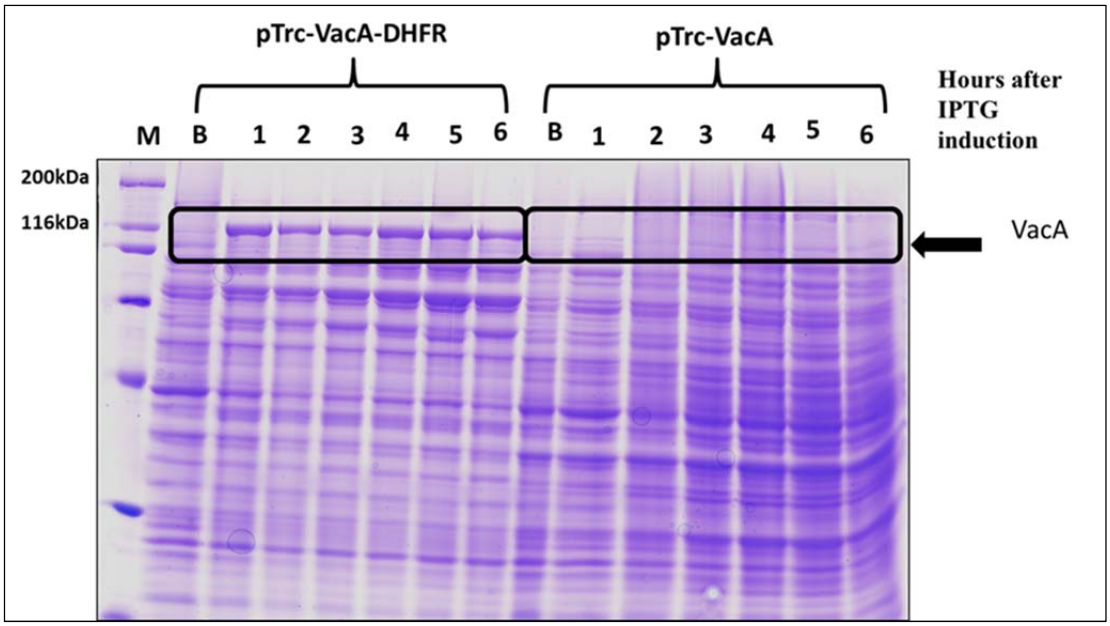

Figure 11: Comparison of expression profiles between DHFR-VacA-His and VacA-His constructs. M-Markers, B-before induction, 1-6: hours after IPTG induction. The target protein expression was markedly reduced with clones lacking the N-terminal DHFR tag. All clones were subjected to sequencing before expression.

Contrary to our expectations, purification by using MTX-Agarose (SIGMA) as affinity matrix was not successful as the target protein appeared to be unable to bind to the MTX column under a number of different experimental conditions (data not shown). Therefore, we have used the 6×His tag at the N-terminus as affinity tag for purification by Ni-NTA chromatography. The HPLC purification profile and SDS gel pictures for protein in the soluble fraction using Ni-NTA column (GE) can be seen in figures 12 and 13. Samples were injected at 1ml min^{-1}. Binding buffer was 20 mM HEPES, pH 7.4, wash buffer was 20 mM HEPES, pH 7.4 and elution buffer was 20 mM HEPES, 250 mM Imidazole, pH 7.4. Fractions of 1 ml min^{-1} were collected.

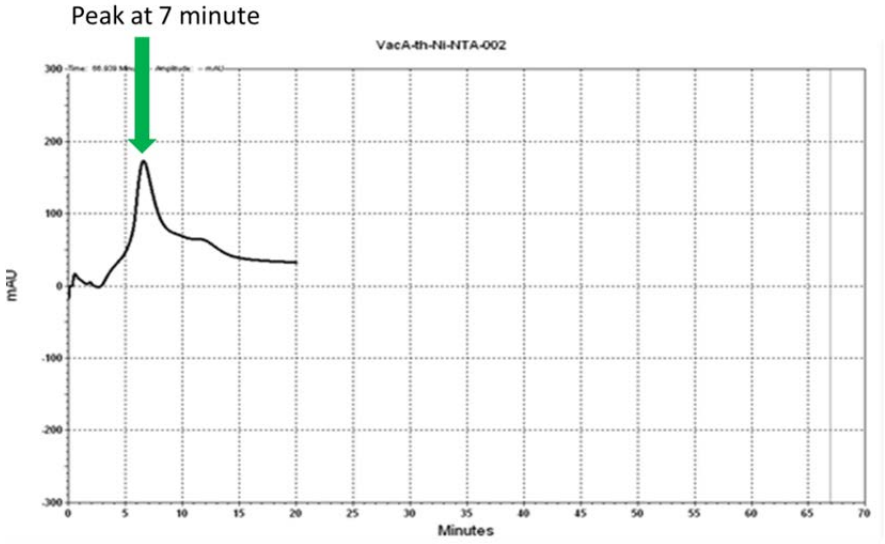

Figure 12: Elution profile of DHFR-VacA, target protein was eluted at 50 mM imidazole.

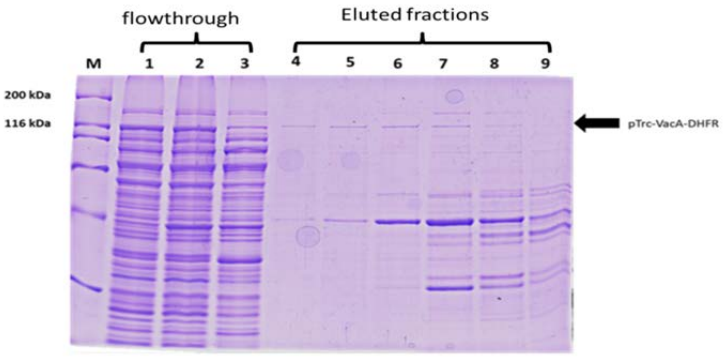

Figure 13: Comassie blue-stained 10% SDS-PAGE gel of fractions collected after Ni-NTA affinity chromatography. M, molecular weight markers; 1-3: flowthrough; 4-6: 50 mM imidazole elution fractions.

