Investigating the Mechanism of Action of Potato Extract against *Helicobacter pylori*

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List of Abbreviations

Amp	Ampicillin
APS	Ammonium persulfate
bp	base pair
BSA	Bovine serum albumin
cag PAI	cag pathogenicity island
CagA	Cytotoxic associated antigen A
CFU	Colony forming unit
Cla	Clarithromycin
DNA	Deoxyribonucleic acid
EDTA	Ethylendiaminetetraacetic acid
FBS	Foetal bovine serum
h	Hour
IL8	Interleukin-8
INF	Interferon
kDa	Kilo daltons
LB medium	Luria-Bertani medium
Le	Lewis antigen
Lev	Levofloxacin
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
Met	Metronidazole
MIC	Minimum inhibitory concentration
min	Minutes
NF-κB	Nuclear Factor-kappaB
OD	Optical density
OMP	Outer membrane protein
P.E	Potato extract
PBP	Penicillin binding protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PI	Propidium iodie
PMN	Polymorphonuclear cells
PPI	Proton pump inhibitor
Rif	Rifampin

RNA	Ribonucleic acid
SD	Shine-Dalgarno Sequence
SDS	Sodium dodecyl sulphate
SHP-2	Protein tyrosine phosphatase
T4SS	Type IV secretion system
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TEMED	N,N,N’,N’, tetramethylethylenediamine
Tet	Tetracycline
Th	T helper cell
TNFα	Tumor necrosis factor-alpha
Tris	Tris(hydroxymethyl)aminomethane
UDP	Uridine diphosphate
V	Volts
v/v	Volume per volume
VacA	Vacuolating cytotoxin A
w/v	Weight per volume
WT	Wild type
µ	Micro
µg	Microgram
µl	Microlitre

Abstract

Submitted by TEMITOPE ADENIKE ADEYEMI for the Degree of Doctor of Philosophy at The University of Manchester and entitled 'Investigating the Mechanism of Action of Potato Extract against *Helicobacter pylori*' in July, 2015.

Helicobacter pylori is a Gram-negative bacterium that is the major cause of many upper gastrointestinal diseases such as gastritis and peptic ulcer disease. It has the unique ability of colonising the human gastric mucosa. Failure in complete eradication of *H. pylori* in infected patients, mainly due to antibiotic resistance, has necessitated the development of better therapeutics, especially from natural sources.

In this study, extract of Maris piper potatoes were obtained and evaluated for antibacterial activity against *H. pylori in vitro*. Antibacterial activity was carried out against antibiotic-sensitive and clinical antibiotic-resistant *H. pylori* strains, as well as a range of Gram-negative bacteria including *Helicobacter* and *Campylobacter* species, using the viable count method. Result of the antibacterial assays indicated that potato extract is bactericidal against *H. pylori* lab strain as well as clinical antibiotic-resistant strains, with minimum inhibitory concentration at 15.6 mg/ml. Potato extract also showed minimal antibacterial activity against other Gram-negative bacteria tested, with minimum inhibitory concentration at 250 mg/ml. The effect of the extract on the morphology of *H. pylori* was also observed by transmission electron microscopy (TEM). TEM analysis of potato extract-treated *H. pylori* cells showed disruption of the morphology of *H. pylori*, characterized by separation of the outer membrane from the inner membrane and loss of cell shape. Potato extract also caused hyperpolarisation of *H. pylori* plasma membrane; however it is unclear whether the membrane active pumping activity is affected.

Mutants of *H. pylori* that are resistant to potato extract were generated as a means to identify the target of potato extract within the *H. pylori* genome. Genome sequence analysis led to the discovery of a hypothetical protein, encoded by HP0603 gene, which may be involved in inducing resistance to potato extract.

The results obtained in this study provide great insights into the anti-*H. pylori* activity of potato extract. Overall, this study suggests the potential use of potato extract as a source of anti-*H. pylori* agents; and stimulates further studies into identifying its mechanism of action.

Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright Statement

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My deepest gratitude is given to my parents for funding my Ph.D study and for their constant emotional and financial support always.

