

MOLECULAR CHARACTERIZATION OF A SPHINGOMYELIN
BINDING SITE IN THE VACUOLATING CYTOTOXIN OF *H. PYLORI*

BY

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THESIS

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Abstract

The main determinants in toxin cell specificity are often interactions with surface receptors. It has recently been determined that the membrane phospholipid sphingomyelin (SM) is the primary receptor for the *Helicobacter pylori* vacuolating cytotoxin (VacA). While the cellular receptor has been identified, the molecular basis of SM recognition remains unknown. Molecular modeling of unrelated SM-binding proteins suggests that specific binding occurs through tryptophan rich aromatic clusters located in solvent exposed peptide loops between β -sheet domains. Structural comparison of these proteins with VacA led to the identification of a similar tryptophan containing aromatic cluster in the mid-region of the p55 subunit of VacA. Additionally, sequence alignment of this putative binding site exhibited sequence homology with the phosphorylcholine binding site of another SM-binding toxin. In this study we tested the effects of mutating each of the 11 tryptophan residues in VacA. While mutating each of the 8 tryptophan residues present in the auto-transporter p33 domain did not cause substantial attenuation in toxin activity, alanine substitution of 2 tryptophan residues within the putative binding site led to significant attenuation. Further, tryptophan to alanine mutation of W603, a residue present in the center of the putative binding site, was found to be attenuated in cell binding to human-derived AZ-521 gastric epithelial cells. Interestingly, a W603A mutation is also present within a *vacA* allele associated with a lessened propensity for gastric disease, decreased toxin binding, and differences in cell specificity. This thesis proposes a novel VacA-SM binding site and identifies a possible cause of allele associated differences in VacA pathogenicity.

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List of Abbreviations

Bc-SMase- *Bacillus cereus* sphingomyelinase
BCA- bicinechonic acid
BME- 2-mercaptoethanol
BSA- bovine serum albumin
BSFB- Bisulphite and sulfite free brucella
EGF- epidermal growth factor
EqII- Equinatoxin II
FBS- fetal bovine serum
GPI- glycosylphosphatidylinositol
Hepes- 4- 2-hydroxyethyl -1-piperazineethanesulfonic acid
i1- intermediate 1
i2- intermediate 2
IPTG- isopropyl- β -D-thio-galactoside
LB- lysogeny broth
m1- middle1
m2- middle 2
MEM- minimum essential media
PBS- phosphate buffered saline
PC- phosphatidyl choline
PE- phosphatidylethanolamine
PI- phosphatidylinositol
PI-PLC- phospholipase C
PMSF- phenylmethylsulfonyl fluoride
RTP- receptor tyrosine phosphatase
S1- signal sequence 1
S2- signal sequence 2
SM- sphingomyelin

SOC- super optimal broth with catabolite repression

StI- sticholysins I

StII- sticholysins II

Streptavidin HRP- streptavidin conjugated horse radish peroxidase

TBE- tris-borate-EDTA

TMB- 3 3' 5 5'-tetramethybenzidine

VacA- Vacuolating Cytotoxin

WHO- World Health Organization

Chapter 1: Introduction

This thesis discusses the characterization of the molecular bases of sphingomyelin binding by the Vacuolating cytotoxin (VacA) of *H. pylori*. As with any toxin, the first step in VacA intoxication is binding to the plasma membrane of eukaryotic cells (Garner et al. 1996). Along with providing a means of cell entry, the mechanism of cell binding plays a key role in determining cell sensitivity, tissue tropism, mode of internal transportation, and toxin activity, making it an important determinant in toxin pathogenicity and overall cellular outcome. VacA is of particular interest as a model system for studying such interactions as a result of the diverse effects it has on varying cell types. In addition to this multifunctionality, VacA is the only known bacterial toxin to utilize the phospholipid sphingomyelin (SM) as a receptor, allowing for the investigation of a novel mechanism of toxin cell binding (Cover et al. 2005, Gupta et al. 2010). Through the investigation of these interactions, a more thorough understanding of the structural motifs involved in binding can be achieved and subsequently applied to analogous systems. In this thesis, the importance of VacA cell binding as a model system and as a primary factor in *H. pylori* pathogenicity will be discussed.

Infectious Disease

Infectious diseases are among the leading causes of death worldwide, with the majority of deaths occurring in developing countries. This problem is of growing severity due to widening transportation networks (Tatem et al. 2006) and the spread of antibiotic resistance (Versluis et al. 1992, Davis et al. 1994, Zhang et al. 2011), which together have contributed to the broader and more rapid spread of pathogens which are resistant to pharmaceutical agents that were once most effective. These problems are of particular concern in gastrointestinal bacteria due to the

increased potential for horizontal gene transfer between bacteria in areas simultaneously colonized by multiple species (Devirgiliss et al. 2011, Megraud et al. 2004, Dorer et al. 2010). The growing demand for an alternative to antibiotics has increased the need for research which will investigate the pathology of human pathogens and identify potential targets for novel therapeutic drugs.

Drug Design

Understanding the fundamental basis of bacterial toxin binding interactions is a potentially important factor in the development of inhibitor drugs and vaccines. Cell binding is typically the first interaction involved in cellular internalization, which makes it a very attractive target for pharmaceutical agents. In addition to inhibiting a toxin in the initial stage of intoxication, the extracellular location of toxin binding allows therapies to be effective without the added complication of entering cells. One main strategy employed in the development of inhibitors as well as vaccines the rational design based on the location and molecular composition of a receptor binding site (Tanrikulu et al. 2008, Liu et al. 2004). While inhibition of VacA receptor binding would not necessarily kill *H. pylori* directly, there is substantial evidence that suggests that VacA is necessary for *H. pylori* colonization and persistence (Schmitt et al. 1994, Salama et al. 2001, Atherton et al. 1995, Kidd et al. 1999). In this study, characterization of the VacA binding pocket defines a conceptual framework for the design of novel therapies targeting SM-binding toxins and grants new insight into a never before investigated binding motif.

H. pylori

H. pylori, is a microaerophilic, Gram negative, spiral shaped bacteria that is the only known species to persistently colonize the human stomach (Cover et al.2005). While most bacteria are killed by the acidic environment of the stomach, *H. pylori* is able to survive by utilizing flagellar motility and chemotaxis to move up a pH gradient toward the gastric mucosa, where it buries itself within the mucous, attaches to the gastric epithelia, and produces urease, an enzyme that modulates the pH of the colonization site by neutralizing gastric acid through the production of ammonia from urea (Cover et al. 2009, Atherton et al. 2009, Schweintizer et al. 2008, Parsonnet et al. 1995, Schreiber et al. 2004, Kim et al. 2004, Kuwahara et al. 2000). *H. pylori* then produces a number of factors such as VacA, which serves to remodel the host tissue and assist in immune evasion through effects on the gastric epithelium as well as immune cells (Stingl et al. 2002).

The main outcome of *H. pylori* infection is chronic inflammation of the gastric epithelium. In most cases gastritis persists for the duration of the infection, which without treatment often continues for the lifetime of the host. Gastritis is usually subclinical, however, and is not often diagnosed, leading to infection rates over 50% worldwide and in excess of 80% in developing countries (Cover et al. 2009, Atherton et al. 2009). Despite a lack of acute symptoms, 20-30% of *H. pylori* infections lead to the development of more serious illnesses such as peptic ulcer disease, gastric lymphoma, and gastric adenocarcinoma, which is the second leading cause of cancer death worldwide, with over 800,000 deaths each year attributed to gastric adenocarcinoma (Amieva et al. 2008, Atherton 2006, Tetzlaff et al 2008, Montecucco et al. 2001). *H. pylori* has been classified as a class I carcinogen by WHO and estimates suggest that *H. pylori* infection increases the risk of developing gastric cancer approximately 10 fold (IARC

1994). These estimates do not, however, differentiate between the risk associated with each of the well-defined *H. pylori* phenotypes, which suggest that cancer risk is strongly correlated with the production and allelic variation in virulence factors such as VacA (Schmitt et al. 1994, Salama et al. 2001, Atherton et al. 1995, Kidd et al. 1999). This correlation suggests the possibility that *H. pylori* associated carcinogenicity is at least partly a result of the production of toxins such as VacA, rather than a result of infection alone.

VacA

VacA is produced as a 140 kD protoxin which is cleaved into an 88 kD mature protein. VacA is then secreted by a 33-kD C-terminal β -barrel autotransporter domain, which is separated from a 55-kD N-terminal domain by a protease-sensitive loop (Fischer et al. 2001) (fig. 1A) (Patrice et al. 2012). VacA is secreted into the extracellular space as a soluble protein which can oligomerize to form an active anion-selective pore forming toxin (IARC 1994). Evidence of the role of VacA in *H. pylori* virulence comes from the presence of the *vacA* gene in every *H. Pylori* human isolate, the association of specific VacA alleles with increased risk of developing gastroduodenal diseases, the decreased gastric ulceration observed in gerbil models treated with *vacA* knockout strains of *H. pylori*, and the in vivo treatment of mice models with VacA alone causing necrosis and inflammation in the gastric epithelium (Schmitt et al. 1994, Salama et al. 2001, Atherton et al. 1995, Fischer et al. 2001, Cover et al. 1992). The most visually evident activity of VacA and the basis of toxin's name is the formation of large acidic vesicles in the cytoplasm of cells intoxicated in vitro (Tombola et al. 1999).

When VacA binds to epithelial cells, individual monomers oligomerize and the toxin is inserted into the membrane (Wang et al. 2001) (2A). The mechanism of VacA internalization

has yet to be fully characterized, but it is known that cell entry is dependent on temperature, ATP, and actin, but independent of clatherin based vesicle formation (Ogura et al. 2000, Leunk et al. 1988). Following internalization, proton pump ATPase activity in the endocytic vesicles leads to vesicle acidification (fig. 2B). VacA anion-selective pore forming activity then causes an influx of chloride ions, which neutralizes the vesicle and stimulates further ATPase activity (Willllhite et al. 2004, de Bernard et al 1995, Pagliaccia et al. 1998, Telford et al. 1994). Membrane permeable weak bases, such as ammonia, then transverse the membrane, are protonated in the acidic environment, and are trapped within the vesicle. Increased concentration of protonated bases results in hypertonic conditions within the vesicle, causing vesicle swelling and vacuole formation within the cytoplasm of cells.

This channel-dependent activity is present in several other actions of VacA, including the formation of channels in the plasma membrane, which cause the leakage of ions and small molecules from the cell, and in the mitochondrial membrane, which leads to a loss of the mitochondrial membrane potential ($\Delta\Psi$) (Willllhite et al. 2004, Galmiche et al. 200). When $\Delta\Psi$ is lost, cytochrome C is released, causing the activation of caspase 9 and the initiation of the cellular apoptosis pathways (Gebert et al. 2003, Cover et al. 2001). In addition to these VacA membrane channel-dependent activities, VacA can influence signaling pathways in a channel-independent fashion to suppress immune cell activation, resulting in an increase in intracellular calcium, or by acting as an adhesin for *H. pylori* attachment to the gastric epithelia (fig. 2A) Cover et al. 2001, Czajkowsky et al. 1999).

Allelic Variation in VacA

One major determining factor for VacA functionality is cell specificity, which has been shown to change depending on allelic variations in the *vacA* gene (Iwamoto et al. 1999). The *vacA* gene is largely conserved, but there are 3 regions with substantial allelic diversity. Characterization of these regions based on differences in *H. pylori* virulence and VacA activity has led to the identification of three families of allelic variants in the signal sequence (s1 and s2), the intermediate region (i1 and i2), and in the mid-region (m1 and m2) of *vacA* (fig. 1B) (Gangwer et al. 2010). The signal sequences (s1 and s2) are located within the p33 portion of the gene, and differ predominately as a result of the presence of a hydrophilic region consisting of 12-amino-acid region present in s2 but not s1 toxins. This region is responsible for the absence of activity in s2 *vacA* compared to s1, and has been shown to act as a dominant negative mutation, causing a loss of activity in s1 *vacA* even when the s2 variant is at a fourth of the concentration. Further, it has been shown that mutation of this area results in inactive hetero-oligomeric complexes which are still able to form anion selective channels (McClain et al. 2001; Ivie et al. 2008).

As with the signals sequence, the intermediate (i) region encodes for a portion of the p33 gene, which has been shown to be important for VacA activity. The intermediate region is classified into i1 (vacuolating) and i2 (non-vacuolating) regions, with i1 strains being shown to significantly increase VacA infiltration and inflammation activity compared to *vacA* i2 strains (Rhead et al. 2007, Basso et al. 2008). Interestingly, only s1/m2 strains show i-type variation, with s1/m1 and s2/m2 strains exclusively having i1 and i2 strains, respectively, suggesting binding activity dependent variations within the intermediate region.

The final region of substantial allelic variation is the middle (m) region, which is located within the p55 domain of *vacA*. The m region of the p55 domain consists of the pathogenic m1 allele as well as the less pathogenic m2 strain. The corresponding increased cytotoxicity of m1 *vacA* in HeLa cells is believed to be indicative of the increased propensity for m1 strains to bind to gastric epithelia, cause inflammation, and ultimately increase the susceptibility for developing gastric adenocarcinoma in humans infected with *H.pylori* expressing the m1 allele compared to m2 type strains.

In an investigation of the cell binding characteristics of m1 and m2 *vacA*, allelic variations within the mid-region have been shown to lead to differences in cell binding as well as cell specificity, suggesting that different mid region alleles may have mutations in residues determining VacA cell binding and receptor dependent differences in tissue tropism. Further, it has been shown that cellular internalization and tissue tropism are dependent on the middle (m) region allele, with m2 strains showing activity in RK-13 cells but not HeLa cells, and the more virulent m1 strain showing greater activity in both RK-13 as well as in HeLa cells (Cristina et al. 1998) (fig. 2A-B). While it remains possible that m2 *vacA* has specifically attenuated binding to certain cell types, in vivo expression and decreased colonization of m2 *vacA* knock-out strains, suggesting differences in tissue tropism.

In addition to the identification of differences in cell binding between middle region m1 and m2 alleles, there are a number of studies which have indicated that p55 contains the VacA cell binding site (1, 36, 48-49) (Jean-Marc et al. 1999; Moll et al. 1995). One experiment supporting this hypothesis showed VacA vacuolating activity in cells following the sequential addition of p55 followed by p33 subunits of VacA, but no activity when the same domains were added in the opposite order (Torres et al. 2005). This hypothesis was further tested by Wang et

al. through the development of truncation mutants and the evaluation of mutant vacuolating and cell binding activity. Results of this study strongly indicated that the p55 domain played the predominant role in cell binding, and further suggested that the c-terminal 100 residues of the p55 domain played a critical role in cell binding (Wang et al 2000). While this study provided early indications of the role of p55 in cell binding, the study did not account for the loss of binding as a result of the removal of progressively larger portions of the VacA gene, which could have resulted in cooperative effects rather than the direct contributions of each of the removed portions.

To account for some of the shortcomings of the previous deletion studies, Ivie et al. introduced far smaller deletion mutations within the p55 domain of VacA which were limited to a single β -helical turn (Fig. 3A). The adoption of this approach allowed for the analysis of much smaller regions of p55 while simultaneously reducing the probability of toxin misfolding (Megraud et al. 2004). Analysis of mutant toxins indicated that the most drastic ablation of vacuolating activity was observed in mutations $\Delta 559-579$, $\Delta 580-607$, and $\Delta 608-628$, which were each hypothesized to be caused by disrupted toxin folding. Each of 3 β -helical turns $\Delta 484-504$, $\Delta 511-536$, and $\Delta 517-544$ retained vacuolating activity, indicating that these regions are dispensable for VacA activity. Finally, a mutation in the N-terminal region $\Delta 433-461$ of VacA showed activity in RK13 and AZ521 cells but not in HeLa cells, indicating a region of potential receptor specificity (Fig. 3 B-D).