Although the DHFR-VacA protein contained in the soluble fraction of the cell lysate shows successful binding to Ni-NTA column, the final amount of protein after elution was too low for further studies. However, inclusion bodies washed with 20 mM Hepes, pH 7.4, ostensibly contain the target protein in a relatively pure form (figure 14).

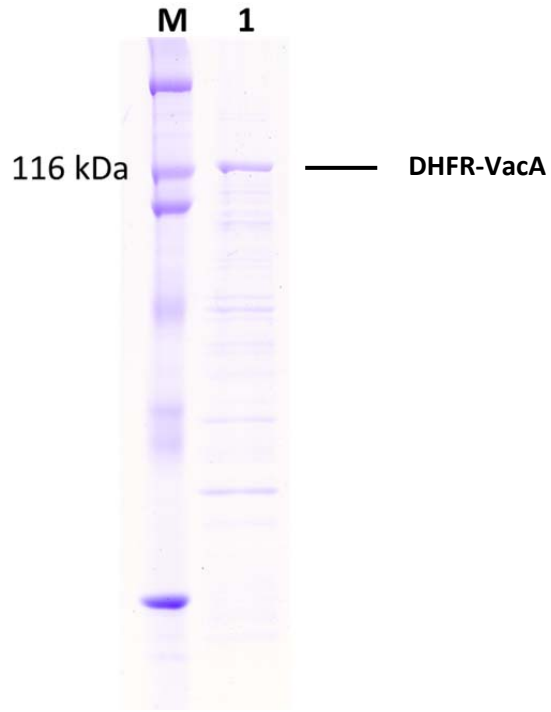

Figure 14: Comassie blue-stained 10% SDS-PAGE gel of DHFR-VacA after washed with 20 mM HEPES, pH 7.4.M, markers; 1 washed inclusion bodies.

The washed fractions were pooled and used for HPLC purification by Ni-NTA chromatography. The chromatography profile and corresponding SDS gel can be seen in (figures 15 and 16). Sample was injected at 1 ml per minute. Binding buffer was 20 mM HEPES,

pH 7.4, wash buffer was 20mM HEPES, pH-7.4 and elution buffer was 20 mM HEPES, 250 mM Imidazole, pH 7.4. Fractions of 1 ml were collected per minute.

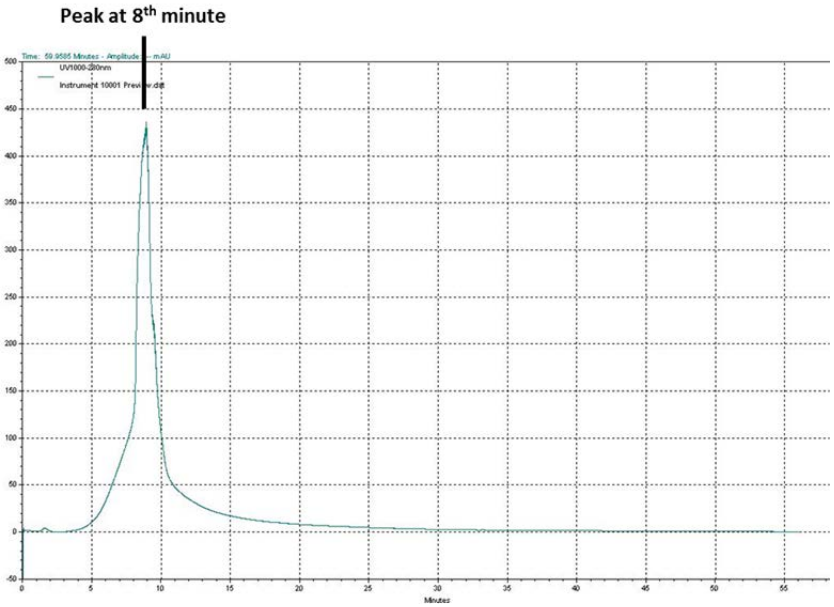

Figure 15: Elution of DHFR-VacA, elution of target protein at 250 mM imidazole.

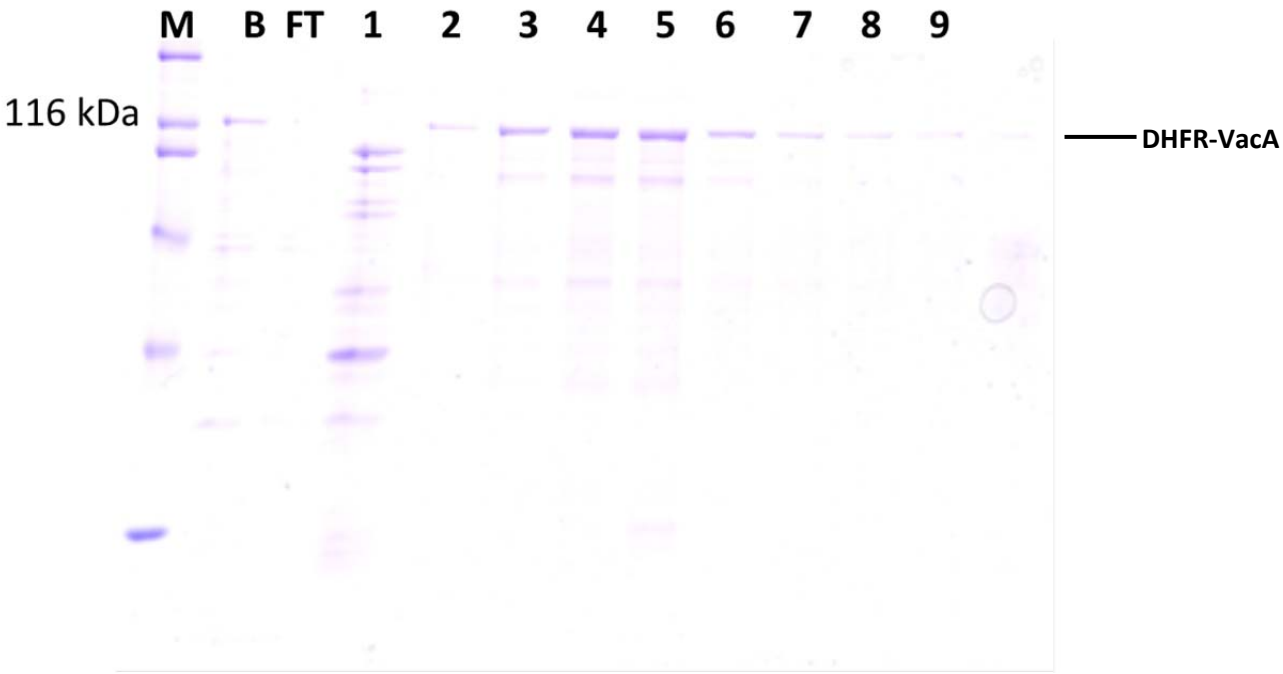

Figure 16: Comassie blue-stained 10% SDS-PAGE gel of fractions collected after Ni-NTA affinity chromatography. M, molecular weight marker; B, before injection onto the Ni-NTA column; FT, flowthrough; 1-9: 250 mM imidazole elution fractions.