Chapter 1

Introduction

1.1 *Helicobacter pylori*

The genus *Helicobacter* belongs to the epsilon (ε) sub-division of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae* (Kusters et al., 2006). *Helicobacter* species are subdivided into; the gastric and enterohepatic (non-gastric) species. The gastric *Helicobacter* species colonise the gastric mucosal surface while the enterohepatic species colonise the lower gastrointestinal tract (Kusters et al., 2006). *Helicobacter pylori*, which is a gastric species, was initially referred to as *Campylobacter pyloridis* or *C. pylori* and was re-classified upon identification of features different to that of *Campylobacter*, such as sheathed flagella, distinct outer membrane structures, fatty acid composition and a unique 16S rRNA sequence (Goodwin et al., 1989). *H. pylori* is phylogenetically closely related to Gram-negative bacteria such as *Wolinella* and *Campylobacter* genus (Wesley et al., 1995) as well as other *Helicobacter* species such as *H. acinonychis*, *H. nemestrinae*, and *H. felis* as shown in Figure 1.1 (Percival et al., 2004; Solnick et al., 2006).

1.1.1 Genetics

The complete genome of several *H. pylori* strains, including strains 26695 and J99 have been sequenced and are available on the NCBI genome database. *H. pylori* strain 26695, originally isolated from a patient suffering from gastritis in the United Kingdom in the 1980s (Eaton et al., 1989), was the first to be sequenced by the Institute for Genomic research. Genome sequence reveals a circular chromosome of approx. 1.67 M base pairs in

size, 1,590 predicted coding sequences and an average G + C content of 39 mol% (Tomb et al., 1997). In comparison, strain J99 isolated in 1994 from a patient with duodenal ulcer in the United states, has 1.64 Mbp with 1,495 predicted open reading frames (ORF), and equal G + C content as strain 26695 (Alm et al., 1999). By comparing genomes of these unrelated *H. pylori* isolates, Alm et al (1999) found 117 and 89 genes unique to strain 26695 and J99, respectively. In both genomes, about 60 % of ORFs have been assigned a predicted function. By comparison to the genome sequence of *C. jejuni* NCTC11168, *H. pylori* has 24 % of its genes conserved in other genomes and 17 % of its genes unique to it with no homologues in the database (Alm and Noonan, 2001).

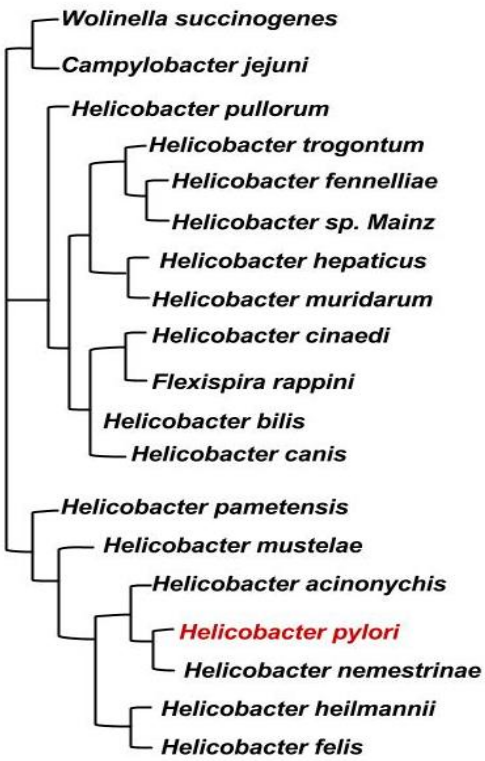


Figure 1.1 Tree showing *H. pylori* in relation to other bacteria in the epsilon subdivision of *Proteobacteria* (The length of the branches does not indicate the phylogenetic relationship). (Adapted from: Percival et al., 2004).

By sequencing of 16S rRNA genes and analysis of protein profile, *H. acinonychis* is considered the most closely related species to *H. pylori*. *H. nemestrinae* is considered a junior heterotypic synonym of *H. pylori* as 16S rRNA sequencing shows that it is less than 1 % different from most *H. pylori* strains (Solnick et al., 2006).

Sequence analysis of *H. pylori* genome reveals that it has well-characterised systems for DNA processing, pH tolerance, motility, as well as genes encoding numerous outer membrane proteins, urease, and *cag* pathogenicity island (Tomb et al., 1997). This has provided some insights into its pathogenicity.