Following the discovery that the middle region of VacA played a role in cell binding and allele dependent tissue tropism, a number of studies were done to identify which portion of the middle region was predominantly responsible for this specificity. To evaluate the effects of m1 and m2 regions on VacA virulence, chimeric vacA consisting of portions of both the m1 and m2

alleles were constructed (fig. 4A-C)(Ji et al. 2000). Once formed, mutant vacuolating activity assayed in various cell types suggested that the primary region which determines m1 and m2 vacA tissue tropism is present within the first 148 amino acids of the p55 middle region, as indicated by the loss of activity in HeLa cells present within the m2 substitution mutants PJ2 and G27::AB25 mutants. While the entirety of this region was shown to affect vacuolating activity, results indicated that the first 35 amino acids of this region must correspond to the m1 sequence to achieve HeLa cell cytotoxicity, as indicated by the complete loss of activity by the PIV6 mutant. Further, these findings validated the previous theories suggesting that the C-terminal portion of the mid-region are not involved in cell binding by showing that the amino acid from 657 onwards plays no role in the phenotypic difference between the m1 and m2 vacA.

To further elucidate the region important for tissue tropism in m1 and m2 strains, Skibinski et al. constructed a series of chimeric vacA mutants of m1 vacA with m2 substitutions starting from progressively further distance from the n-terminal end of the middle region (Fig. 4D-F)(Skibinski et al. 2006). Toxin m1 activity was measured using neutral red uptake assays for determining the extent of vacuolation in RK13 and HeLa cells when exposed to *H. pylori* growth supernatants. Results showed a much more graded loss of HeLa cell binding than previously suggested, indicating that the entire region spanning from amino acid 501 to 696 played an important role in m1 HeLa cell phenotype with no one specific region causing a complete loss of activity. Knowing that vacA likely has multiple binding site receptors, this result was hypothesized to suggest that multiple binding sites may be involved in tissue tropism. A secondary non-exclusive possibility is that binding site residues are positioned on multiple β -sheets which are proximal in physical location, but distal in linear sequence. In addition to

understanding the molecular basis of VacA binding by characterizing the binding site of VacA with the more virulent m1 phenotype, identification of binding site residues may give insight into the molecular basis of differences in toxin cell binding, pathogenicity, and cell specificity between VacA m1 and m2 alleles.

Identification of a VacA Cell Receptor

Though the numerous activities of VacA are relatively well understood, a thorough understanding of VacA cellular receptors has remained elusive, a factor which is of specific interest as a result of the multiple activities of VacA, including its role in adhesion, in stimulating inflammation, as well as in inducing vacuolation (Boquet et al. 2012). Major reasons for the in identifying the receptor include multiple receptors present in different cell lines, alternative receptor binding characteristics for VacA expressed by different *H. pylori* strains, or even multiple or alternative binding on the same cells in order to mediate alternative VacA activities simultaneously. One of the earliest receptors identified was the epidermal growth factor (EGF) as a receptor in HeLa cells. This was achieved through antibody-mediated inhibition of VacA cell binding as well as the immunoprecipitation of VacA along with the EGF receptor (Seto et al. 1998).

A second receptor identified was the receptor tyrosine phosphatase (RPTP) beta, which was discovered using indirect immunofluorescence and flow cytometry as well as through the observation of increased sensitivity in AZ-521 cells following overexpression of RPTP beta (Hirayama 2012; Nakayama et al. 2006). Further, studies have shown that RPTPalpha is bound by VacA (Yahiro et al. 2004). Consistent with previous evidence suggesting alternative tissue tropism of m1 and m2 type VacA, immunoprecipitation experiments in m2 VacA in AZ-521

cells showed attenuated binding to RPTP alpha from HeLa cells compared to G401 cells (De Guzman et al. 2005). Despite nearly identical amino acid sequence, a slightly increased RPTPalpha molecular mass in G401 cells suggested that differences in binding may be a result of differences in post-translational modifications. This finding provides even further evidence of the complexity of VacA binding and tissue tropism.

While EGFR and RPTP are believed to be involved in the cellular vacuolation activity of VacA, they do not explain the anti-inflammatory activity of VacA on immune cells. In 2010, Sewald et al. identified the immune cell specific receptor CD18 as an m1 VacA receptor in leukocytes (Sewald et al.). This was shown through the lack of activity present in CD18 knock out leukocytes, as well as through direct immune-precipitation. In addition to identifying CD18 as the primary receptor for leukocytes, these studies suggested a number of secondary receptors involved, which interact with CD18 to form heterodimeric complexes. Further, these studies showed that receptor interactions are specific to human CD18, casting substantial skepticism on murine models of *H. pylori* intoxication.

Still other studies suggest that the identity of the high affinity receptor in some cell lines is a glycosylphosphatidylinositol (GPI) -anchored protein. Evidence for VacA association with GPI-anchored proteins is supported by the attenuation of VacA cell binding following cell treatment with phospholipase C (PI-PLC) (Vittorio et al. 2008; Ricci et al. 2000). Upon further experiments, however, it was found that VacA binding to cell lipid rafts associated with GPI-anchored proteins, rather than to the proteins themselves (Schraw et al. 2002). In conjunction with the effects of PI-PLC on VacA cell binding, this result suggested that VacA association with phospholipids present within ordered domains likely plays an important role in cell binding and toxin internalization.

While the protein receptors discussed above are likely the primary receptors for VacA, it is known that in addition to specific high affinity m1 type receptors, there is a distinct secondary receptor which plays a role in VacA binding (Wen-Ching et al. 2001). The identification of the importance of lipid rafts in VacA binding and internalization along with the effects of PI-PLC incubation strongly suggested that a phospholipid may play a role as this secondary receptor for VacA. Evaluation of the effects of exogenous lipid addition on VacA vacuolating activity in HeLa cells showed increased sensitivity following the addition of sphingomyelin (SM), but no alteration in cellular activity following the addition of phosphatidyl choline (PC), phosphatidylethanolamine (PE), or phosphatidylinositol (PI) (fig. 5A)(Gupta et al. 2010). Further, cleavage of the phosphorylcholine head group of SM by sphingomyelinase C prior to intoxication drastically decreased vacuolating activity, suggesting that VacA specifically recognizes the phosphorylcholine head group. Activity could, however, be rescued by the subsequent addition of exogenous SM to the growth media (fig. 5C). To ensure that this attenuation was a result of decreased binding, flow cytometry was used to show that membrane binding is dependent on the presence of the SM phosphorylcholine head group, indicating that SM is a cell membrane receptor for VacA (fig. 5D). This finding was of additional significance because no other known bacterial toxin utilizes SM as a receptor.

In addition to determining that SM is a receptor for VacA, it was found that VacA binds to PC to a similar extent as SM but does not induce cellular vacuolation, giving further evidence that the phosphorylcholine head group present in both SM and PC is important for VacA binding (fig. 5B). This conclusion was supported by the fact that ceramide, which has a structure similar to SM without the phosphorylcholine head group, does not have increased VacA binding compared to other lipids (fig. 6)(Gupta et al. 2010). Further, it was found that VacA SM binding

was substantially higher than binding to PE, which contains a phosphoethanolamine head group identical to phosphorylcholine except for the substitution of a primary amine in the place of the phosphorylcholine quaternary amine. Together these findings suggested that the quaternary amine of SM is the most likely molecule involved in VacA phosphorylcholine specific binding.

SM binding in toxins

While SM was only recently identified as a receptor for VacA, the involvement of SM in lipid rafts and protein trafficking is very well characterized (Bhat et al. 2013). Along with cholesterol, SM forms characteristic lipid raft domains in cell membranes which can act as platforms for the recruitment and organization of cellular membrane proteins as well as those produced by viral and bacterial pathogens. Further, it is known that lipid rafts play an integral role in cellular events including membrane transport and signal transduction (Ikonen 2001; Simons and Toomre 2000). Lipid rafts have also been suggested as the point of entry for a large number of viruses, bacteria, and toxins.

Among the most thoroughly studied sphingomyelin binding proteins which are believed to interact with lipid rafts are those of the actinoporin family, a very well conserved protein family expressed in sea anemonies (Alvarez et al. 2009). Like VacA, actinoporins are extracellular pore forming toxins which are known to bind SM on the surface of cells prior to oligomerization and membrane insertion. The most well studied members of this family are equinatoxin II (EqII) and sticholysins I (StI) and II (StII) from the sea anemones *Actinia equina* and *Stichodactyla helianthus*, respectively. Each actinoporin is known to bind individual SM molecules through a conserved motif containing aromatic residues Trp-112 and Trp-113.

Since its initial discovery, actinoporins have been found to have sequence homology with a multitude of proteins produced by animals and other plants (Kawashima et al. 2003; Gutiérrez-Aguirre et al. 2006). In each instance, the predominant area of homology was located within the C-terminal region known to be involved in SM binding in actinoporins. Further, this region has been identified as a membrane binding site with a conserved P-[WYF]-D pattern located within an unstructured loop between ordered domains. While this region has been shown to be critical for SM binding, it is also known that a secondary cluster of 3 tyrosine residues plays a critical role in binding (Zhang et al. 2011).

As with VacA, Actinoporins have been previously shown to bind specifically to SM without substantial binding to phosphatidylcholine, ceramide, or sphingosylphosphorylcholine, indicating specificity for the phosphocholine head group as well as H-bonding amine and hydroxyl groups (Bakrac et al. 2008). While it is believed that the quaternary amine is bound through cation- π interactions with active site aromatic groups, the means of conveying additional specificity had not been investigated until Maula et al. To determine the molecular mechanism of this specificity, methylated SM-analogues 2NMeSM and 3OMeSM were used to determine if H-bonding to active site tyrosine residues was important for toxin SM binding. Results indicated that tyrosine residues played a key role in SM binding through H-bonding between phosphate oxygens as well as the secondary amine of the SM group (Maula et al. 2013).

Another SM binding toxin, lysenin, was identified in the earthworm *E. foetida*. As with VacA and the actinoporin family, lysenin oligomerizes to form a pore through interactions with membrane lipid raft domains (Yilmaz et al 2013). Recent studies have shown that lysenin undergoes a conformational change, which results in a membrane insertion (De Colibus et al. 2012). As with the actinoporin family, lysenin contains a SM binding domain and is a pore

forming protein targeted to lipid rafts of the cell membrane (Pawel et al. 2011). An investigation of the lysenin SM binding site suggested that, as with actinoporins, the phosphorylcholine is bound through aromatic interactions and that the phosphate moiety is bound through hydrogen bonding with active site residues.

Yet another SM binding protein is the pleurotolysin of edible mushroom *Pleurotus Eryngii*. As with the other SM binding toxins, pleurotolysin A2 is an extracellular A/B type pore forming toxin which binds SM present within membrane lipid rafts. PlyA2 contains an aromatic cluster which is conserved between all related strains, and tryptophan residues which have been shown to be important for cell binding. Further, the pleurotolysin binding site contains a central proline residue as well as secondary tyrosine residues believed to play a role in hydrogen bonding to the phosphate moiety (Bhat et al. 2013).

In addition to toxins produced by eukaryotic organisms, there are several known bacterial proteins which bind SM. Among the most thoroughly characterized of these proteins is that Sphingomyelinase (SMase) from *Bacillus cereus* (Bc-SMase) (Ago et al. 2006). Bc-SMase contains an aromatic domain which is conserved in proteins from related bacteria including *S. aureus* and from *L. ivanovii*. Consistent with the trend in other SM binding proteins, these aromatic residues are solvent exposed, located in a hydrophobic region believed to interact with the membrane, and located in a beta hairpin motif. Replacement of these conserved aromatic residues with alanine through site directed mutagenesis has been shown to result in drastic attenuation in activity as well as plasma membrane binding, confirming their role in binding, and providing further evidence of the consistent role of aromatic residues in SM binding.

Cation- π interactions

In the investigation of other SM binding proteins, the emergence of a multitude of consistencies within binding site residues suggests a mechanism of binding which is largely conserved even between bacteria and complex eukaryotic organisms. Among the most evident of these trends is the presence of a cluster of conserved aromatic amino acids, which normally contain tryptophan residues. Further, it is known that many of these proteins achieve SM specific binding to membranes which also contain similar phospholipids including PE, PI, and PC, a result which suggest that the quaternary amine of SM is the most likely moiety involved in VacA phosphorylcholine specific binding.

Quaternary amines such as that present in the head group of phosphorylcholine are cationic molecules regardless of the pH of a solution, making them very good ligands for electrostatic binding. In biological systems, one of the most common interactions with quaternary amines are strong non-covalent interactions with the electron rich pi system of aromatic rings called cation- π bonding (Ji et al. 2000, Doughtry et al. 2007, Schiefner et al. 2004). Aromatic amino acids provide a surface of negative electrostatic interaction through pi orbitals, resulting in a negative charge on the face of an aromatic ring. In order for cation- π bonding to occur, this face must be positioned specifically to allow for the appropriate orientation for electrostatic interactions to occur (Dogherty et al. 2007).

Of the aromatic amino acids which play a role in Cation- π interactions, by far the most conserved is tryptophan. This is likely a result of the indol ring electrostatic potential being substantially larger than that of phenylalanine or tyrosine. These cation- π interactions usually occur within a distance of 6 Å and are strongest with tryptophan and phenylalanine residues, but

also occur with the tyrosyl group of tyrosine. While phenylalanine is believed to have a greater potential for strong cation- π interactions, the H-bonding capability of tyrosine can frequently result in stronger overall binding. Further, when the hydrogen acts as a donor, electrostatic interactions substantially increase resulting in greater charge density and stronger cation- π bonding (Dogherty et al. 2007).

Cation- π interactions are a very common form of non-covalent interaction present in nearly half of protein complexes and a third of homodimers investigated (Cowley et al. 2005). Cation- π binding has been demonstrated using x-ray crystallography to show protein ligand interactions. One of the earliest demonstrations of recognition of this type was binding of the neurotransmitter acetylcholine by acetylcholine esterase, wherein a quaternary amine group like the one in SM is recognized (Dougherty et al. 1990). Similar interactions have been observed in the recognition of both GABA and serotonin (Lummis et al. 2005; Beene et al. 2002). Cation- π interactions have also been shown to play a critical role in sphingomyelinase recognition of the phosphorylcholine head group specifically (Parrill et al. 2011).

Though no known bacterial toxin other than VacA uses SM as a cell surface receptor, there are proteins such as sphingomyelinase, equinatoxin, and actinoporins that specifically bind SM (Beene et al. 2002, Hong et al. 2002, Kiyokawa et al. 2004, Ago et al. 2006, Gallivan et al. 1999). Using site-directed mutagenesis, the SM-binding site of each of these proteins has been characterized. Unsurprisingly, a SM binding motif rich in aromatic amino acids with conserved tryptophan residues has been shown to recognize ligands through cation- π interactions.

Considering the evidence presented above, I hypothesize that:

The vacuolating cytotoxin of *H. pylori* contains a SM binding pocket composed of aromatic residues which specifically bind SM through cation- π interactions with the quaternary amine of the phosphorylcholine head group of SM.