Purified fractions from HPLC were pooled and dialyzed to remove imidazole. Proteins were first dialyzed against 20 mM HEPES, 150 mM NaCl, pH 7.4 and subsequently against a 100-fold volume of 10 mM potassium acetate, pH 4.5, 150 mM NaCl. Liposomes were

prepared in PE: PC: cholesterol at a ratio of 10: 10: 1, and concentrations of liposomes were determined by a spectrofluorometric assay using Na_2HPO_4 as a standard. Liposome concentration within the assay mixture was estimated to 10 μM; the protein sample was used at the following amounts: 1 μM and 8 μM in HEPES pH 7.4; and 40 nM in acetate buffer pH 4.5. A relatively low concentration of protein was used at pH 4.5 because acid or alkali activation of VacA was reported earlier to greatly enhance binding to mammalian cell lines or lipid bilayers. Sulforhodamine B was used as an indicator dye to measure the release of the fluorescent reporter dye during membrane disintegration of liposomes caused by membrane channel formation of p33. The following formula was used to calculate the percentage of fluorescence recovery after addition of DHFR-VacA in selected concentrations followed by addition of triton-X to reach 100% release of dye from liposomes:

$$F_t = (I_f - I_o) / (I_{max} - I_o) \times 100$$

F_t = %age of fluorescence recovery; I_o = Initial fluorescence; I_f = fluorescence observed after adding toxin; I_{max} = total fluorescence observed following addition of Triton-X100.

At low pH VacA was able to form pores in liposomes (table 1) leading to substantial dye leakage from the liposomes. This would be in agreement with earlier reports by *Tombola et al.* and *Yahiro et al.* having shown that VacA activity is substantially increased in the presence of highly acidic or alkaline conditions.

	HEPES buffer, pH-7.4		**Acetate buffer, pH-4.5**
DHFR-VacA	**1uM**	**8uM**	**40nM**
% of fluorescence recovery	4.76%	1.77%	92.5%

Table 1: Percentage of fluorescence recovery after addition of DHFR-TVD.

Finally, we attempted to determine DHFR enzymatic activity by a spectrophotometric assay of the VacA-DHFR fusion protein using a DHFR assay kit (SIGMA). The assay is based on the conversion of dihydrofolate to 5,6,7,8-tetrahydrofolate (THF) utilizing NADPH as cofactor. Activity of DHFR was calculated following the following formula:

$$\text{Units/mg P} = \frac{(\Delta\text{OD/min(sample)} - (\Delta\text{OD/min(blank)} \times \text{d}}{12.3 \times \text{V} \times \text{mg P/ml}}$$

ΔOD/min blank: ΔOD/min/min(blank) for the blank, from the spectrophotometer readings; ΔOD/min(sample): ΔOD/min-for the reaction, from the spectrophotometer readings; 12.3- extinction coefficient (, mM^{-1} cm^{-1}) for the DHFR reaction at 340 nm; V- enzyme volume in ml (the volume of enzyme used in the assay); d- The dilution factor of the enzyme sample; mg P/ml: enzyme concentration of the original sample before dilution; Units/mg P: - specific activity in mmol/min/mg protein.

The measured DHFR activity was calculated to 14.22 μmol/min/mg protein. MTX at a final concentration of 50 nM failed completely to block DHFR activity of VacA-DHFR, suggesting that DHFR is contained in an enzymatically non-active form in the fusion protein. This correlates with the observation that VacA-DHFR exhibits no detectable binding to the MTX affinity column, a finding which would suggest that the active site of DHFR is conformational masked in the fusion protein. We are currently employing further experiments to elucidate the mechanism by which the VacA-DHFR protein is capable to induce dye release from artificial liposomes.

CONCLUSIONS

1. We described the first isolate of *H. pylori* vacuolating cytotoxin VacA from a clinical sample in Thailand. The gene was sequenced and the VacA phenotype was identified to s1/m2. Subsequently a procedure for the overexpression and purification of recombinant VacA was developed. The sample was assayed for biological activity through the induction of apoptosis using 2 epithelial cell lines. We were able to demonstrate that the VacA Thai isolate was able to induce apoptosis in both cell lines as revealed by nuclear staining.

2. A detailed analysis of expression indicated adverse effects of VacA full-length (p88) and subdomains such as p33 and p511 containing the p33 moiety on host cell growth. The effect was less prominent with the p55 domain which could also be efficiently purified. The use of an alternative expression system (pET Blue) did not lead to improved protein yields.

3. As possible approach to overcome the expression barriers seen with VacA clones, a fusion protein consisting of VacA N-terminally linked to DHFR was constructed and successfully expressed. Protein yields were substantially improved and the protein could be purified to >95% purity. The VacA-DHFR fusion protein shows activity in a fluorescence dye-release assay using artificial liposomes.