H. pylori is highly polymorphic having several genetically diverse strains, with strain variation observed within the gastric niche of a single host (Israel et al., 2001). Sequencing studies have shown genetic diversity of orthologous genes between *H. pylori* isolates, showing that it is rare for orthologous genes from *H. pylori* strains to possess the same DNA sequence (Garner and Cover, 1995; Kansau et al., 1996). In addition to this, a high occurrence of recombination between gene alleles of *H. pylori* strains has been reported (Suerbaum et al., 1998; Achtman et al., 1999). Genetic diversity within *H. pylori* populations has been attributed to point mutations, insertions, deletion or large substitution of one or more genes or gene segments (Blaser and Berg, 2001). As a consequence, each *H. pylori* host carries a distinct *H. pylori* strain (Kansau et al., 1996), possibly exhibiting different genetic and phenotypic characteristics.

1.1.2 Morphology, growth and cultural characteristics

H. pylori is a Gram-negative, spiral shaped or curved bacterium measuring 0.2 – 1.2 µm in diameter and 1.5 – 3.5 µm in length (Jones et al., 1985; Goodwin et al., 1985). Coccoid forms of *H. pylori* have also been reported in old cultures (Solnick and Vandamme, 2001; Chan et al., 1994). This coccoid form is thought to be a mechanism enabling *H. pylori* to survive unfavourable growth conditions (Azevedo et al., 2007); however its viability is still questionable (Kusters et al., 1997; Cellini et al., 1994; Cellini, 1996; Eaton et al., 1995). *H. pylori* cells are motile with a corkscrew-like motion

aided by the presence of multiple sheathed flagella (Geis et al., 1993). The cells are microaerophilic, growing best in low oxygen conditions of 2 – 5 % and requires 5 – 10 % CO_2 at an optimum temperature of 37°C (Kusters et al., 2006). Despite inhabiting the acidic gastric lumen, *H. pylori* is a neutralophile growing in conditions between pH 6.0 – 8.0 (Sidebotham et al., 2003), and optimally at neutral pH (Scott et al., 2002). In culture, *H. pylori* appears as small (0.5 – 1 mm), circular, smooth translucent to pale greyish colonies on blood agar (Andersen and Wadstrom, 2001). Like other gastric *Helicobacter* species, it is urease, catalase and oxidase positive (Kusters et al., 2006).

1.2 *Helicobacter pylori* as a disease pathogen

H. pylori is known to be the only bacterium with the unique ability of successfully colonising the human gastric mucosa (Blaser and Atherton, 2004). It is able to achieve this, partly, by secreting urease which functions to neutralize the stomach acidic content, thereby providing itself a suitable environment for survival (Nakamura et al., 1998).

Since its identification in 1983, *H. pylori* has been identified as the cause of common upper gastrointestinal diseases such as gastritis and peptic ulcers (Marshall and Warren, 1984). It has also been implicated in causing gastric cancer (Forman et al., 1991); and as a result, it has been classified as a type 1 human carcinogen by the World Health Organisation (Blanchard et al., 2004). *H. pylori* colonises about half of the world population, with less than 40 % prevalence in the developed countries and more than 80 % prevalence in developing countries (Pounder and Ng, 1995; Rothenbacher and Brenner, 2003; Perez-Perez et al., 2004; Ford and Axon, 2010). This variation in prevalence is thought to be a result of improved socioeconomic

status, high living standard and improved hygiene in the western world (Malaty and Graham, 1994). Though the exact mode of transmission is largely unknown (as reviewed in: Kusters et al., 2006), colonisation with *H. pylori* normally occurs during early childhood and persists for a lifetime with the absence of treatment (Malaty and Graham, 1994). In infected individuals, *H. pylori* causes active chronic gastritis which progresses in 10 – 20 % of affected persons to peptic ulcer disease, mucosa-associated lymphoid tissue (MALT) lymphoma or gastric adenocarcinoma, which is the second-leading cause of cancer-related deaths worldwide (Blaser and Berg, 2001; Israel and Peek, 2001; Peek and Blaser, 2002). *H. pylori* infection is mostly asymptomatic due to the fact that the ability of *H. pylori* to cause gastric diseases is dependent on host genetic factors, virulence of the colonising *H. pylori* strain, and host environmental factors such as hygiene and diet (Atherton, 2006).