To test this hypothesis, UCSF Chimera molecular modeling software and ClustalW2 sequence alignment were used to determine regions of structural similarity with other SM binding sites as well as amino acid sequence homology with previously characterized SM-binding proteins. Upon identification of a putative binding site, site-directed mutagenesis was used to develop tryptophan to alanine point mutants of residues in each of the VacA domains. An *E. coli* based recombinant expression system was then developed for the independent production of the p55 and p33 subunits of VacA. The purified toxin was then screened for the ability to induce the formation of cytoplasmic vacuoles in cultured gastric epithelial (AZ521) cells. Two attenuated tryptophan mutants were identified within a putative binding site, and the residues were then shown to also have attenuated cell binding. Following initial screens for mutant activity, non-tryptophan mutants present in the putative binding site within the middle region of p55 were selected for further mutagenesis. Following the screen of mutant vacuolating activity, CELISA and SM ELISA will be used to further characterize the putative binding site. Upon the completion of this project, this research will provide additional information on the mechanism of bacterial toxin SM binding, will be the first study to identify specific residues involved in a VacA cell binding site, and will provide the framework for determining if allelic variation in the VacA SM-binding site is a determinant in the altered cell specificity and toxin virulence observed between VacA mid-region alleles.

Chapter 2: Materials and Methods

Bioinformatics

BLAST, ClustalW2, and GeneDoc were used to compare the VacA peptide sequence to other VacA alleles as well as sphingomyelin binding proteins equinatoxin II (PDB ID: 2L2B) and *Bacillus cerus* sphingomyelinase (PDB ID: 2DDT). Protein sequences were acquired from PubMed protein database and all sequences were compared to the VacA allele present in the *H. pylori* strain 60190 (PDB ID: 2QV3). Molecular modeling was performed using UCSF CHIMERA and Swiss-Pdb Viewer.

Plasmid purification

Plasmids were purified using a QIAprep Spin Miniprep Kit and the suggested procedure. In short, 3 mL of LB media supplemented with 100 ug/mL ampicillin was inoculated with the DH5 α cells and incubated for 6-12 hrs. Following this incubation, cells were pelleted using a table top centrifuge (IEC MicroMax) for 10 min at 5000 x *g*. The supernatant was then removed, the cell pellet was re-suspended in 250 uL P1, and the solution was transferred to a microcentrifuge tube. 250 μ L Buffer P2 was then added, and the tube was inverted 4–6 times. Once the solution was a uniform blue color, 350 μ L Buffer N3 was added and mixed immediately by inverting the tube 4–6 times. Tubes were then centrifuged for 10 min at 18,000 x *g*. The supernatant present following this spin was then applied to a QIAprep spin column and a spun for 1 min at 18,000 x *g*. The column was then washed by adding 0.75 mL Buffer PE and again spinning down at 18,000 x *g* for 1 min. Finally, the column was added to a new centrifuge tube and elution buffer, EB, was added and allowed to sit for 1 min. The column was then centrifuged for 1 min to remove the eluate, which was stored at 4°C (Figure 8).

Site-directed mutagenesis

Primers (Table 1) were designed and analyzed for hairpin formation and melting temperature using the OligoAnalyzer 3.1 by Integrated DNA Technologies and were synthesized by the same company. Lyophilized Primers were suspended in sterile molecular biology grade water to an estimated final concentration of 100 nM. PCR was performed using PfuUltra high-fidelity DNA polymerase (Agilent Technologies) using the suggested protocol. Each 50 μ L reaction mixtures were made with 5 μ mol of pET20b-vacA-p55 (Fig. 7A; Novagen) containing the p55 or p33 *vacA* insert, 1x Pfu reaction buffer, 250 μ M of each dNTP, 2.5 U PfuUltra DNA Polymerase (Stratagene), and 100 pmol of each primer. Sterile molecular grade water was added to bring volume to 50 μ L, and reactions were performed in sterile, DNase treated, thin-wall PCR tubes. PCR was carried out on a PTC-200 Peltier Thermal Cycler (MJ Research) with the following operations: 1) 95°C for 2 min, 2) 95°C for 20 s, 3) 42-66 °C for 20 s, 4) 72 °C for 2:15 min, 5) Go to step 2 18x, 6) 72 °C for 3 min, 7) 4°C forever, 8) end.

Following PCR, 1 μ L of 2000 U/mL DpnI (New England Biolabs) was added to each 50 μ L reaction along with 5.7 μ L 10x DpnI buffer. Tubes were then incubated at 37 °C for 3 h to degrade methylated template DNA. PCR products were analyzed using DNA electrophoresis with a 0.9% agarose gel. Following electrophoresis, the agarose gel was incubated for 20 min in a 100 mL solution of TBE buffer containing 0.5 μ g/mL ethidium bromide and DNA was visualized using the UV setting on a ChemiDoc XRS Molecular Imager and Quantity One 1-D Analysis software (BioRad)(fig. 7B).

Transformation

Chemically competent *E.coli* DH5 α cells (University of Illinois Cell Media Facility) were thawed for 20 min at 4 °C after being stored at -80 °C. 45 μ L of competent cells were then added to 5 μ L of DPN1 digested PCR product. Cells were allowed to incubate with DNA for 5 min at 42°C, and were then added to a water bath at 42°C for 45 s. Following heat shock, 1 mL of SOC broth was added to each tube and cells were grown in a rotary incubator (Fischer Scientific) at 37 °C for 90 min. Cells were then pelleted by centrifugation at 2000 x *g* for 2 min, were suspended in 200 μ L SOC media, and were spread on LB plates supplemented with 100 μ g/mL ampicillin (Sigma) and incubated at 37 °C for 12-16 h. Following incubation, the number of colonies was determined and used to calculate transformation efficiency of competent cells. Individual colonies were picked with a pipette and used to prepare stock cell lines, which were stored at -80 °C. Plasmids were purified from DH5 α cells using the procedure previously discussed.

Mutants were confirmed by DNA sequencing (Core Sequencing Facility at the University of Illinois at Urbana-Champaign). Following sequence confirmation, chemically competent BL21 cells (University of Illinois Cell Media Facility) were transformed with vector DNA using the procedure previously described. Transformants were selected on LB plates containing 100 μ g/mL ampicillin, and 5 colonies from each plate were used to inoculate 5 mL cultures of LB media. Cultures were grown in a rotary incubator until an OD₆₀₀ of .5 was reached. VacA expression was then induced using IPTG at a final concentration of .125 mM and cultures were then allowed to grow for an additional 3 hours. 1 mL of each growth was then centrifuged at 5000 x *g* for 10 min using a table top centrifuge (IEC MicroMax), and the pellet was re-suspended in SDS PAGE buffer. Tubes were then heated for 5 min at 100 °C and cell

lysates were analyzed using SDS-PAGE. Cell lines expressing the greatest amount of recombinant protein were used to make stocks that were stored at -80°C (fig. 8A).

VacA recombinant protein expression and purification

Cell pellets were removed from -80 °C and re-suspended in 10 mL of a 1% PMSF solution made with PBS and lysed using sonication (Fischer Scientific Model: 550 Sonic Dismembrator) done with samples on ice. Samples were subjected to 2 rounds of sonication consisting of 2 second intervals of sonication followed by 5 seconds without sonication until a total sonication time of 3 min was reached. Cell lysates were then centrifuged at 16,000 x g for 20 min at room temperature to pellet debris. The supernatant containing soluble protein was then removed and the remaining pellet, including insoluble VacA within inclusion bodies, was solubilized with Buffer B (8 M urea, 0.1 M sodium phosphate buffer, 0.01 M Tris-Cl, pH 8.0). Re-suspended inclusion body were then centrifuged at 16,000 x g for 20 min at 4 °C. Following centrifugation, supernatant containing recombinant protein was added to 2 mL of Talon metal affinity resin (Clontech), which had been previously washed and equilibrated with Buffer B. The protein was allowed to bind the resin for at least 30 min at room temperature or overnight at 4 °C.

The resin-protein mixture was then loaded into a column the solution was allowed to flow through the column and was collected. Resin was then washed twice with 8 mL of Buffer C (8 M urea, 0.1 M sodium phosphate buffer, 0.01 M Tris-Cl, pH 6.3) and the flow through was collected. Protein was then eluted with 4 mL of Buffer D (8 M urea, 0.1 M sodium phosphate buffer, 0.01 M Tris-Cl, pH 4.5) and was collected. Fractions were then analyzed by SDS PAGE

and stained with Coomassie brilliant blue to analyze purity. Protein was then quantified using a Bradford assay and stored at -80 °C (fig. 8B).

Refolding of Recombinant VacA

Protein was then dialyzed at 4°C using Spectra/Por[®] membrane (MWCO 6-8,000) for 18 h with 20x the protein volume of Dialysis Buffer #1 (10 mM Tris-Cl, 1 M urea, 0.1 mM β -mercaptoethanol, 1 mM L-cysteine, 5% glycerol, pH 11.0). The protein was then dialyzed with fresh Dialysis Buffer #1 for 6 h followed by dialysis with Dialysis Buffer #2 (10 mM Tris-Cl, 0.1 mM β -mercaptoethanol, 1 mM L-cysteine, 5% glycerol, pH 11.0) for 18 h, fresh Dialysis Buffer #2 for another 6 h, Dialysis Buffer #3 (10 mM Tris-Cl, 0.1 mM β -mercaptoethanol, 5% glycerol, pH 8.8) for 18 h, and finally with fresh Dialysis Buffer #3 for 6 h. Debris was pelleted by centrifugation at 16,000x *g* for 20 min at 4°C, and 3 kD pore size Amicon Ultra Centrifugal Filter Unit were used to concentrate protein to 2 μ M or higher. Protein was then stored at 4°C.

Purification of native VacA

VacA was produced and purified from *H. pylori* as previously described (35). *H. pylori* was grown in BSFB media in culture flasks with 5 μ g/mL vancomycin on a rotary platform shaker for 48 h at 37 °C with an atmosphere of 5% CO₂. After incubation, *H. pylori* cultures were harvested by centrifugation at 8,000 RPM with a Beckman Coulter Avanti J-E centrifuge for 30 min at 4°C. After centrifugation, the supernatant was collected and protein was precipitated using 90% ammonium sulfate and overnight incubation at 4°C. Precipitated protein was collected by centrifugation at 8,000 RPM for 30 min at 4°C then re-suspended in 10 mM phosphate buffer at pH 7.0. Samples were then dialyzed using the Spectra/Por membrane (MWCO 50,000) three times into 10 mM phosphate buffer pH 7.0 at 4 °C. After overnight

dialysis, the crude concentrated extract was filter sterilized using a 0.2 μ m filter. VacA was then purified by anion exchange chromatography with Diethyl aminoethyl Sephacel resin (Sigma). 10 mM phosphate buffer containing 200 mM sodium chloride pH 7.0 was used to elute VacA. All eluted fractions were collected and analyzed by SDS-PAGE followed by Coomassie brilliant blue staining and immunoblot analysis with rabbit anti-VacA polyclonal antibodies. Fractions with the greatest purity were pooled and stored at 4°C until use.

Visual Vacuolation Assay

Individual recombinantly expressed VacA subunits (p33 and p55) were mixed in a 1:1 molar ratio for 1 h at room temperature. Following subunit mixing, pH was lowered to 2.0 through the addition of 3 M HCl, and samples were incubated for 30 min at 37 °C. VacA was then be neutralized using .3 M NaOH and added to AZ521 cells at concentrations between 1-200 nM and allowed to incubate for 8 h in MEM (Sigma) supplemented with 2 mM glutamine (Sigma), 100 U/mL penicillin(Sigma), 1 mg sulfate/mL streptomycin (Sigma), 10% FBS (JRH Biosciences), and 5 mM ammonium chloride (EM Science). Phase contrast microscopy was used to count 50 cells in each well to determine the percent of cells containing one or more vacuoles.

Cell culture

AZ-521 cells (3940; Japan Health Science Foundation) were maintained in MEM (Sigma) supplemented with 2 mM glutamine(Sigma), 100 U/mL penicillin(Sigma), 1 mg/mL streptomycin (Sigma), and 10% fetal bovine calf serum (FBS; JRH Biosciences). Cells were grown at 37 °C with a 5% CO₂ humidified atmosphere.

CELISA

Recombinant VacA was purified and refolded as previously described. Following dialysis, p33 protein concentration was determined using a Bradford assay, and 10 moles of EZ-Link Sulfo-NHS-LC-Biotin was added to each sample for every 1 mole of p33. The reaction was allowed to proceed for 30 min at room temperature, then was halted using Tris hydrochloride at a final concentration of 20 mM. Samples were then added to pre-equilibrated dialysis tubes (MWCO: 6-8 kD, Spectrum Labs) and dialyzed into PBS (pH 7.4). After 4-6 h, dialysis buffer was switched. After another 4-6 h, samples were removed from dialysis and quantified a second time using a Bradford Assay.

Biotinylated p33 was recombined with p55 and acid activated as previously discussed then stored at 4 °C until addition to cells. AZ521 cells were prepared in a 96-well plate until 80% confluent, and were removed from growth conditions and placed on ice 30 min prior to the addition of VacA. After 30 min, cells were washed with 200 µL of a 3% BSA solution in PBS, and VacA was neutralized with .3 M NaOH at 4 °C then added to cells PBS (pH 7.4) with 3% BSA. After a 30 min incubation at 4 °C, VacA was removed and cells were washed 3 times with PBS (pH 7.4). Cells were then fixed by adding of 100 µL of a 2% paraformaldehyde(sigma), 0.2% glutaraldehyde (calbiochem), PBS solution (pH 7.4) and allowing the solution to incubate for 30 min at room temperature. Cells were then washed 3 times again with a 3% BSA solution in PBS. Following washes, streptavidin-HRP (Fisher Scientific) was added to cells with a 1:15,000 dilution for 30 min at room temperature. After 30 min, cells were washed 3 times with 200 µL PBS supplemented with 0.1% tween 20 (promega). 100 µL of TMB ultra (Thermo Scientific) was then added to each well and samples were incubated at room temperature for 30 min. Following incubation, 100 µL 2 M sulfuric acid was added to each well containing the

TMB ultra. Plates were read with microplate reader (Spectronic 20 Genesys spectrophotometer, Sigma-Aldrich) at a wavelength of 450 nm.

Chapter 3: Results

Bioinformatics

It has been shown previously that VacA binds the phosphorylcholine head group of SM and PC preferentially over other sphingolipids and that VacA cell binding to SM on the surface of eukaryotic cells is necessary for toxin activity (Gupta et al. 2010). These findings suggest that SM acts as a receptor for VacA on the surface of cells and that binding occurs specifically through the phosphorylcholine head group. Site-directed mutagenesis has been used in studies with other SM binding proteins to show that SM binding occurs through interactions of tryptophan and tyrosine rich binding motifs (Hong et al. 2002, Kiyokawa et al. 2004, Ago et al. 2006). As a primary method for initially identifying a VacA putative SM binding pocket, the amino acid sequence and molecular structure of the previously characterized SM-binding proteins were compared to that of VacA in order to determine structural similarities between phosphorylcholine binding sites in other proteins and VacA aromatic clusters.

UCSF chimera based molecular modeling was used to compare the crystal structure of the p55 domain of VacA to that of the sphingomyelinase of *B. cereus* (BC-SMase) as well as the eukaryotic pore forming toxins equinatoxin II, an actinoporin-like protein produced by sea anemones (Hong et al. 2002), and lysenin, a toxin secreted by the earthworm *E. foetida* (Kiyokawa et al. 2004, Kwiatkowska et al. 2007). Molecular modeling of equinatoxin II indicated that the previously identified binding site aromatic residues were located primarily in solvent exposed loops connecting β -sheet domains (fig. 9A)(Athanasiadis et al. 2001)(PDB ID: 1IAZ). A similar binding motif was observed in Bc-SMase (fig. 9B)(Ago et al. 2006)(PDB ID: 2DDT) with tryptophan, phenylalanine, and tyrosine residues organized in a solvent exposed

cluster present on beta hairpins between ordered domains with residues from multiple turns contributing to SM binding. Though no crystal structure for lysenin yet exists, predictions of secondary structure suggest that a SM binding aromatic cluster is present in turns between β -sheets (Jamroz et al. 2010). The consistent location of SM binding domains within protein loops is likely due to higher solvent exposure for residues within turns as well as increased flexibility compared to more structured domains such as α -helices or β -sheets, allowing for the mobility to adopt favorable binding site orientation while interacting with ligands (Shakor et al. 2003).