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5. OUTPUT

1. Junaid, M., Al-Gubare, S., Yousef, M., Na Ubol, M., Leetachewa, S., Muanprasat, C., Angsuthanasombat, C., Chaicumpa, W., Ali, N. and **Katzenmeier, G.** (2014). Sequence and apoptotic activity of VacA cytotoxin cloned from a *Helicobacter pylori* Thai clinical isolate. *BioMed Research International* http://dx.doi.org/10.1155/2014/398350 **(impact factor 2.880)**

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Research Article

Sequence and Apoptotic Activity of VacA Cytotoxin Cloned from a *Helicobacter pylori* Thai Clinical Isolate

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Th vacuolating cytotoxin VacA produced by *Helicobacter pylori* induces the formation of large cytoplasmic vacuoles in host gastric epithelial cells as well as a release of cytochrome C from mitochondria resulting in cell apoptosis. Considerable sequence diversity in VacA relating to different degrees of disease severity is observed with clinical samples from a multitude of geographic places. In this study we describe expression in *Escherichia coli*, purific tion to homogeneity and *in vitro* assay of its apoptotic activity of a VacA toxin from a *H. pylori* isolate of a Th i patient with gastrointestinal lymphoma. Sequencing revealed that the deduced amino acid sequence of the cloned Th i isolate VacA is similar to *H. pylori* s1/m2 type strains. The percent sequence similarity to the model strain 60190 was lower due to the presence of extra amino acids in the mid (m) region. Th purifie VacA toxin exhibited signific nt apoptotic activity on both T84 and MDCK epithelial cell lines, as revealed by DAPI staining, whereby the observed activity was signific ntly higher on MDCK cells. These findings could relate to a modulation of VacA activity on host cells in the Th i isolate-VacA toxin that may differ from those of the model strain.

1. Introduction

Helicobacter pylori is a Gram-negative spiral-shaped bacterium, which causes serious chronic infectious diseases that damage gastric structure, and function and lead to gastric atrophy, peptic ulcer disease, gastric adenocarcinoma and primary gastric cell lymphoma [1, 2]. About half of the world's population is infected with *H. pylori* making this organism the most prevalent bacterial pathogen which is known to date [3, 4].

Th vacuolating cytotoxin A (VacA) is one of the major virulence factors released by *H. pylori*. VacA targets not only epithelial cells, but also cells of the immune system where it induces immunosuppression [5]. Th *vacA* gene is present in all strains and encodes a ~140-kDa protoxin [6] which upon proteolytic processing produces the mature ~90 kDa (~821 amino acids) toxin [7]. Th mature toxin can undergo proteolytic cleavage into fragments of 33 and 55 kDa which represent 2 domains of VacA [8–10]. Th p33 domain is involved in membrane insertion and ion-channel formation, whereas the p55 domain has a role in receptor binding and toxin oligomerization [11, 12]. Two receptor-like protein tyrosine phosphatases, RPTPα and RPTPβ, were identifi d as cellular receptors involved in VacA uptake [13, 14].

The production of cytotoxin activity can vary considerably among *H. pylori* strains due to extensive sequence

polymorphisms in the *vacA* gene [15]. Th most variable region corresponds to a ~800-bp sequence located in the middle of the p55 domain ("mid" or "*m*" region). Various *m* sequences have been grouped into two families of alleles, *m*1 and *m*2, whereby strains carrying the *m2* phenotype appear to have limited vacuolating activity [16]. Additional sequence variations are located in the "*s*" region which includes the N-terminal signal sequence of the mature VacA toxin [17]. Two main allelic families are recognized, designated *s*1 and *s*2. All possible combinations of these polymorphic regions (*s*1/*m*1, *s*1/*m*2, *s*2/*m*1, and *s*2/*m*2) have been detected in clinical isolates of *H. pylori*, although *s*2/*m*1 forms occur rarely. Strains containing a type *s2* allele reportedly are unable to induce vacuolation with specific cell types [17].

A third polymorphic site within the *vacA* sequence was recently identifi d and designated "*i*" (intermediate) region [18]. Th "*i*" region is located within the p33 domain and 2 types, *i*1 and *i*2, were commonly found in clinical isolates of *H. pylori*. Vacuolation assays demonstrated that vacuolating activity of *s*1/*m*2 VacA was dependent on the presence of the "*i*" region, thereby suggesting a pivotal role for VacA activity.

Vacuolating cytotoxin purifie from *H. pylori* causes mitochondrial damage leading to apoptotic cell death and it was speculated that differences in gastric mucosal cell apoptosis among *H. pylori*-infected persons may be caused by differences in the structure and activity of VacA allelic forms [19].

In the present study, we constructed an expression system to obtain sequence information and to analyze the apoptotic activity of a *H. pylori* VacA toxin isolated from a Tha patient with gastric lymphoma ("*Th i isolate*"). We were seeking to understand how genetic differences and sequence polymorphisms between *m*1 and *m*2 types of *vacA* alleles are related to the Thai isolate VacA and how these could possibly influence the apoptotic activity of this protein.

2. Materials and Methods

2.1. Cloning and Sequence Analysis of vacA Gene from a Thai Patient Isolate. *H. pylori* was cultured from a gastric biopsy sample from a Thai patient with diagnosed gastric lymphoma (62-year-old male) obtained at Vichaiyut Hospital, Bangkok, Thailand, on the surfaces of horse blood agar plates (10% horse blood in Casman agar base (BBL Microbiology Systems, Cockeysville, MD)), which were incubated in an atmosphere of 5% oxygen and 10% carbon dioxide for 72 h at 37°C for up to 5 days [20–22]. Chromosomal DNA was extracted as described previously [23] and used as template for PCR amplification of *vacA*. The following pair of primers derived from the sequence of *H. pylori* model strain 60190 was used:

SJ_*vacA*1F: 5′-CATGCCATGGCCTTTTTTACAACC-GTGATCA-3′ (underlined sequence represents the *Nco*I restriction site) and SJ_*vacA*821R: 5′-TGCACTGCAGAG-CGTAGCTAGCGAAACGC-3′ (underlined sequence represents the *Pst*I restriction site). Oligonucleotides were purchased from Sigma-Aldrich, Singapore.

PCR was performed with *Pfu* DNA polymerase (Fermentas Life Sciences, USA) using a thermal cycler GeneAmp PCR system Model 2400 (Perkin Elmer Cetus, USA), with predenaturing (98°C, 2 min), 25 cycles of denaturing (98°C, 0.1 min), annealing (65°C, 0.3 min), extension (72°C, 1.3 min), and final extension (72°C, 7 min). Th resulting PCR products were subjected to agarose gel electrophoresis, and the 2.5-kb fragment representing the mature full-length *vacA* toxin gene (p88) was excised and purifie by QIAquick gel extraction kit (QIAGEN, Germany).