1.3 Colonisation and virulence factors

H. pylori has the ability to resist and evade innate host defence mechanism against bacterial colonisation such as production of gastric acid, peristalsis and constant shedding of the gastric mucus layer. It is also able to cause diseases and evade host immune response. It achieves this through intrinsic mechanisms such as motility, urease production, adherence to gastric epithelial cells, and via cytotoxic activities of virulence factors.

1.3.1 Motility

With the pH of the gastric lumen at pH < 2.0 and that of the gastric mucus layer ranging from pH 2.0 to neutral pH (Quigley and Turnberg, 1987), *H. pylori* escapes the acidic pH of the lumen by entering into the gastric mucus

layer. Also, in order to remain in the gastric mucosa, *H. pylori* requires motility to escape being flushed out as a result of constant renewal and secretion of the mucus gel. It is able to do this with the presence of a tuft of flagella (Geis et al., 1993), which aids its movement in viscous solutions like the gastric mucus layer of the human stomach (Hazell et al., 1986). It was previously believed that the flagella facilitate the movement of *H. pylori* by propelling the bacteria into the mucus layer in a corkscrew-like motion. However, recent findings have shown that *H. pylori* is able to move freely in the mucus layer at high pH facilitated by the production of urease, which elevates the mucus pH thereby altering the viscosity of the gastric mucin gel (Celli et al., 2009). The flagella are made up of 4 – 6 polar-sheathed flagellum with terminal bulbs that are thought to protect each flagellum from acid depolymerisation (Geis et al., 1993). The flagella are made up of 3 components; filament, hook and basal body. The flagella filament is comprised of two polypeptide repeating flagellin subunits: FlaA- the major component; and FlaB- the minor component (Kostrzynska et al., 1991; Suerbaum et al., 1993; Josenhans et al., 1995). Both of which play essential roles in motility and pathogenesis (Habeeb et al., 2001). Eaton et al (1996) has demonstrated the importance of motility in colonisation and infection by *H. pylori*. They showed that in gnotobiotic piglets, FlaA-FlaB double mutant persisted for only 2 days; isogenic FlaA and FlaB mutants persisted for no more than 4 days; while the wild-type strain was still persistent 10 days after inoculation. As reviewed by Cover et al (2001), the flagella filament is connected to the hook which connects the filament to the basal body, a protein structure which serves as a proton motive force-driven motor that brings about rotation. The motor and base of the filament are linked to a molecular signal transduction cascade which senses chemical gradients and transmits signals of attraction or repulsion to the

flagella motor which then responds by changing the direction of rotation (as reviewed in: Josenhans and Suerbaum, 2002). This suggests the role of the flagella in the chemotaxis of *H. pylori* towards certain chemical gradients such as urea and bicarbonates, as motility and chemotaxis work collectively to enable bacteria to detect and move towards nutrients as well as maintaining a colonization niche (Josenhans and Suerbaum, 2002).

1.3.2 Urease activity

H. pylori produces a cytoplasmic enzyme, urease, which is a nickel metalloenzyme that is made up of two distinct subunits; UreA and UreB (Hu and Mobley, 1990; Dunn et al., 1990; Labigne et al., 1991). It catalyses the hydrolysis of urea, a nitrogenous human waste, to 2 moles of ammonia and 1 mole of carbon dioxide (Sachs et al., 2001). The principal function of urease is to protect *H. pylori* from acidic damage by utilizing ammonia, produced from urea hydrolysis, which directly neutralize the low gastric pH of < 2 (as reviewed in: Cover et al., 2001). Urease maintains the periplasmic pH of *H. pylori* at pH 6.2 as *H. pylori* is only able to survive, in the absence of urea, at pH 4.0 – 8.0 (Scott et al., 1998). Other functions attributed to urease are acid resistance through alteration of bacterial intra-cytoplasmic pH (Cover et al., 2001), and as a source of ammonia for nitrogen metabolism (Tomb et al., 1997). Ferrero et al (1992) has shown that urease is essential for successful *H. pylori* colonisation of the gastric mucosa by assessing the ability of wild-type strains and urease-negative mutants to colonise the gastric mucosa. Urease negative mutants have also been found to be unable to colonise the gastric mucosa after neutralization of the gastric pH by omeprazole treatment (Eaton and Krakowka, 1994), thereby suggesting an additional role of urease in colonisation. Nakamura et al (1998) has demonstrated the role of urease in the chemotaxis of *H.*