Comparison of the structural location of SM binding sites in other proteins suggested that the VacA binding site would consist of an aromatic tryptophan containing cluster located in solvent exposed loops between ordered domains. The crystal structure of the p55 subunit of VacA was examined to determine if a similar motif was present within the p55 domain of VacA believed to be responsible for cell binding. Of the 3 tryptophan residues present in p55, 2 were found to be located in unstructured surface exposed peptide loops between the β -sheet domains which form the core structure of p55 (Fig. 10)(PDB ID: 2Qv3). The tryptophan residues identified (W577 and W603) were predicted to be within 3.3 Å of each other and within 14 Å of 5 other aromatic residues within the mid-region of VacA (Table 2)(Pagliaccia et al. 1998). While cation- π interactions normally occur within a range of 6 Å, it is important to consider that the large degree of 3-dimensional flexibility present within peptide loops, which likely allows for far closer proximity than indicated by the unbound orientation of the VacA crystal structure (Kiyokawa et al. 2004).

Peptide sequence alignment was used to further characterize the similarities between other SM binding proteins and VacA through alignment with other SM binding proteins. Though no substantial homology was found between VacA and the peptide sequence of Lysenin or BC-

SMase, a short homologous region was identified between VacA and equinatoxin II, which has previously been reported as a conserved phosphorylcholine binding motif present in Actinoporin domains present in three animal and two plant phyla (Monastyrnaya et al. 2010, Gutiérrez-Aguirre et al. 2006) (fig. 11).

Among the conserved residues within the region homologous to the phosphorylcholine binding domain were 3 of the aromatic residues initially hypothesized to be part of the putative VacA SM binding site based on three dimensional molecular modeling. Though VacA does not contain the exact consensus sequence P-[WYF]-D determined from the sequence alignment of actinoporins-like proteins, residues are functionally conserved to a large extent (Gutiérrez-Aguirre et al. 2006). Additionally, there is some variation between the peptide sequences in actinoporins, suggesting that there is a degree of latitude in the sequence required for phosphorylcholine binding. The lack of significant homology between VacA and the other SM binding proteins was unsurprising due to secondary and tertiary structural elements normally playing a more substantial role in binding site configuration than primary structure.

A second sequence alignment based strategy used to identify binding site residues was the analysis of the mid-region of different VacA alleles. Previous studies have shown that VacA virulence is dependent on mosaicism in the *vacA* gene (Telford et al. 1994), and that allelic variation in the mid-region of *vacA* leads to changes in toxin cell specificity through alterations in cell binding. Pagliaccia et al. demonstrated that m2 strains have decreased virulence in HeLa cells compared to m1 strains, but both have comparable activity when VacA of the m2 allele is expressed intracellularly (Pagliaccia et al. 1998), suggesting that the differences in cell specificity are a result of changes in cell binding. While a far less pronounced attenuation of m2 VacA activity is observed in RK13 and AZ521 cells, alterations in key SM binding residues

would be a strong indicator of a potential difference in tissue tropism and cellular trafficking between middle region alleles.

Peptide sequence alignment was used to compare m1 and m2 alleles throughout the mid-region in order to determine if the different activities observed between alleles could be a result of mutated aromatic residues and attenuated SM binding in VacA of the m2 subtype (fig. 12). Using this technique, 8 aromatic residues were found to consistently vary in the m2 allele relative to the m1 subtype (table 2). It was also found that a F555Y allele was consistently present, which is notable due to the substantially decreased strength of tyrosine cation- π interactions relative to those of tryptophan and phenylalanine (Dougherty 2007). Of the residues that were consistently altered in the m2 VacA allele, the aromatic residues F555, Y574, W603, and F626 were found to be located within the range of possible cation- π interactions with SM in the solvent exposed putative SM binding pocket previously identified (table 2). Notably, it is difficult to predict the degree of conformational change during binding or the movement of flexible β -helical turn regions, so it is possible that any of the screened residues are involved in the same SM binding pocket. In addition to suggesting a possible location of a SM binding site on VacA, this result suggests that attenuation in SM binding may account for some of the differences in virulence between *vacA* alleles as well as the differences in pathogenicity observed between *H. pylori* strains.

Site-directed mutagenesis

To test the hypothesis that the VacA SM binding site is composed of an aromatic cluster with tryptophan residues playing an important role, scanning mutagenesis was employed to mutate each tryptophan within the p55 and the p33 domain of VacA. Each residue selected was

mutated to alanine in order to remove all interactions that are dependent on polar or aromatic groups. Following mutagenesis, VacA was produced using a recombinant expression system, purified, refolded, and assayed for activity by intoxicating AZ521 cells with mutant and wild type toxin.

The p33 autotransporter domain of VacA is known to interact with the cell membrane, but structural analysis was not performed because no crystal structure exists for this domain. Though no structural homology could be identified, the large number of tryptophan residues in close proximity to one another within the peptide sequence suggested the possible presence of SM binding domains (fig. 13). To test the importance of p33 tryptophan residues for VacA activity, each tryptophan within p33 was individually mutated to alanine and the resulting mutant proteins were screened for vacuolating activity. The screen of p33 tryptophan residues using isual vacuolation assays done using phase contrast microscopy and AZ521 gastric epithelial showed that mutation of any of the 8 tryptophan residues within the p33 subunit of VacA led to only minor decreases in activity, which were likely a result of minor changes in mutant protein structure (fig. 17, table 3). This finding suggested that the mutation of p33 VacA tryptophan residues did not cause substantial attenuation of VacA activity (fig. 14A and 14B). This finding was consistent with previous studies, which suggested that the p55 domain of VacA is responsible for cell binding, as well as with molecular modeling, which suggested that p55 contained a putative SM binding site.

Unlike the p33 domain, however, the mutation of each of the 3 tryptophan residues in the p55 domain led to significant attenuation in vacuolating activity (fig. 14, table 3). As expected, mutation of both tryptophan residues present within the putative binding site (W577A and W603A) caused significant attenuation in vacuolating activity and resulted in a 4.5 and 7.3 fold

attenuation for W577A and W603A, respectively (fig. 14C). Surprisingly, W773A, a mutation located in a separate loop between β -sheets, showed a 3-fold attenuation in activity when mutated. This finding could indicate the presence of a second SM binding domain, but could also be the result of the loss of a secondary activity such as dimerization.

Binding site residues were investigated further through site-directed mutagenesis of non-tryptophan residues proximal to the putative binding site. Considering evidence from molecular modeling and sequence alignment of VacA, 10 novel mutants were selected for mutagenesis based on their location relative to the putative binding site and homology with other SM binding proteins (table 2). In addition to selecting aromatic residues, R552 and K607 were selected for mutagenesis based on the possibility of ionic interactions between the positive side groups and the negative charge of the phosphorylcholine phosphate group. So far, 4 of the 10 mutants have been screened for activity, and results of visual vacuolation assays indicated that mutation of K607A caused a 2 fold attenuation in activity, suggesting that this residue may be involved in ionic interactions with the phosphate group of SM (fig. 14D).

The observation of substantial attenuation in cell activity as a result of a single point mutation was consistent with other studies, which showed that removal of complete β -helical turns through mutations $\Delta 559-579$, $\Delta 580-607$, and $\Delta 608-628$ resulted in a full loss of activity (Fig. 3A) (Ivie et al. 2010). While Ivie et al. explained this finding by hypothesizing that this region was important for protein folding, the present study suggests that attenuation was a result of ablated binding activity. Notably, three out of the four mutant residues found to be significantly attenuated in this study were located within the $\Delta 559-579$ and $\Delta 580-607$ β -helical turn deletions in this study. While this finding is consistent with our results, it is notable to

observe that improper protein folding in these regions remains a possibility, though this would not be expected as a result of a single point mutation.

Further validation of experimental results comes from prior studies of m1 and m2 variations within the p55 domain, which here suggested that the major region influencing middle region tissue tropism is located within the 35 most N-terminal amino acids of the p55 domain (fig. 4)(Ji et al. 2000; Skibinski et al. 2000). In these studies, only modest differences in activity were observed for other m1 to m2 substitution mutations in HeLa cells, with no significant change observed in RK13 or AZ-521 cells following middle region m1 to m2 substitutions. Contrary to these previous results, however, K607A and W603A showed significant decreases in activity when mutated, despite having mutations vary similar to those present in m2 VacA. While this could possibly be explained by the identity of the amino acids substituted in the m2 allele, position 603 is an alanine in m2 VacA, as in the present mutation. Position 607 in m2 type VacA is a glycine residue. This mutation would not normally be expected to cause large differences in activity as a result of the large degree of similarity between glycine and alanine residues. These results suggested an alternative phenotype than previously observed.

Identification of mutant residues within the putative SM binding site confirmed that these tryptophan residues were necessary for toxin activity, but did not directly show that they were involved in cell binding. To determine if the attenuated activity observed in the p55 tryptophan mutations was a result of decreased cell binding, CELISA binding assays were conducted using the p55 mutants W577A and W603A. As suspected, the results of CELISA suggested that the attenuation in toxin activity was caused by decreased cell binding. Compared to wild type toxin, which had a K_d of 54 nM, W577A and W603A VacA had K_d values of 142 nM and 248 nM, respectively (fig. 15). These findings indicate that the putative binding pocket identified in the

mid region of the p55 domain of VacA is a cell binding site (Garner et al. 1996). Though the mid-region of p55 was previously suspected to contain a VacA binding site, this finding is the first to identify specific amino acids involved in cell binding interactions. The attenuated binding shown for W603A is of particular significance because this same mutation is present in the less virulent m2 *vacA* allele, suggesting that this single amino acid substitution could largely account for the decreased pathogenicity of *H. pylori* strains expressing this allele (fig. 15).

Chapter 4: Conclusions

As one of the primary virulence factors and a main determinant in *H. pylori* associated disease risk, understanding the pathology of VacA is of great importance to understanding the fundamental cause of *H. pylori* associated disease (Salama et al. 2001). The identification of VacA binding site residues is of particular interest due to the strain associated differences in *H. pylori* virulence that may be a result of deviations in tissue tropism between VacA binding site alleles (Iwamoto et al. 1999). Beyond contributing to the understanding of *H. pylori* pathogenicity, a characterized VacA sphingomyelin(SM)-binding site would be the first instance of such a binding motif in a bacterial toxin and could serve as a basis for the investigation of other systems.

In this study, molecular modeling suggested that SM-binding in other proteins occurs primarily through tryptophan rich solvent exposed motifs present in unstructured loops between ordered domains (fig. 9). A similar putative binding pocket was identified within the mid-region of the p55 domain of VacA and molecular distances between aromatic residues within the identified aromatic cluster were consistent with the cation- π interactions believed to be responsible for binding the quaternary amine present in the phosphorylcholine head group of SM (fig. 10, table 2). Further, core residues within the VacA putative SM binding site had substantial sequence homology to a previously identified phosphorylcholine binding site in actinoporin-like proteins (fig. 11).

To elucidate the contribution of the putative binding site residues to VacA cell binding, sequence alignment was done using VacA alleles known to have differences in cell binding (fig. 12). Unsurprisingly, many of the putative binding site residues were mutated in the less virulent

m2 VacA allele shown to have attenuated binding to HeLa cells. Beyond supporting the hypothesis that the putative binding site is involved in cell binding, this finding suggested that the residues identified within the site may represent important differences between alleles and could play a role in determining VacA cell specificity. While AZ521 cells are not the most sensitive to m1/m2 allelic variation, the investigation of differences in other cell lines presents an exciting opportunity for future investigation.

In order to test the hypothesis that the SM binding site is composed of a tryptophan rich aromatic cluster, scanning mutagenesis was used to mutate all tryptophan residues within VacA. Results of vacuolation and CELISA assays showed that mutants of tryptophan residues present within the putative binding pocket (W577A and W603A) showed a 4.5 and 7.3 fold attenuation in activity, respectively, as well as substantial attenuation in cell binding (Fig. 14-15). This result was of particular significance as a result of W577 being present within a region shown to have sequence homology with a known actinoporin phosphorylcholine binding site (Fig. 11).

To further characterize the putative SM-binding pocket of VacA, 10 new mutants were selected based on molecular modeling and sequence comparison (table 2). In addition to residues present within the putative binding site, several residues were selected from another aromatic cluster on the same face to exclude the possibility of a secondary binding site (fig. 13D). Vacuolation assays on the novel mutants suggested that mutation of residues within the secondary site did not attenuate toxin activity, but mutation of a positive residue within the putative binding site, K607A, led to a 2 fold loss of activity when mutated, suggesting that the cationic side chain may be involved in interactions with the negatively charged SM-phosphate group (fig. 14).

Chapter 5: Future Work

Recombinant expression systems are a terrific tool for studying mutated proteins, but refolded proteins do not always have the same structure as the native form. To confirm that the attenuation observed in mutant proteins is not a result of improper folding, protease sensitivity assays will be used to show similar degradation profiles between native, wild type recombinant, and mutant recombinant proteins. Further confirmation of recombinant protein structure can be achieved through the use of circular dichroism to indicate protein secondary structure (Clarke 2011). In addition to confirming that no improper folding is observed between wildtype and recombinant VacA, these same techniques can be used to confirm that point mutations are not causing changes in protein folding. Further, it must be shown that the C-terminal His-tag does not affect VacA activity. This will be accomplished by measuring the activity of VacA following thrombin cleavage of the His-tag. Determining that recombinant proteins have similar structure to native VacA and confirming that the His-tag does not affect activity are both important quality control steps to ensure that any difference in activity observed is solely a result of induced mutations.

To further characterize the SM binding site of VacA, additional novel mutants generated close to the putative binding pocket will be screened for activity using visual vacuolation assays (Table 2). These experiments will further elucidate residues within the binding site which are involved in interactions with SM, and results will be used to generate double and triple mutants. While a full loss of activity has not yet been observed in mutants, this strategy will allow the cumulative contributions of active site residues to be determined. To confirm that any attenuation observed is a result of decreased cell binding, CELISA will be performed on each mutant with attenuated activity. While CELISA indicates the extent of cell binding, it is not a direct measure

of SM binding. To prove that mutants are attenuated in SM binding, a SM ELISA will be performed to directly measure the binding of VacA SM adhered to the surface of a 96-well plate. A final CELISA will be done following incubation of cells with varying concentrations of sphingomyelinase in order to degrade cell membrane SM, or following the addition of different concentrations of exogenous SM in order to determine the dependence of toxin cell binding on the presence of membrane SM (Gupta et al. 2010).

Along with determining the location of the VacA SM-binding site, the molecular interactions involved in this binding will be investigated. Previous studies have shown that cation- π interactions often occur between aromatic residues and quaternary amines such as the one present on the head group of SM (Doughtry 2007). While data from CELISA and ELISA can be used to show that mutants are attenuated in SM binding, tryptophan to alanine mutations are not sufficient to conclude that binding occurs through cation- π interactions. To test whether or not cation- π interactions are necessary for SM binding, the mutation of binding site residues from one aromatic residue to another can be used. This strategy is based on the fact that there are distinct differences in the strength of cation- π interactions between aromatic functional groups, with tryptophan having the strongest binding followed by phenylalanine and tyrosine (Gallivan et al., 1999). If cation- π interactions are being used in SM binding, then tryptophan and phenylalanine to tyrosine mutations of binding site residues will decrease SM binding less than the same mutation to alanine. A similar strategy can be employed for determining whether a charged group such as the lysine residue K607 is involved in ionic interactions through the mutation of this residue to a neutral or acidic amino acid such as glutamate.