Th purifi d *vacA* PCR product and pTrcHisA (4.4 kb) vector (Invitrogen, USA) were digested with restriction enzymes *Nco*I and *Pst*I and purifie by QIAquick purific tion kit. Insert DNA and pTrcHisA vector were combined at a 10 : 1 molar ratio in a ligation reaction containing 1× ligation buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 25% (w/v) polyethylene glycol 8000) and five units of T4 DNA ligase (Gibco BRL, USA) in a fin l volume of 20 μL and incubated overnight at 14°C, resulting in pTrcHisA/VacA carrying a C-terminal $(His)_6$ tag and a translation initiation methionine residue at the N-terminus. Th correct sequence of the recombinant pTrcHisA/VacA construct was verified by restriction digestion and DNA sequencing analysis (Macrogen Inc., South Korea).

2.2. Expression and Purification of the Recombinant VacA Protein. Recombinant plasmid containing the *vacA* insert (pTrcHis2A/VacA) was transformed into *Escherichia coli* TOP10 (GIBCO BRL, USA). To determine optimum conditions for the expression of the VacA protein, the *E. coli* cells were incubated at 37°C in one liter LB medium containing 100 μg mL^{-1} ampicillin. At OD_{600} = 0.5, expression was induced by isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM final concentration). Protein biosynthesis was assayed at different incubation times (0 h, 1 h, 4 h, and 6 h and overnight) and temperatures (18, 25, 30, and 37°C). Cells were harvested by centrifugation (6000 ×g, 4°C, 10 min) and the pellet was resuspended in 30 mL lysis buff r (0.1 M Tris-HCl, pH 7.5, 0.3 M NaCl, 0.25 mg mL^{-1} lysozyme, 10 mg mL^{-1} DNase, and 5 mM $MgCl_2$). Cells were lysed on ice by sonication using an Ultrasonic Processor XL (Misonix Inc., USA). Th cell lysate was subjected to centrifugation (15000 ×g, 4°C, 30 min), insoluble material was pelleted by centrifugation (15000 ×g, 4°C, 20 min), and the soluble fraction was filtered through a 0.22 micron pore-size filter (Pall Corporation, USA).

Histidine-tagged VacA protein was purifie by immobilized metal ion affi ty chromatography (IMAC), using nickel-sepharose HisTrap HP 5-mL columns (GE Healthcare, Sweden). The columns were preequilibrated with 5–10 column volumes of sample buffer containing 100 mM Tris-HCl, pH 7.5, 300 mM NaCl, and the sample was loaded at a fl w rate of 1 mL min^{-1}, using a FPLC pump (AKTA FPLC system, GE Healthcare). Th column was washed with 10 column volumes of degased washing buffer (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10 mM imidazole) and the protein was eluted with elution buffe (100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 100 mM imidazole) at fl w rate of 1 mL min^{-1}. Elution

was monitored by absorbance at 280 nm using a UV detector (AKTA FPLC system, GE Healthcare) and fractions of 2 mL were collected. From each fraction, 20 μL were analysed on SDS-PAGE (10% gel). Western blotting was performed using anti-VacA rabbit antiserum (Invitrogen, USA) at 1:2000 dilution with alkaline phosphatase color detection.

Fractions containing VacA were desalted by stepwise dialysis at 4°C by using SPECTRA/POR dialysis membranes (6–8 kDa MWCO) (Spectrum Medical Industries, Inc. MA, USA), against three batches of a 100-fold sample volume buffer A (100 mM Tris-HCl, pH 7.5, 200 mM NaCl), one batch of a 100-fold volume buffer B (100 mM Tris-HCl, pH 7.5, 100 M NaCl), and one batch of a 100-fold volume of buff r C (50 mM Tris-HCl, pH 7.5). Purifie VacA was further concentrated to 1.0 mg mL^{-1} by centrifugal filter devices (Centricon 15mL, 30-kDa MWCO, Millipore, USA) at 4°C. Protein concentrations were determined with a Bradford protein microassay (Bio-Rad, USA) using bovine serum albumin (Sigma, USA) as calibration standard. Samples were used immediately or stored in 50 mM Tris-HCl, pH 7.5, 50% (v/v) glycerol, at −20°C.

2.3. Assay of VacA Apoptotic Activity. Human colonic adenocarcinoma (T84) and Madin-Darby canine kidney (MDCK) epithelial cells, originally purchased from the American Type Culture Collection (Manassas, USA), were grown as monolayers in a 1:1 mixture of Dulbecco's modifi d Eagle's Medium/Nutrient Mixture F-12Ham (DMEM-Ham) (Sigma, USA) supplemented with 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, and 5% foetal bovine serum. Th culture medium was replaced every other day. Monolayers were subcultured by trypsinization with 0.25% (w/v) trypsin and 5.3 mM EDTA in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) and plated on coverslips at a density of 10^5 cells mL^{-1} to study apoptosis caused by VacA. T84 and MDCK cells were seeded in 75 mL fl sks at 37°C in a humidifie atmosphere of 5% CO_2.

Apoptosis of the colonic epithelium was assessed using a nuclear stain, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma, USA). Cells were placed on coverslips at a density of 5×10^5 cells per well in DMEM medium and kept at the incubator. At the time of experiment, old medium was removed and cells were incubated with either 150 μg mL^{-1} VacA-containing medium or serum-free medium (as a control) for 24 h. Cells were washed in PBS and fixed with 60 μL of 4% paraformaldehyde for 8 min at 4°C followed by three-time washing with 60 μL of 1× PBS. Cells were incubated with 60 μL of 0.1% Triton X-100 for 10 min and nonspecifi binding sites were blocked by adding 2% skimmed milk powder for 1h. Cells were washed in PBS three times, 10 min each. Finally, cells were stained with 50 μL of DAPI (1:1000 dilution in blocking buffer) for 15 min and mounted using 50% glycerol. Th signals were visualized at wavelength 350/460 nm (excitation/emission) by using a fluorescence microscope (model IX71, Olympus, Japan). Binding of DAPI to dsDNA produced a ~20-fold fluorescence enhancement and a minimum of 200 cells was counted for each sample and the control by visual inspection of microscopic images. Results are the mean of three independent experiments and data are represented as mean ± standard error of the mean.