pylori under conditions that mimic the gastric mucus layer. They showed that the chemotactic motility of urease-positive strains towards attractants, such as urea, markedly increased in a highly viscous environment when compared to urease-negative strains; thereby suggesting a possible role of urease in facilitating the rotation of the flagella motor (Yoshiyama and Nakazawa, 2000). Urease production is said to be higher in *H. pylori* as compared to other microbes that synthesize it, with its synthesis accounting for 10 – 15 % of total protein synthesis in *H. pylori*(Bauerfeind et al., 1997). A large amount, 90 %, of urease is located intracellularly while it is claimed, 10 % is located on the cell surface, the origin of which is a controversial issue (Sachs et al., 2001). It is claimed that the origin of this extracellular urease is by secretion (Vanet and Labigne, 1998) while other studies claim it is by lysis of *H. pylori* cells (Scott et al., 1998; Marcus and Scott, 2001) due to the fact that urease was found to be located in the cytoplasm of freshly cultured cells and found on the outer membrane of cells in stationary growth phase. Though beneficial to *H. pylori*, urease can be detrimental as studies have shown that ammonia, produced in urea hydrolysis, generates certain NH_3-derived compounds such as monochloramine which has a direct cytotoxic effect on the gastric epithelial cells (Suzuki et al., 1992).

1.3.3 Outer membrane components

The cell envelope found in Gram-negative bacteria is composed of an outer membrane and an inner (cytoplasmic) membrane, and is different from the cell wall in Gram-positive bacteria. The outer membrane mainly comprises the lipopolysaccharide which is classified as an endotoxin (Moran, 1995b), outer membrane proteins, and porins. Outer membrane components in *H. pylori*, mainly lipopolysaccharide and membrane proteins have been

implicated and shown to contribute to the colonisation and virulence of *H. pylori*(Amieva and El-Omar, 2008; Moran, 2001b).

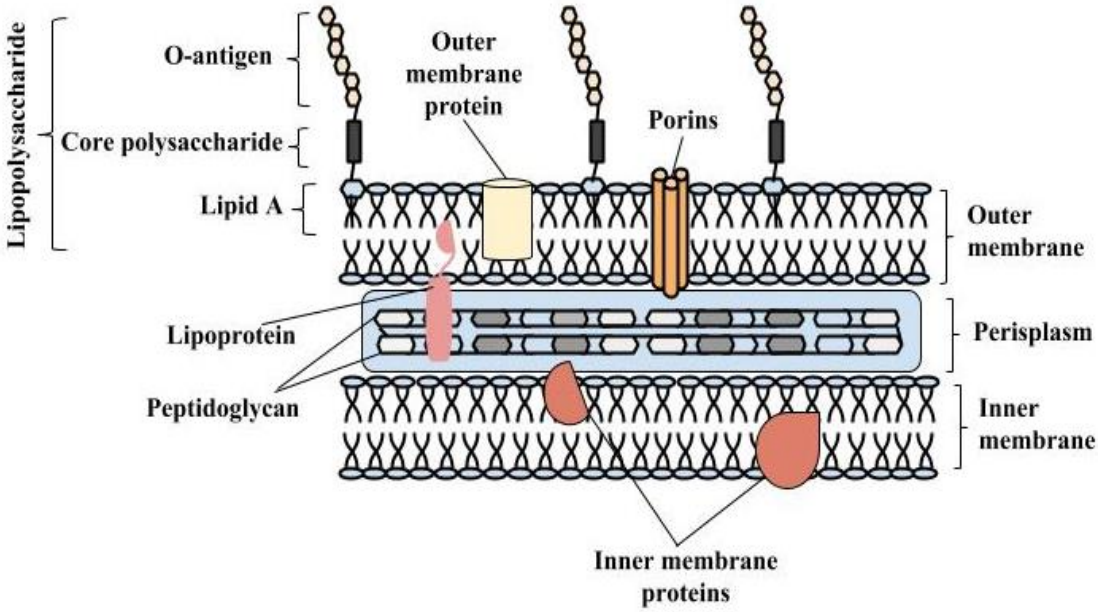


Figure 1.2 Diagrammatic representation of the cell wall in Gram-negative bacteria