The finding that several of the mutants identified as a potential part of the putative SM binding site (F461, F555, F572, Y574, F594, W603, and F626) are mutated in m2 VacA

alleles suggests that changes in the SM binding site may be responsible for allelic variation in VacA activity, changes in VacA cell type specificity, as well as differences *H. pylori* strain pathogenicity. AZ-521 cells are not as sensitive to differences between m1 and m2 VacA alleles, and subsequent studies of these mutants on alternative cell lines would be of extreme interest. To further validate whether these mutations are responsible for the decreased activity and binding observed in m2 VacA alleles, site-directed mutagenesis could be used to mutate m1 aromatic residues to the m2 analogue amino acid. Following mutagenesis, the cell specific activity can be tested using visual vacuolation assays and binding can be tested in wild type and mutant VacA using CELISA.

Though the VacA SM-binding site has yet to be fully characterized, initial activity and binding assays are consistent with structural and sequence alignment based predictions for the location of the SM-binding site. The aromatic cluster identified in VacA is the first SM-binding site found in a bacterial toxin as well as the first time a cell binding site has been characterized in VacA. This finding confirms previous studies that have implicated p55 as the binding domain of VacA, gives important structural information about the interaction of VacA with the plasma membrane of eukaryotic cells, and provides the first evidence of an actinoporin like phosphorylcholine binding site in a bacterial toxin. In addition, this study suggests that differences in the SM-binding site between VacA alleles may account for deviations in tissue tropism, toxin virulence, and overall pathogenicity between *H. pylori* strains.

Figures and Tables

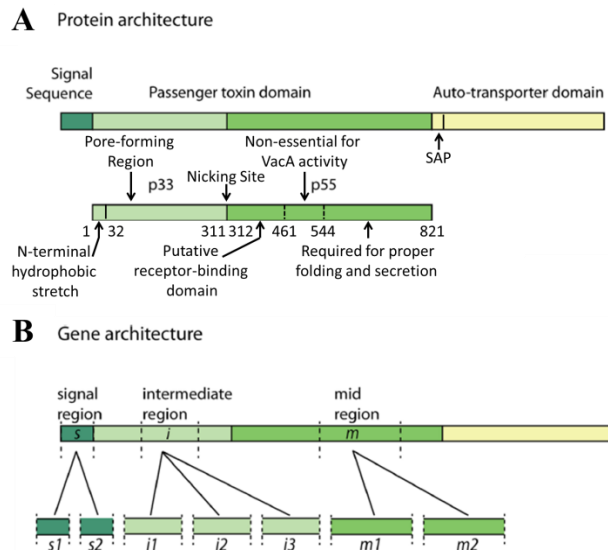


Figure 1. A. VacA gene organization. VacA is initially expressed as a 140-kD protoxin that is cleaved into a mature 88 kD toxin. The 88 kD mature VacA toxin is susceptible to proteolytic cleavage, which yields 33 kD and 55 kD putative domains. Each domain is further characterized based on hypothesized functional role. **B. VacA allelic variation.** The *vacA* gene has 3 main regions where substantial diversity is observed, the signal recognition region (s-region), the intermediate region (i-region) and the mid-region (m-region). Each regions has a number of families of alleles (s1/s2, i1/12/13, and m1/m2), which form mosaic forms of *vacA* through homologous recombination between strains. (Figure adopted from Palframan et al. 2012)

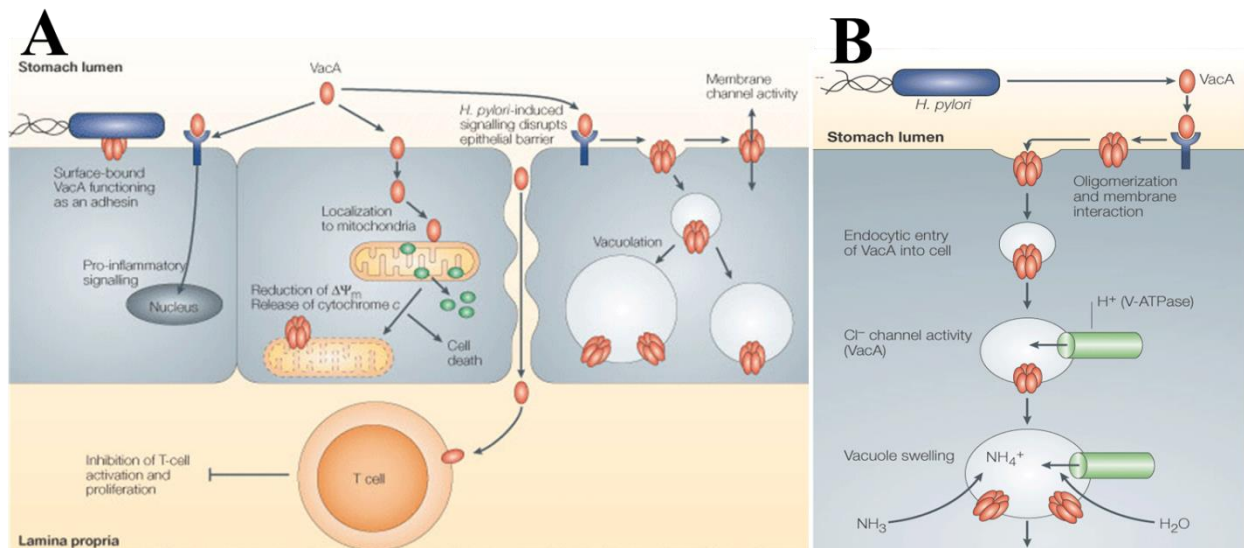


Figure 2. A. Multifunctional effects of VacA. The effects of VacA include anion selective channel dependent processes including permeabilization of the plasma membrane, causing the release of cytosolic factors, permeabilization of the mitochondrial membrane, which leads to the release of calcium and cytochrome C, and vacuole formation. VacA also has channel-independent activities including the stimulation of pro-inflammatory signaling and acting as an adhesin for *H. pylori* attachment to cells. In addition, *H. pylori* produces a number of factors that disrupt the tight junctions between cells and could allow VacA to pass through the epithelial barrier to the lamina propria, where it inhibits the activation and proliferation of T lymphocytes (Cover et al. 1997, Fiocca et al. 1999, Patal et al. 2002, Szabo et al. 1999, Willhite et al. 2004, Galmiche et al. 2000, Gebert et al. 2003). **B. Mechanism of VacA induced vacuole formation.** Following cell binding, VacA oligomerizes, inserts into the membrane, and uses a clathrin-independent pathway to enter the cell. As the endocytic vesicle progresses to a late endocytic vesicle, VacA begins allowing anions such as chloride to enter the vesicle. The increased anion concentration stimulates vacuolar proton pump ATPase activity, which lowers the pH of the vacuole (Cover et al. 2001, Czajkowsky et al. 1999). With a decreased pH, membrane permeable weak bases such as ammonia are protonated within the vesicle and trapped. The increase in concentration of these protonated bases leads to a hypertonic condition within the vesicle and an influx of water, which causes swelling of the vesicle and the formation of vacuoles. (Figure adopted from Cover, T. and Blanke, S., 2005)

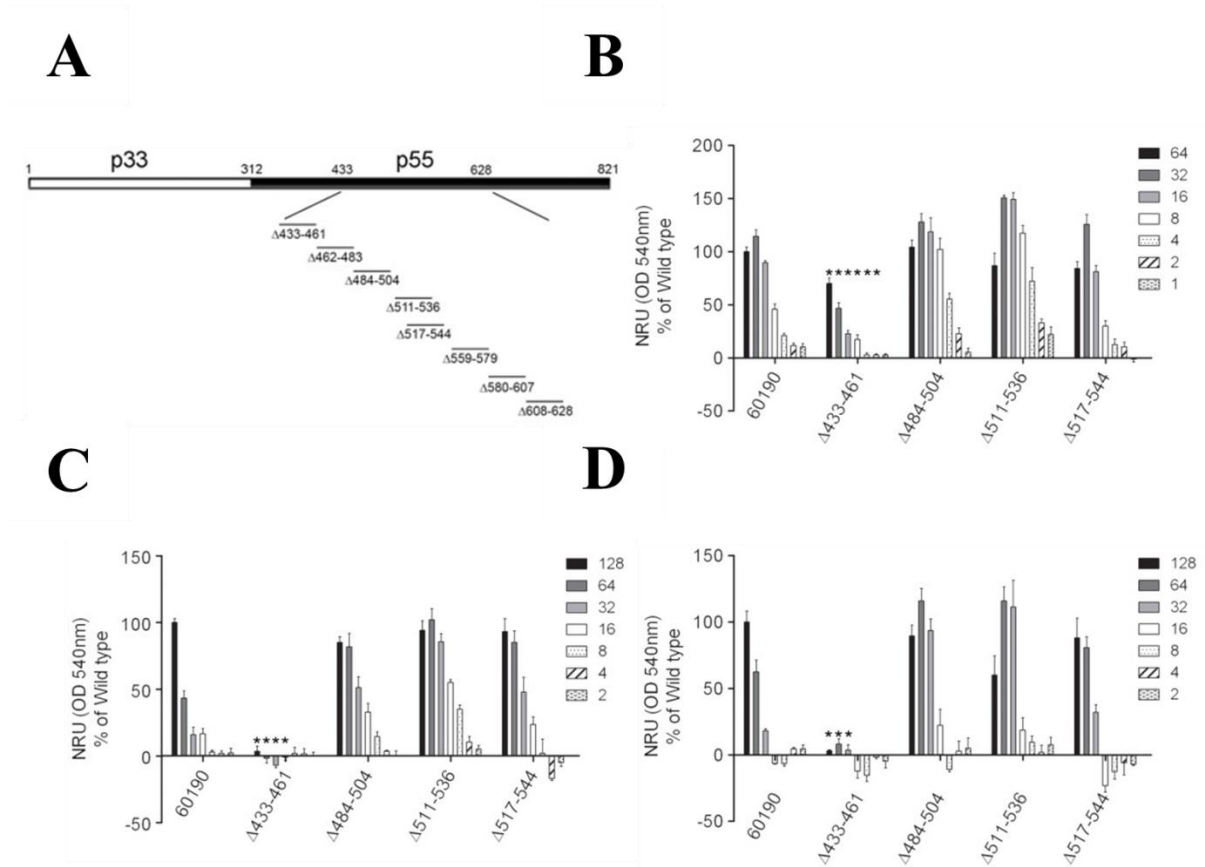


Figure 3. A. Diagram of the full-length 88 kDa. Mutations encoding single coil deletions within the β -helix of the p55 domain were introduced into the *H. pylori* chromosomal *vacA* gene by natural transformation and allelic exchange. The relative position of each coil deletion is indicated. **B-D. Vacuolating cytotoxic activity of mutant proteins.** Wild-type *H. pylori* strain 60190 and transformed mutant strains were grown in culture. Secreted VacA proteins were normalized as described in Methods. Serial two-fold dilutions of VacA-containing preparations were added to HeLa cells (B), RK13 cells (C), and AZ-521 cells (D). Neutral red uptake assays were used to measure relative vacuolating activity. Results represent the mean $\pm$ SD from triplicate samples, expressed as a percent of wild-type VacA neutral red uptake. * indicates that $p \leq 0.02$ as determined by Student's t-test compared to wild type VacA. (Figure adopted from Ivie et al. 2010)

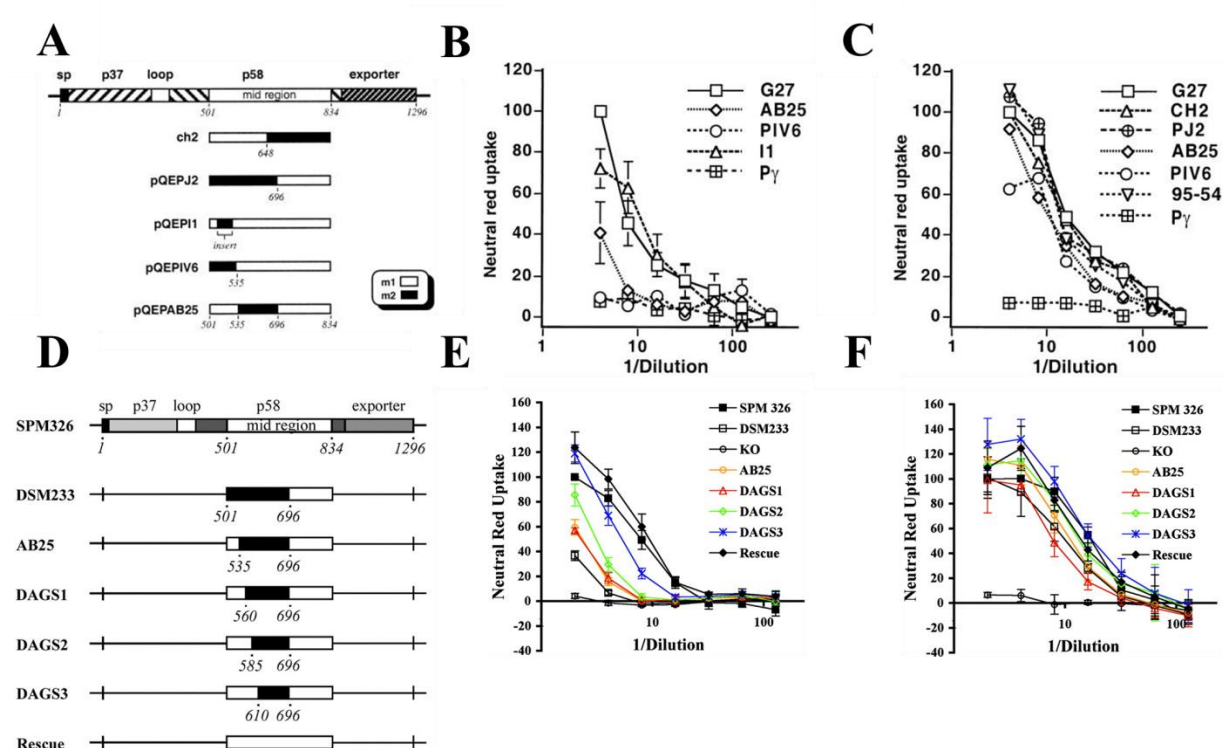


Figure 4. A. Schematic representation of the constructed chimeras. The shaded boxes show replacement of m1 vacA sequence by m2 sequences. Amino acid positions are numbered starting from the initiation codon of the vacA gene (11638). Substitution mutations of m1 to m2 sequences are shown by dark boxes representing these regions. **B. Neutral red uptake assays of HeLa.** Cells were treated with water extracts containing the chimeric VacA proteins. Results of the vacuolation of HeLa cells are normalized based on wild type activity. **C. Neutral red uptake assays of RK13 cells.** (parts A-C adopted from Ji et al. 2000) Results of the vacuolation of RK13 cells are normalized based on wild type activity **D. Schematic representation of chimeras.** Substitution mutations of m1 to m2 sequences are shown by dark boxes representing these regions. The VacA amino acid numbering is based on the initiation codon of the vacA gene from strain SPM326. **E. Neutral red uptake assay of RK13.** HeLa cells treated with growth culture supernatants from the VacA chimeric strains concentrated 10-fold using 50% saturated ammonium sulfate. Data is normalized to the lowest concentration and are the averages of four individual assays. Error bars show standard deviation between assays. **F. Neutral red uptake assay of RK13.** RK13 cell neutral red assays were performed as in part E. above. (parts D-F adopted from Skibinski et al. 2000)