3. Results and Discussion

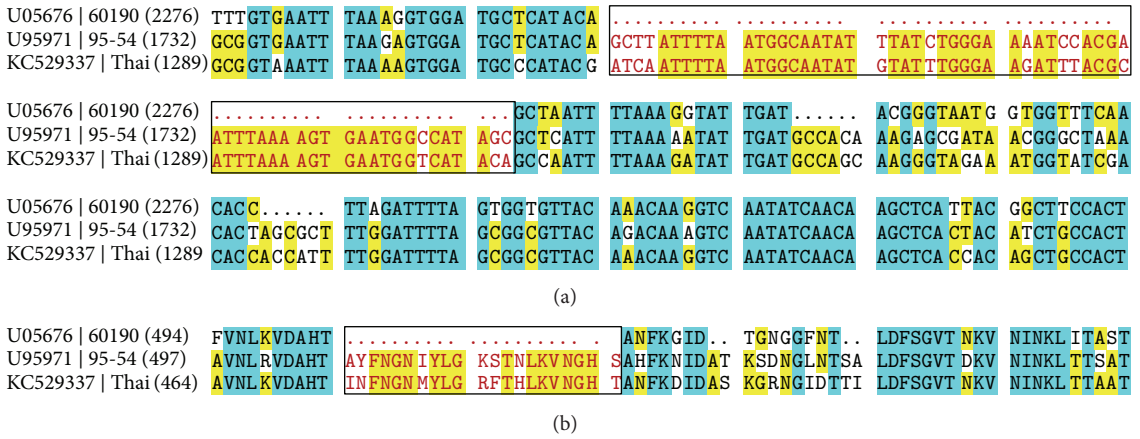
3.1. Sequence and Allele Type of VacA Toxin Gene from Th i Patient Isolate. Th 2.5-kb *vacA* gene sequence encoding the mature VacA toxin was obtained by PCR amplific tion using *H. pylori* genomic DNA extracted from a patient isolate as template. VacA is the product of a single gene that encodes a 140-kDa precursor protein which, upon proteolytic removal of the N-terminal signal sequence, yields the mature 88-kDa toxin containing alanine as N-terminal amino acid residue [7]. An N-terminal methionine residue was included to ensure proper initiation of translation in the recombinant *E. coli* host and a C-terminal $(His)_6$ sequence was added for purific tion purposes.

The construct was analyzed by nucleotide sequencing and alignment with sequences of *H. pylori* strain 60190 (*s*1/*m*1 allelic type) *vacA* sequence (GenBank accession number U05676), and strain 95-54 (*s*1/*m*2 allelic type) (GenBank accession number U95971) (Figure 1). Th sequence of the Th i isolate was deposited in GenBank at accession number KC529337. Sequence analysis revealed that the sequence of the cloned Thai *vacA* gene encompasses 2577 bp encoding a mature VacA toxin of 859 amino acid residues. Th sequence of the Thai isolate *vacA* gene is identical to a recently described strain isolated in China (CHN1811a; GenBank accession number AF050326). It shows ~82% identity to the toxigenic *H. pylori s*1/*m*2 strain 95-54 and identity to the *s*1/*m*1 model strain 60190 is only ~53% (Figure 1(a)). *H. pylori* strain 95-54 encodes an unusually large VacA toxin of 1323 amino acid residues and has been shown to possess an *s*1/*m*2 allelic phenotype [17].

Comparison of deduced amino acid sequences showed a well conserved p33 domain among the surveyed strains containing *m*1 and *m*2 alleles and a diversific tion region which differentiates *m*1 and *m*2 allelic types. Residue D455 is located at the beginning of the mid region in *H. pylori* strain 60190 and it was proposed that this area represents a receptor binding site common to all *m*1 and *m*2 strains [24].

Th most notable difference between *H. pylori* strain 60190 and the Thai strain is the sizable insertion of 21 amino acid residues in the middle region making the Th i isolate an *m*2 type strain (Figure 1(b)). Th structural consequences of this insertion are not well understood at present. In particular, it is unclear whether the inserted sequence directly participates in receptor binding as both RPTPα and RPTPβ are recognized by the *m*2 type of *vacA* alleles [25].

Numerous studies have demonstrated a correlation between the allelic types of "*m*" and "*s*"regions and the occurrence of gastroduodenal diseases in humans; however, the pathophysiological mechanisms involved in these associations are characterized only to a limited extent [17, 26–35]. A study on the clinical relevance of VacA genotypes corroborated that VacA type *s*2 strains are rarely associated with peptic ulceration [17]. As regions of diversity in *vacA* alleles comprise an ample portion of the gene, structural



Figur e 1: Partial nucleotide sequence and deduced amino acid sequence in the *m* region of recombinant *vacA* toxin gene from Thai isolate (rVacA) aligned with sequences of *H. pylori* model strain 60190 (*s*1/*m*1) and strain 95-54 (*s*1/*m*2). (a) Nucleotide sequences identical in the 3 strains are in blue, and nucleotide sequences identical between 2 strains are shown in yellow. Th black box shows the insertion in the middle region of the Thai isolate *vacA* gene. (b) Amino acid sequences identical in the 3 strains are in blue, and amino acid sequences identical between 2 strains are shown in yellow. Th black box shows the insertion in the middle region of the Thai isolate *vacA* gene.

differences between type *m*1 and type *m*2 gene products could give rise to diff rences in cytotoxin phenotypes aff cting receptor recognition, internalization, and vacuolation. Cell-specifi binding has been attributed to differences in the *m*1 and *m*2 alleles. Strains encoding *s*1/*m*1 *vacA* genes typically produce VacA with cytotoxic activity on human cervical carcinoma HeLa cells, whereas *m*2-type VacA could induce vacuoles in primary cultured human gastric cell lines as well as nongastric epithelial RK13cells, but not in HeLa cells [36].

It is interesting to note that the third polymorphic determinant within the Thai isolate *vacA* sequence, the "*i*" region, shows marked differences to strain 95-54, whereas it shares greater homology to the other model strains. How the "*i*" region functions in the context of VacA allelic polymorphisms needs to be elucidated in future studies.

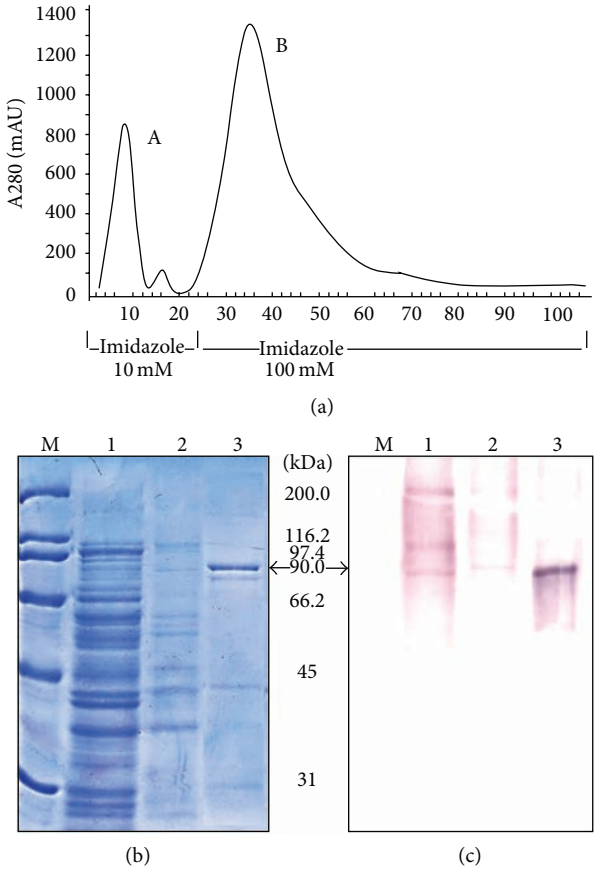
Currently, most *in vitro* studies utilize the s1/m1 type of VacA. Therefore, analysis of the biological effects of the s1/m2 type VacA as described herein could contribute to a better understanding of the relation between toxin activity and their allelic variations. We are currently working on a comparative analysis of toxin activity and structural differences from the s1/m1model strain 60190 and the s1/m2Th i isolate.

3.2. Expression and Purification of the Recombinant VacA Protein. Th recombinant plasmid pTrcHis2A/VacA directed expression of the VacA toxin in *E. coli* TOP10 upon induction with IPTG. Optimization of expression conditions was attempted to achieve increased levels of expression. Time-dependent expression of VacA was analyzed by SDS-PAGE of crude lysate and subsequent Western blotting with commercially available anti-VacA antiserum. It is interesting to note that growth of the recombinant stain carrying pTrHis2A/VacA ceased after addition of IPTG, and over a period of 14 h, no further increase in bacterial growth rate was observed, thereby suggesting cytotoxic effects of the recombinant VacA toxin on *E. coli* cell growth (data not shown). At lower temperatures (18–25°C), reduced amounts of recombinant VacA were detected after incubation for 14 h and, consequently, expression was performed at 37°C.

Th recombinant VacA protein was purifie to near-homogeneity in a single step procedure by metal chelate affi ity chromatography (IMAC). At a concentration of 10 mM imidazole, most host proteins were eluted from the affi ty matrix and recombinant VacA protein was eluted from the column with 100 mM imidazole as a single peak (Figure 2(a)). Elution fractions analyzed on SDS-PAGE revealed a major protein band at 90 kDa and another minor protein band at ~88 kDa (Figure 2(b)). Subsequent Western blotting with anti-VacA rabbit polyclonal antiserum produced a single protein band at ~90 kDa (Figure 2(c)). Th purity of VacA in the elution fraction was estimated to be >95%; however, the yield of the recombinant VacA protein was relatively low (<1 mg L^{-1} of bacterial culture).

3.3. VacA Apoptotic Activity. Apoptosis plays a major role in the pathogenic action of *H. pylori* [37]. Previous studies have established that a correlation exists between the development of duodenal ulcer in *H. pylori* infection and the level of apoptosis in antral mucosal epithelium [38]. Literature reports have also shown that the vacuolating toxin from *H. pylori* can increase the epithelial permeability of T84 and MDCK monolayers independent of its vacuolating activity and that *in vitro* infection of T84 intestinal epithelial cells with *H. pylori* can result in apoptosis [39–41]. Moreover, earlier studies have shown that exposure to VacA induces the degradation of tight junctions in MDCK cells [39]. Th T84 and MDCK cell lines thus appear to represent interesting models to study the interaction of *H. pylori* with epithelial monolayers leading to apoptotic cell death.

Herein, apoptotic activity of the purifie recombinant VacA toxin was detected by incubation of T84 and MDCK cells for 24 h in the presence of 150 μg mL^{-1} VacA protein



Figur e 2: Purific tion and Western blot analysis of VacA. (a) Purific tion of *H. pylori* VacA toxin by Ni^{2+}affi ty chromatography. Th column was washed with 0.1M Tris-HCl, pH 8.0, 0.3 M NaCl, 10mM imidazole (*peak a*). VacA toxin was eluted with elution buffer (0.1M Tris-HCl, pH 8.0, 0.3 M NaCl, 100 mM imidazole) (*peak b*). (b) SDS-PAGE (10% gel) analysis of *H. pylori* VacA toxin after IMAC purific tion. Lane M: broad range protein marker; lane 1: flow-through; lane 2: 10 mM imidazole wash; lane 3: 100 mM imidazole elution fraction of the protein. (c) Corresponding Western blot of *H. pylori* VacA toxin after IMAC purific tion. Western blot profile of the gel as seen in (a) using anti-VacA antibody on 10% SDS-PAGE. Lane M: broad range protein marker; lane 1: fl w-through; lane 2: 10mM imidazole wash; lane 3: 100 mM imidazole elution fraction of the protein.

and subsequent nuclear staining with DAPI analyzed by fl o-rescence microscopy. The DAPI-nuclear staining revealed an increase in the number of nuclei which showed cytopathic symptoms typical of apoptosis such as chromatin condensation as well as DNA fragmentation (Figure 3).