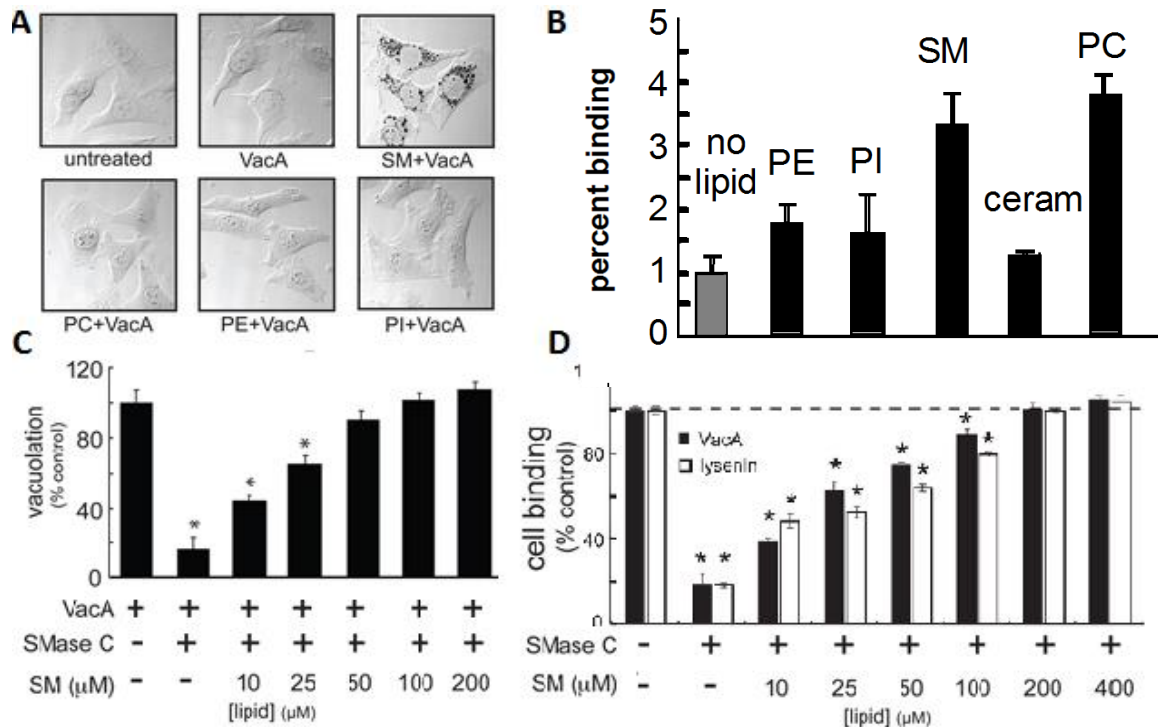


Figure 5. A. VacA activity increases in the presence of extracellular SM. HeLa cells were treated with 200 μ M PC, PE, PI, or SM for one hour, then were inoculated with 50 nM VacA and incubated for 24 hrs. Cells were then dyed with neutral red and visualized through DIC imaging. **B. VacA binds to SM and PC preferentially.** In brief, the wells of a 96 well plate (costar) were coated with 50 μ l of phospholipid antigen (50 fmol). Wells were then blocked with PBS containing 30% BSA, and were incubated with VacA in PBS with 3% BSA for 2 h at room temperature. Wells were then washed with PBS, and bound VacA was detected using primary antibodies diluted 1/1000 with 3% BSA-TBS incubated for 2 h, followed by incubation with anti-rabbit IgG bound to streptavidin for an additional 2 h. TMB ultra (Thermo Scientific) was then added to each well and samples were incubated at room temperature for 30 min, and 2 M sulfuric acid was added to each well. Plates were read with microplate reader (Spectronic 20 Genesys spectrophotometer, Sigma-Aldrich) at a wavelength of 450 nm. Binding was normalized to the percent binding relative to control wells without any lipids. **C. The addition of extracellular SM following SMaseC incubation rescues VacA activity.** HeLa cells were pretreated with 100 mU/mL SMase C for 1 h, were washed, and were incubated with 0–200 μ M SM for 1 hr. Cells were then washed and incubated with 100 nM VacA for 24 hr. Neutral red uptake assay was used to measure vacuolation. Asterisks indicate statistically significant differences in neutral red uptake. **D. Extracellular SM rescues lysenin and VacA cell binding following SMaseC incubation.** HeLa cells were pretreated with 100 mU/mL SMase C at 37 $^{\circ}$ C and under 5% CO₂ for 1 hr. Cells were then washed and chilled to 0 $^{\circ}$ C. 0–200 μ M SM was then added and incubated for 1 hr. Cells were then incubated with 10 nM Alexa Fluor 488-labeled VacA or 1 μ M lysenin-GFP at 4 $^{\circ}$ C. After a 1 h incubation, protein binding was quantified using flow cytometry. Asterisks indicate statistically significant differences in VacA binding to cells treated with SMase C and mock-pretreated cells. (Figures adopted from Gupta et al., 2010)

Ceramide

N-acyl-sphing-4-ene

Sphingomyelin (SM)

N-acyl-sphing-4-ene-1-phosphocholine

Phosphatidylcholine (PC)

1,2-dihexadecanoyl-sn-glycero-3-phosphocholine

Phosphatidylethanolamine (PE)

1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine

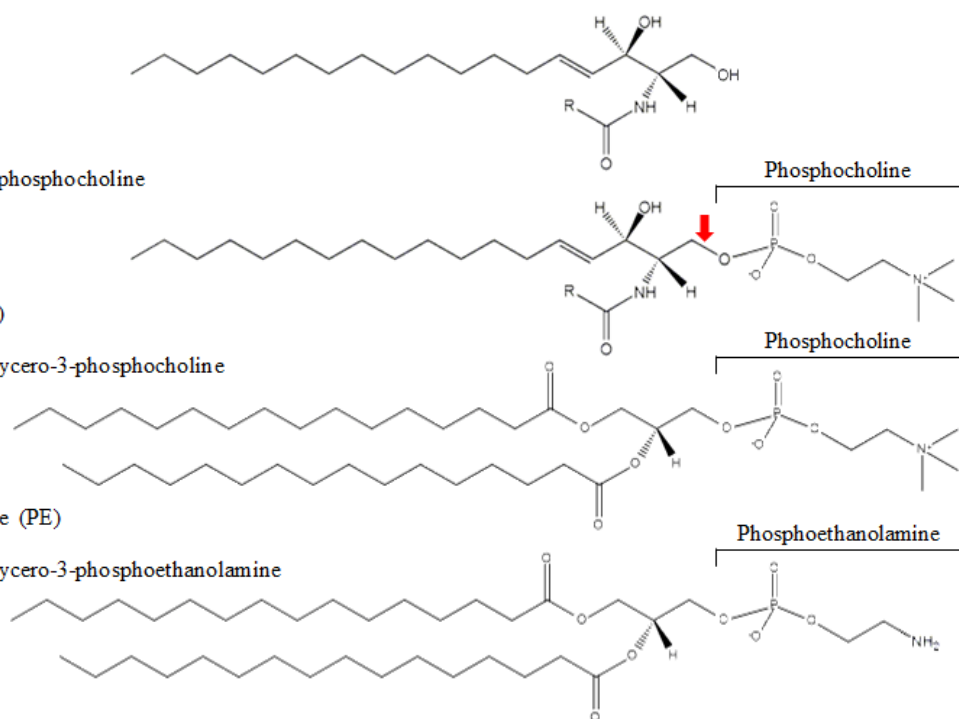


Figure 6. Membrane lipid structure. The structure of pertinent membrane lipids were found using the lipidomics gateway (<http://www.lipidmaps.org/data/structure/>). The red arrow indicates the site of SMaseC cleavage.

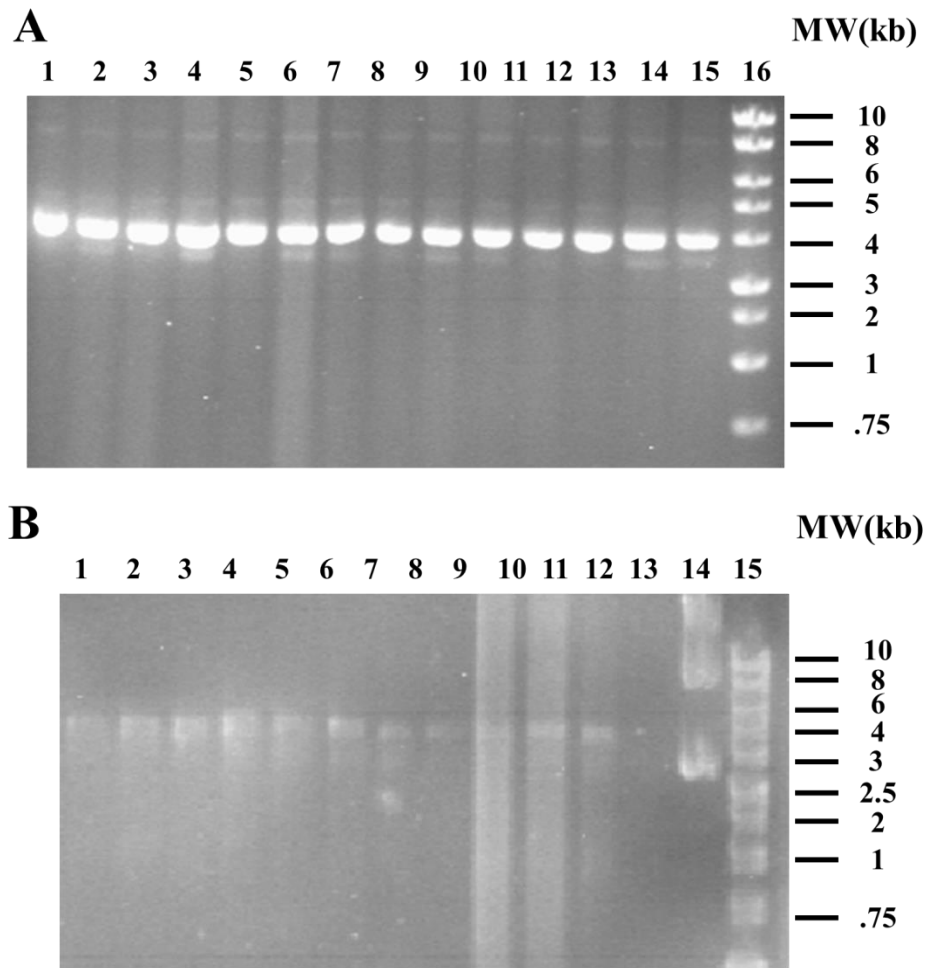


Figure 7. A) Plasmid purification. Purified pET20b-vacA-p55 was run on a 1% agarose gel in TBE buffer for 50 min at 100 V. A 1 kb DNA ladder (Promega) was used as a standard, and ethidium bromide was used to visualize DNA. Lanes were loaded as follows: 1) Y428A, 2) F461A, 3) F483A, 4) R552A, 5) F555A, 6) Y573A, 7) Y574A, 8) W577A, 9) Y579A, 10) W603A, 11) K607A, 12) F626A, 13) W773A, 14) wild type, 15) 1 kb DNA ladder. **B) PCR products of site-directed mutagenesis.** PCR was performed using PFU-Ultra high fidelity polymerase (Agilent Technologies) and desalted primers from Integrated DNA technologies. Purified pET20b-vacA-p55 was used as template for each reaction. PCR products were run on a 1% agarose gel in TBE buffer for 50 min at 100 V following a 3 h DNP1 digestion. A 1 kb DNA ladder (Promega) was used as a standard, and ethidium bromide was used to visualize DNA. Lanes were loaded as follows: 1) W603A, 2) Y428A, 3) Y579A, 4) Y573A, 5) F626A, 6) Y574A, 7) R552A, 8) F461A, 9) F483A, 10) K607A, 11) F555A, 12) pET20b-vacA-p55 with DNP1 digest, 13) pET20b-vacA-p55 without DNP1, 14) 1 kb DNA ladder. The pET20b-vacA-p55 in lane 13 is not representative of normal plasmid purity (Figure 8).

Mutation	Sequence
W49A	5'CCCCAGATAAACCCGATAAAGTTGCGCGCATTCAAGCAG 3' 5'CTGCTTGAATGCGCGCAACTTTATCGGGTTTATCTGGGG 3'
W80A	5'CCAGTAAGATTGATGGAGGTGCGGATTGGGGGAATGC 3' 5'GCATTCCCCCAATCCGCACCTCCATCAATCTTACTGG 3'
W82A	5'GATTGATGGAGGTGGGATGCGGGGAATGCCGC 3' 5'GCGGCATTCCCCGCATCCCAACCTCCATCAATC 3'
W90A	5'GAATGCCGCTACGCATTATGCGATCAAAGGCGGGC 3' 5'GCCCCGCTTTGATCGCATAATGCGTAGCGGCATTTC 3'
W96A	5'GGATCAAAGGCGGGCAAGCGAATAAGCTTGAAGTGG 3' 5'CCACTTCAAGCTTATTCGCTTGCCCGCCTTTGATCC 3'
W225A	5' GTTAAATTAAATGGCAATGTGGCGATGGGCGTGGTTCGCAATACG 3' 5' CGTATTGCAAACGGCCCATCGCCACATTGCCATTTAATTTAAC 3'
W283A	5' GGCACACTGGATTTGGCGCAAAGCGCGGGG 3' 5' CCCC GCGCTTTGCGCCAAATCCAGTGTGCC 3'
W301A	5'CAGGGCTTCTTGGCGCGGGGCTCAAACAAGC 3' 5'GCTTGTTTGAGCCCCGCGCCAAGAAGCCCTG 3'
W577A	5' GTTATAGATGAGTTTTACTATAGCCCTGCGAATTATTTTGACGCTAGG 3' 5' CCTAGCGTCAAAATAATTCGCAGGGCTATAGTAAAACTCATCTATAAC 3'
W603A	5' CGCTTCTTCAACCCAGAAAACCCTGCGGGCACATCAAAAC 3' 5' GTTTTGATGTGCCCCGAGGGTTTTCTGGGGTTGAAGAAGCG 3'
W773A	5' CAAGTATCTTATCGGTAAAGCAGCGAAAAATATAGGCATCAGTAAAACG 3' 5' CGTTTTACTGATGCCTATATTTTTCTGCTGCTTACCGATAAGATACTTG 3'
F555A	5' CACTAGGTCAATCGCGTCTGGGGGTGTC 3' 5' GACACCCCCAGACGCGATTGACCTAGTG 3'
Y428A	5' CAAGTGGGTGGCGCGGCTTTGGCAGG 3' 5'5' CCTGCCAAAGCCGCGCCACCCACTTG 3'
Y579A	5' CCCTTGAATGCTTTTG CGC 3' 5' GCGTCAAAAGCATTCCAAGGG 3'
Y573A	5' GGT ATAGATGAGTTTGCCTATAGC 3' 5' GCTATAGGCAAACTCATCTATAACC 3'
F626A	5' GCGGTCATGGACTATAGTCAAGCGTCAAATTTAACCATTTCAGGGG 3' 5' CCCCTGAATGGTTAAATTTGACGCTTGACTATAGTCCATGACCGC 3'
Y574A	5' GGTTATAGATGAGTTTTACGCGAGCCCTTGGAATTATTTTG 3' 5' CAAAATAATTCCAAGGGCTCGCGTAAAACTCATCTATAACC 3'
R552A	5' GCGTTTGAAACTGGCACTGCGTCAATCTTTTCTGGGGG 3' 5' CCCCCAGAAAAGATTGACGCAGTGCCAGTTTCCAAACGC 3'
F461A	5' CAATAACGATATTAGTCTGGGAAGAGCGGTGAATTTAAAGGTGGATGCTCAT 3' 5' ATGAGCATCCACCTTTAAATTCACCGCTCTTCCCAGACTAATATCGTTATTG 3'
F483A	5' GTATTGATACGGGTAATGGTGGTGCGAACACCTTAGATTTTAGTGTTG 3' 5' CACCACTAAAATCTAAGGTGTTTCGCACCACCATTACCCGTATCAATAC 3'
K607A	5' CCAGAAAACCCTTGGGGCACATCAGCGCTCATGTTTAATAATCTAACCCTG 3' 5' CAGGGTTAGATTATTAACATGAGCGCTGATGTGCCCCAAGGGTTTTCTGG 3'

Table 1. PCR primers. PCR primers were synthesized by IDT technologies and were analyzed for melting temp and the formation of secondary structures using OligoAnalyzer. Primers were designed with the mutated codon at the center of each complimentary primer pair.