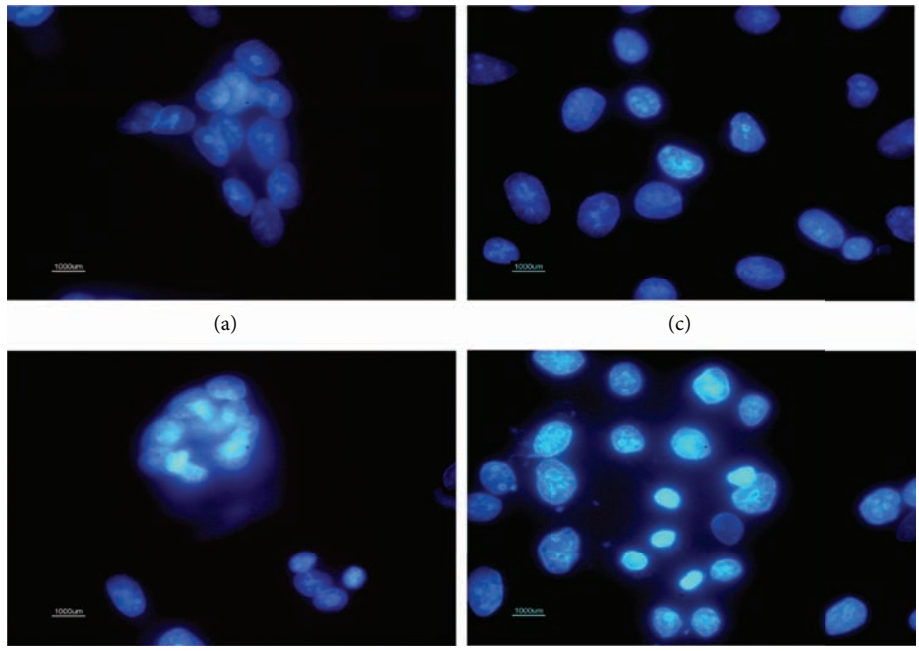
Incubation of T84 cells with VacA resulted in an increase in apoptosis of T84 cells with 8.2 ± 1.6% in VacA treated cells (mean ± SEM) compared with 4.2 ± 1.4% in the control cells with a signific nce $P < 0.05$ (Figure 4). VacA induced a marked apoptosis in MDCK cells with an increase of 25.4 ± 3.1% when compared to 3.6 ± 1.1% in control cells with $P < 0.001$ signific nce. Similar to T84 cells, presence of VacA induced apoptotic symptoms in treated MDCK cells even at a higher degree. Thus, MDCK cells appear to be significantly more sensitive to VacA than T84 cells.

An earlier study using HeLa cells suggested the existence of a nonapoptotic mechanism for Vac-induced cell death [42]. It is important to note that the experimental approach used herein does not allow conclusive differentiating between the induction of apoptosis or a programmed necrosis mechanism caused by VacA as proposed in a study using the gastric cell line AZ-521 [43]. While numerous reports have accumulated evidence that VacA-induced cell death involves the activation of caspases and the proapoptotic proteins Bax and Bak as well as the release of cytochrome C [44], Radin et al. suggested the existence of a necrotic pathway based on an observed release of LDH and the proinflammatory protein HMGB1 in VacA-treated AGS cells. It is noteworthy, however, that we have not observed an apparent rupture of the plasma membrane as typically seen with necrotic cells. It remains to be investigated in detail whether the cell-type specifi release of proinflammatory proteins by gastric epithelial cells is caused by pyronecrosis or an apoptotic mechanism of cell death.

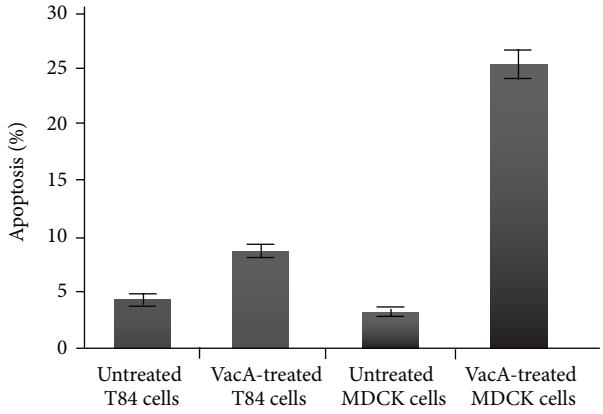
Differentiated T84 monolayers display high transepithelial resistance (TER) [45], a well-organized brush border, and the capacity to release IL-8 at the basal cell surface under adhesion with *H. pylori* [46]. Previous studies demonstrated that stimulation of T84 monolayers with *H. pylori* soluble extracts has dramatic effects on epithelial physiological balance and integrity [47]. Apical, but not basolateral, exposure of confluent monolayers of T84 cells to *H. pylori* extracts induces 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. It was previously proposed that during an infection with *H. pylori* physiological gastric secretion in the antrum is impaired, eventually leading to the subsequent development of duodenal ulcer [47]. Although these findings suggest a correlation between the development of duodenal ulcer and the level of apoptosis in the antral mucosal epithelium, the precise molecular mechanism of the events leading to an accelerated disease development remains to be investigated.

4. Conclusion

We have successfully established the construction of a prokaryotic expression system for *vacA* gene from a clinical isolate and purific tion of the biologically active toxin. This is the first reported analysis of a VacA toxin from a patient sample obtained in Thailand. The isolate represents an s1/m2 allelic type and purified VacA toxin was able to induce apoptosis in two types of epithelial cell lines. This study could serve as an entry point to future investigations on genetic diversity within the *vacA* gene and their role for the diff rential pathogenicity of diff rent strains of *H. pylori*.



Figur e 3: DAPI staining of intestinal epithelial cells (T84) and Madin-Darby Canine Kidney (MDCK) cells. (a) Control of untreated T84 cells showing normal nuclei. (b) T84 cells treated with VacA (150 μg mL^{-1}) cells showing chromatin condensation and DNA fragmentation. (c) Control of untreated MDCK cells. (d) MDCK cells treated with VacA (150 μg mL^{-1}) showing chromatin condensation and DNA fragmentation. DAPI was used at 1000-fold dilution in buffer and the magnific tion of images is 1000×.



Figur e 4: Percentage of apoptotic cells as observed after DAPI staining in T84 and MDCK cells. A mean of six pictures from each sample with at least 200 cells was counted for apoptosis-positive cells and the mean percentage of apoptotic cells was compared with the untreated control. Error bars represent SEM.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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