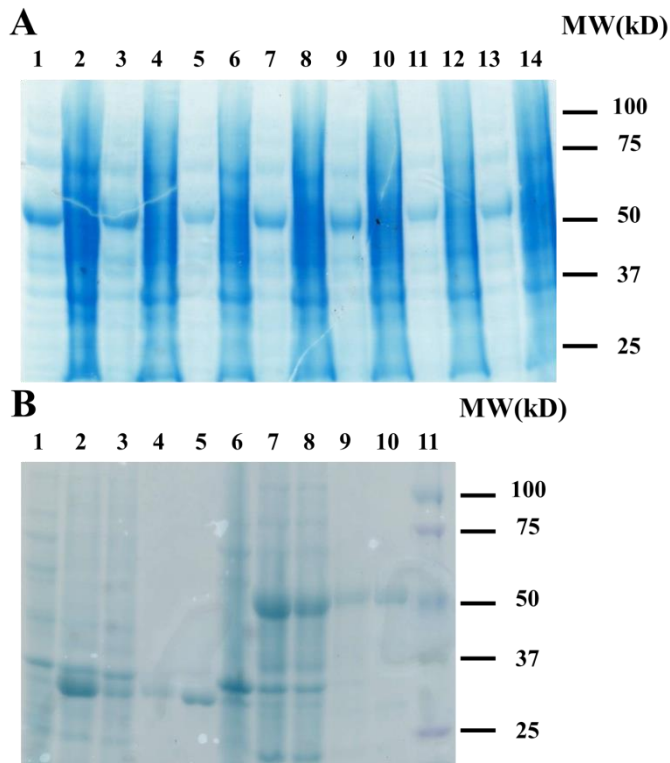


Figure 8. A. p55 expression gel. BL21 transformed cells were selected for maximum p55 production following induction with .125 mM IPTG. Samples were lysed, centrifuged, and inclusion bodies were solubilized in an 8 M urea solution. Proteins were run on a 10% polyacrylamide gel at 100 V for 1 h with solubilized inclusion bodies (SIB) loaded before the soluble cell extract (SCE). (Lane: 1)p55 W461A-1 SIB , 2) p55 W461A-1 SCE, 3)p55 W461A-2 SIB , 4) p55 W461A-2 SCE, 5)p55 W461A-3 SIB , 6) p55 W461A-3 SCE, 7)p55 W461A-4 SIB , 8) p55 W461A-4 SCE, 9)p55 W626A-1 SIB , 10) p55 W626A-1 SCE, 11)p55 W626A-2 SIB ,12) p55 W626A-2 SCE, 13)p55 W626A-3 SIB , 14) p55 W626A-3 SCE) **B. Purification of p55 and p33 VacA.** Cells induced with IPTG and harvested were lysed using sonication, pelleted by centrifugation at 16,000 x *g* for 20 min, and the supernatant containing solubly expressed proteins was collected (S lane 1 and 6). Inclusion bodies containing insoluble VacA were then brought into solution by the addition of a Tris buffer (10 mM) containing urea (8 M) and sodium phosphate (100 mM). Samples were spun down a second time at 16,000 x *g*, and supernatant was collected (IS lane 2 and 7) and incubated with Talon metal affinity resin overnight at 4 °C. VacA bound resin was then added to a gravity flow column and flow through was collected (FT lane 3 and 8). Beads were then washed with the same buffer at pH 9 (W lane 4 and 9) then eluted with buffer at pH 4.0 (E lane 5 and 10). Kaleidoscope (Bio-Rad) protein standard was used as a marker in lane 11. Lanes were loaded with samples as follows: 1) p33 S, 2) p33 IS, 3)p33 FT, 4)p33 W, 5)p33 E, 6)p55 S, 7)p55 IS, 8)p55 FT, 9)p55 W, 10)p55 E, 11) protein standards.

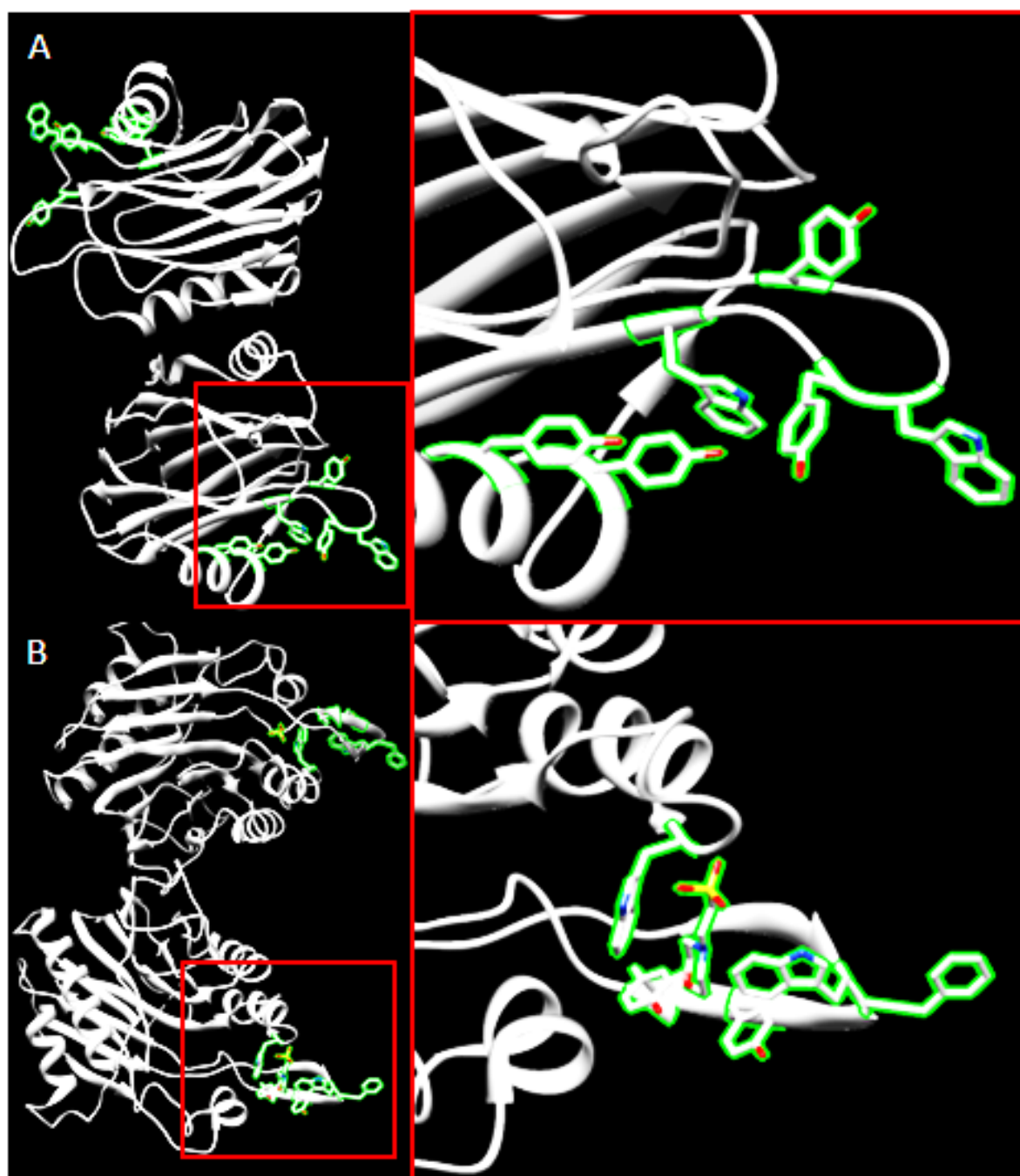


Figure 9. Molecular modeling of SM-binding proteins. UCSF chimera (<http://www.cgl.ucsf.edu/chimera>) was used to visualize the crystal structure of SM-binding proteins. **A. The SM binding site of equinatoxin II.** The SM binding site of equinatoxin 2 is composed of an aromatic cluster located in a solvent exposed loop between β -sheet domains (Kwiatkowska et al. 2007) (PDB ID: 1IAZ). **B. The SM binding site of Bc-SMase.** As with equinatoxin II, the SM binding site of Bc-SMase is composed of an aromatic cluster in a solvent exposed loop connecting β -sheet domains (Monastyrnaya et al. 2010) (PDB ID: 2DDT).

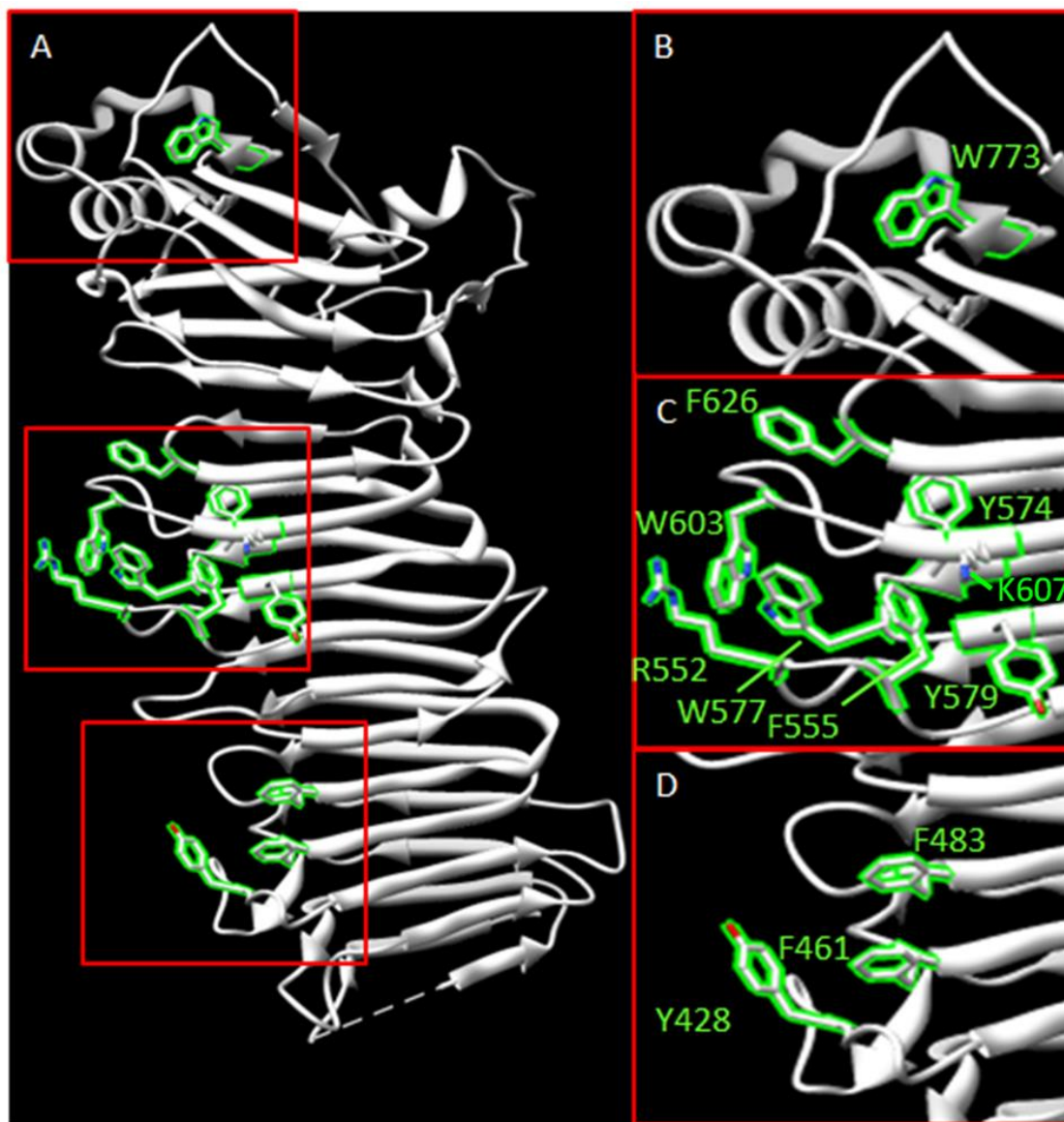


Figure 10. A. Molecular modeling of the p55 domain of VacA. The crystal structure of VacA of the m1 allele from strain 60190 was viewed using UCSF chimera software (PDB ID: 1IAZ) (Kwiatkowska et al. 2007). **B. W773 is not located within the putative SM-binding site.** W773 is located in a loop between ordered domains in a site distinct from the putative SM-binding site. **C. The putative SM-binding site of VacA is a solvent exposed aromatic cluster between β -sheet domains.** The putative SM binding site of VacA includes residues F555, Y573, Y574, W577, W603, and F626, which form an aromatic cluster with residues from several solvent exposed loops, as well as R552 and K607, which may be involved in ionic interactions with the SM phosphate group. **D. A secondary small aromatic cluster is present on a similar face as the putative SM-binding site.** Aromatic residues Y428, F461, and F48 exist in solvent exposed loops between β -sheet domains in a site distinct from the main putative binding site.

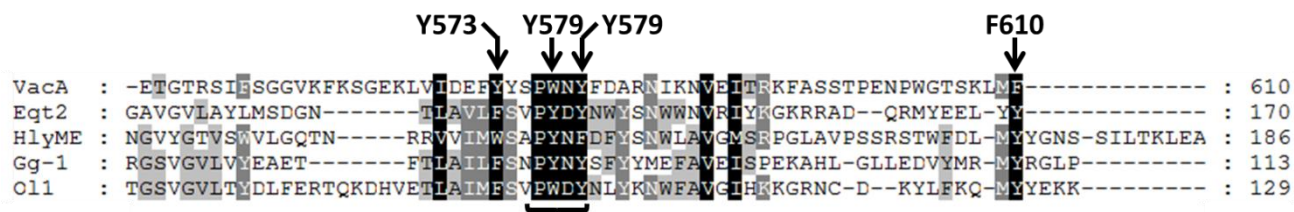


Figure 11. The VacA putative binding site shares homology with actinoporin-like protein SM binding site. This alignment was prepared using GeneDoc (<http://www.psc.edu/biomed/genedoc>). Black shading indicates that amino acids conserved in more than 90% of sequences, dark grey shading with light lettering indicate amino acids are conserved in more than 60% of sequences, and light grey shading with black letters indicate amino acids conserved in more than 40% of sequences. The SM binding domain of actinoporin-like proteins is characterized by a conserved P-[WYF]-D motif (Jamroz et al. 2010), the majority of which is conserved in the mid region of VacA. Residues of interest are indicated include Y573, W577, Y579, and F610, which were identified through structural analysis as part of the putative SM-binding site. The area with sequence homology to the PC binding motif of actinoporins-like proteins is labeled with brackets.

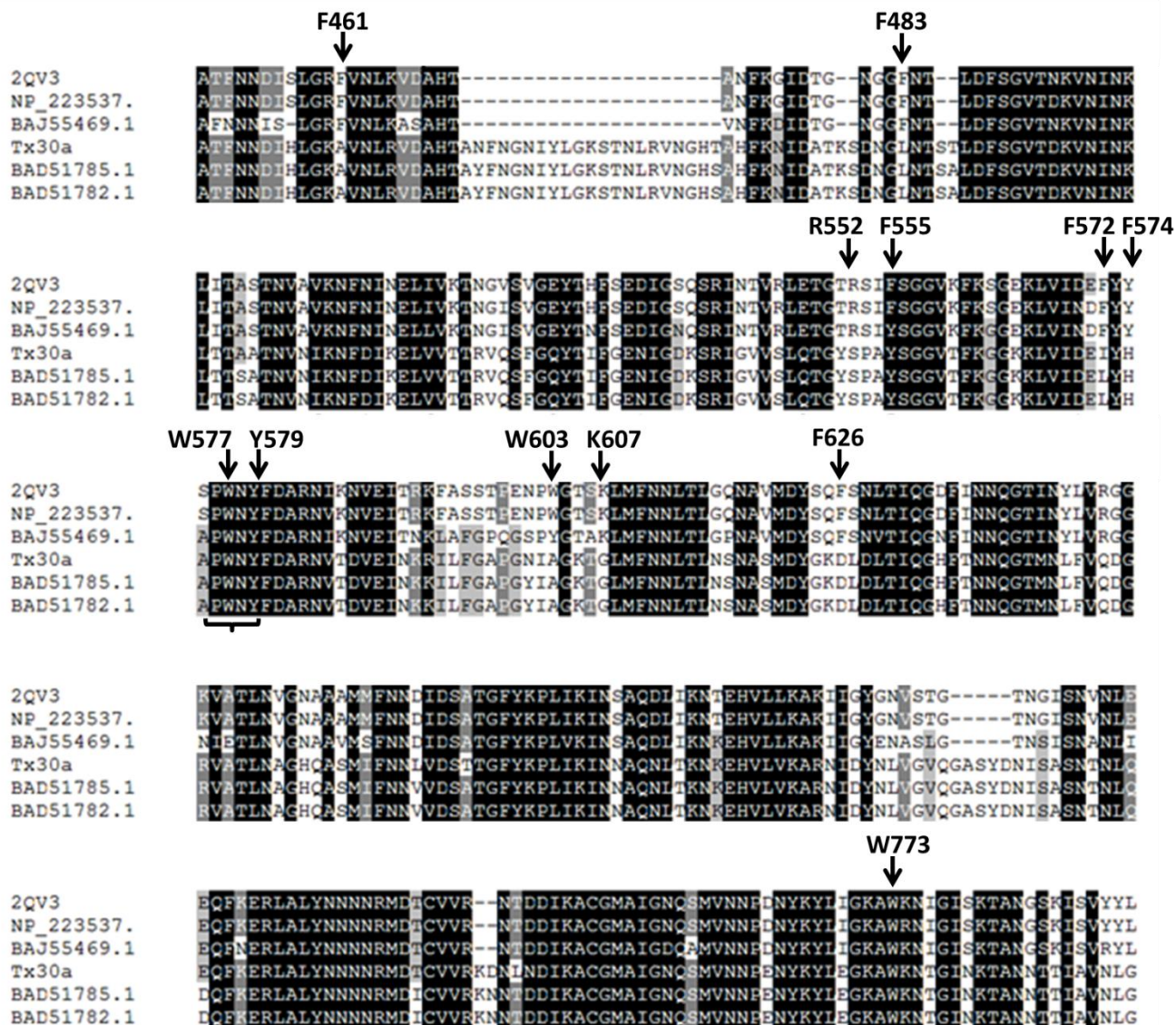


Figure 12. Sequence alignment of VacA mid-region. This alignment was prepared using GeneDoc (<http://www.psc.edu/biomed/genedoc>). Black shading indicates that an amino acid is conserved in more than 90% of sequences, dark grey shading with light lettering indicate amino acids are conserved in more than 60% of sequences, and light grey shading with black letters indicate amino acids conserved in more than 40% of sequences. Residues of interest are underlined below the sequence and labeled according to the residue number of VacA from strain 60190. The area with sequence homology to the PC binding motif of actinoporins-like proteins is labeled with brackets. Aligning m1 (m1b:F16 m1a: J99 and 60190) and m2 (Tx30a, OK144, OK129) alleles of p55 allowed for the identification of aromatic residues mutated in the less virulent m2 strains. Accession numbers at GenBank database with *H. pylori* corresponding strain in parenthesis are: BAJ55469.1(F16), NC_000921.1(J99), 2qv3(60190), AAA86834.1 (Tx30a), BAD51782.1 (OK129), BAD51785.1(OK144).

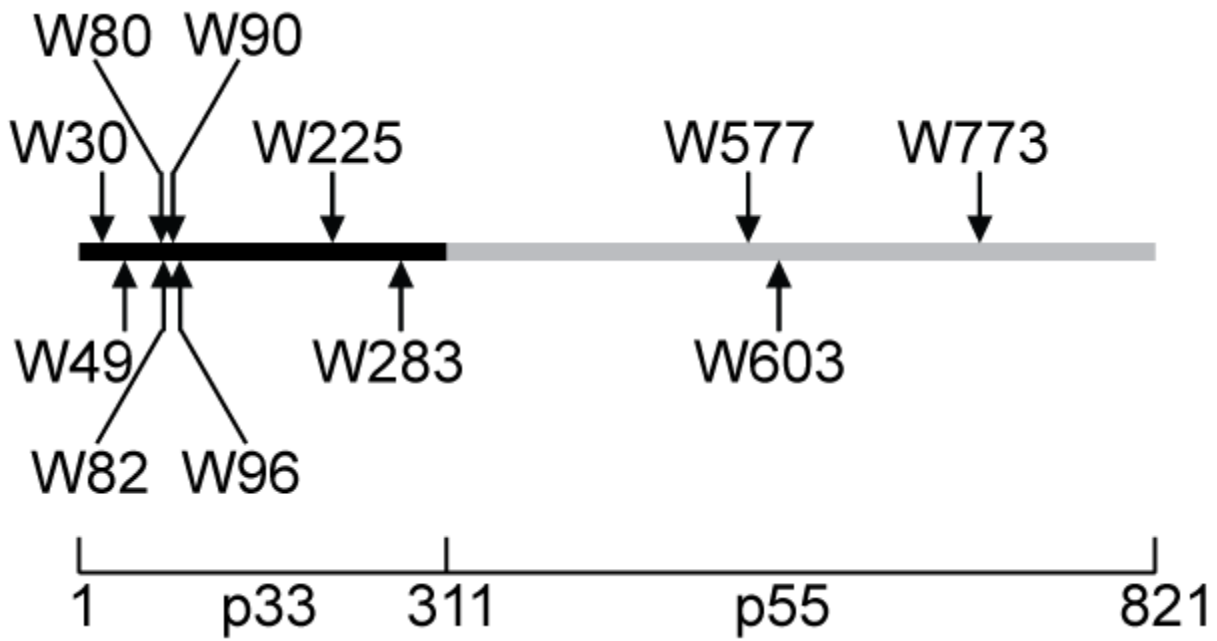


Figure 13. Distribution of VacA tryptophan residues. The p33 subunit (black) contains 8 tryptophan residues, and the p55 subunit (grey) contains 3 tryptophan residues. Amino acids are numbered in accordance with the mature VacA toxin in *H. pylori* strain 60190. (Figure modified from one made by Matt Pierson)

Residue Number	Distance from Primary Site	Consensus m1 Amino Acid	Consensus m2 Amino Acid
Y428	23.6*	Tyr	Tyr
F461	26.3*	Phe	Ala
F483	22.5*	Phe	Leu
R552	6.0	Arg	Ser
F555	7.4	Phe	Tyr
Y573	13.8	Tyr	Tyr
Y574	11.1	Phe	His
W577	1.7	Trp	Trp
Y579	13.1	Tyr	Tyr
W603	1.7	Trp	Ala
K607	13.2	Lys	Gly
F626	8.2	Phe	Asp
W773	40.3*	Trp	Trp

Table 2 Distance and homology analysis of non-tryptophan p55 mutants. UCSF chimera was used to view the p55 domain of VacA from strain 60190 (PDB ID: 2Qv3). The distance to the putative binding site was calculated from the center of aromatic rings or the nucleus of charged atoms to a point within the putative binding site equidistant from W603 and W577. Bolded residues indicate a difference between m1 and m2 alleles. An asterisk indicates that the residue of interest is not within the main putative binding site. Sequence alignments were prepared using GeneDoc (<http://www.psc.edu/biomed/genedoc>) and the sequence of m1 and m2 VacA alleles were compared (fig. 12).

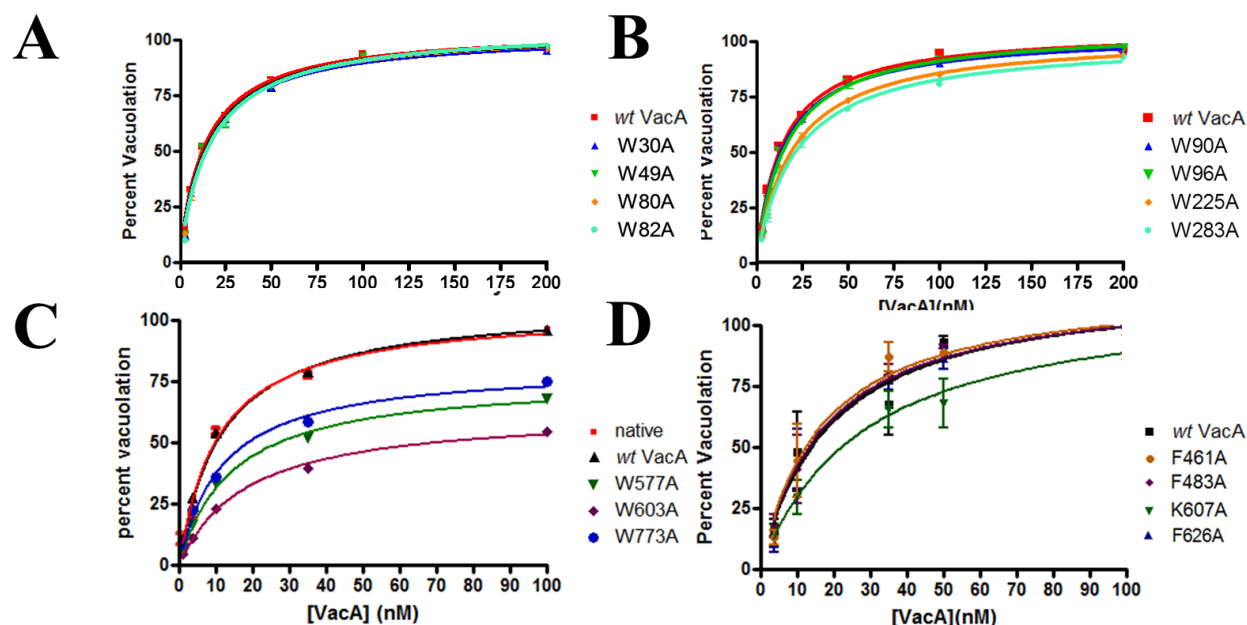


Figure 14. Visual vacuolation assay of VacA mutants. A. Mutation to W30, W49, W80, and W82 does not cause significant attenuation in VacA vacuolating activity. Prior to inoculation, individual recombinantly expressed VacA subunits were mixed in a 1:1 ratio for 1 hat room temperature. Following subunit mixing, .3 M HCl will be used to lower the VacA pH to 2.0 for 30 min at 37 °C. VacA was then be neutralized using .3 M NaOH and added to AZ521 cells at concentrations between 1-200 nM and allowed to incubate for 8 min MEM (Sigma) supplemented with 2 mM glutamine(Sigma), 100 U/mL penicillin(Sigma), 1 mg sulfate/mL streptomycin (Sigma), 10% FBS (JRH Biosciences), and 5 mM NH₄Cl. Phase contrast microscopy was then used to count 50 cells in each well to determine the percent of cells containing one or more vacuoles. **B. Mutation to W90, W96, W225, and W283 does not cause significant attenuation in VacA vacuolating activity.** The protocol described in above was used. **C. Visual vacuolation assay of p55 W577A, W603A, and W773A.** The protocol described in above was used. **D. Visual vacuolation assay of p55 F461A, F483A, K607A, and F626A.** The protocol described in above was used. (Figure 14A and B were rendered from data acquired by Piotr Karwowski)

p55 Mutants		p33 Mutants	
Mutant	Vac ₅₀ (nM)	Mutant	Vac ₅₀ (nM)
native	10 ± 4	wild type recombinant	12 ± 4
F461A	13 ± 5	W30A	13 ± 1
F483A	13 ± 4	W49A	13 ± 1
W577A	30 ± 6	W80A	14 ± 1
W603A	68 ± 7	W82A	13 ± 1
K607A	19 ± 3	W90A	14 ± 1
F626A	14 ± 3	W96A	14 ± 1
W773A	21 ± 4	W225A	19 ± 1
W603A/W773A	86 ± 13	W283A	20 ± 1
W577A/W603A	103 ± 12	W225A/W283A	28 ± 4

Table 3. Vac50 Summary. AZ521 were treated as in Fig. 3. Visual analysis of vacuolation was performed using a phase contrast microscope. Experiments were done with at least 3 biological replicates each done in triplicate. Bold lettering indicates that a significant attenuation was observed. (p33 mutant activity reported from data acquired by Piotr Karwowski)

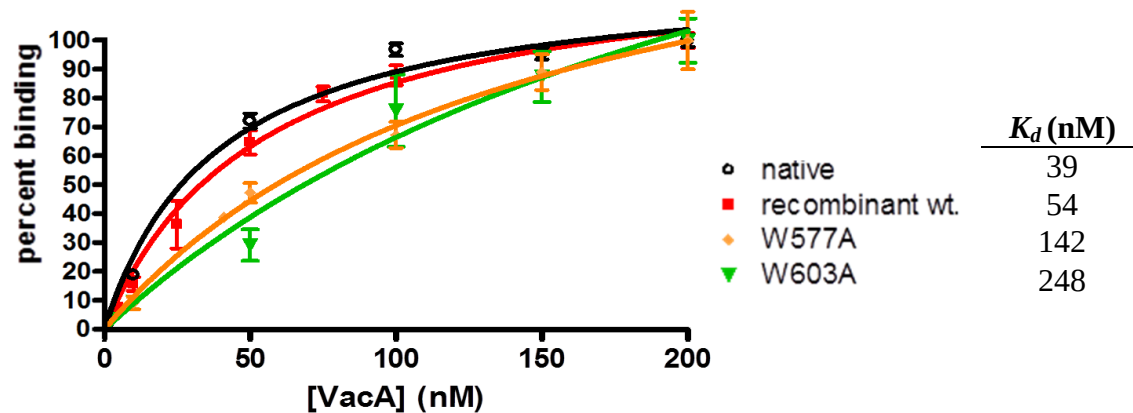


Figure 15. CELISA assay of W577A and W603A cell binding. In short, VacA was biotinylated then added to cells at 4 °C following recombination and acid activation as in fig. 15. Following a 30 min incubation at 4 °C, cells were fixed with 2% paraformaldehyde in PBS for 30 min, and washed with PBS. Streptavidin-HRP was then added at a 1:15,000 dilution to conjugate to biotin bound VacA. Following additional washes, TMB ultra (Thermo Scientific) was added to each well, samples were incubated at room temperature for 30 min, and 2 M sulfuric acid was added to each well. Plates were read with microplate reader (Spectronic 20 Genesys spectrophotometer, Sigma-Aldrich) at a wavelength of 450 nm, and binding was normalized based on the signal observed in Native VacA (200 nM). Experiments were done with at least 3 biological replicates each done in triplicate. The bold K_d value for p55 W603A VacA indicates a statistically significant difference in VacA binding to cells.

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