In Gram-negative bacteria, the cell wall is comprised of phospholipid bilayers that make up the outer and inner membrane. The outer membrane contains lipopolysaccharides which are toxic to the host, and porins through which molecules diffuse. Between the outer and inner membrane is the periplasm, which houses the peptidoglycan layer to which lipoproteins are attached. Membrane proteins are located within the outer and inner membrane.

1.3.3.1 Outer membrane proteins

The ability of *H. pylori* to adhere to gastric epithelial cells is an important requirement for its colonisation of the gastric mucosa (Cover et al., 2001). *H. pylori* invade the gastric epithelial cells so as to avoid clearance due to constant mucus turnover, secretion of gastric juices and peristaltic movement of the gastric walls; thereby remaining mechanically attached and protected from the extracellular environment (Cover et al., 2001; Amieva and El-Omar, 2008). The ability to adhere to host cells is attributed

to outer membrane proteins, mainly adhesins (Falk et al., 1994), and *H. pylori* has been shown to contain over 30 genes expressing outer membrane proteins (OMP) (Amieva and El-Omar, 2008). The major OMPs involved in adherence are those that bind carbohydrate modifications in host cell glycoproteins (Amieva and El-Omar, 2008). These are: blood group antigen-binding adhesin, BabA, which binds to the fucosylated blood group antigen, Lewis-b (Le^b) (Ilver et al., 1998); and sialic-acid binding adhesin, SabA, which adheres to sialylated glycoprotein, sialyl-Lewis-x (sLe^x) (Mahdavi et al., 2002). Other adhesins identified are adherence-associated lipoprotein A and B (AlpA and AlpB), whose mutants were defective in colonising the stomach of guinea pigs (de Jonge et al., 2004); and *Helicobacter* outer membrane proteins, HopZ and HopH, encoding outer inflammatory protein A (OipA) which have been suggested to act as adhesins due to their role in mediating binding of *H. pylori* to AGS gastric adenocarcinoma cells in culture (Peck et al., 1999; Dossumbekova et al., 2006). Apart from the need to avoid clearance and remain in the gastric mucosa, *H. pylori* adheres to cells so as to damage the epithelium, access nutrients released due to epithelial damage, deliver toxins and induce inflammation (Amieva and El-Omar, 2008). Adhesive interactions have been shown to contribute to inflammation and progression of gastric disease (Prinz et al., 2001; Petersson et al., 2006; Necchi et al., 2007). *H. pylori* adhesin, SabA, has been shown to bind inflamed mucosa and activate neutrophils via selectin mimicry (Petersson et al., 2006), thereby confirming the role of adherence in disease progression. Adhesins have also been implicated in severe chronic gastritis, increased bacterial attachment and parietal cell loss (Guruge et al., 1998).

1.3.3.2 Lipopolysaccharide (LPS) outer membrane

Like other Gram-negative bacteria, the outer membrane envelope of *H. pylori* contains lipopolysaccharide (LPS), which is believed to play an important role in its virulence and pathogenesis (Moran, 1995a). LPSs are phosphorylated lipoglycans that are said to possess immune-modulating and immune-stimulating properties (Rietschel et al., 1994; Raetz and Whitfield, 2002). *H. pylori* produces a high molecular mass smooth-form (SF) LPS that is comprised of an outer saccharide moiety, made up of the O-polysaccharide chain and Oligosaccharide (OS) region, and a lipid moiety called lipid A (Rietschel et al., 1994; Moran, 1995b; Raetz and Whitfield, 2002). A low molecular mass rough-form (RF) LPS lacking the O-chain can also be synthesized by *H. pylori* (Rietschel et al., 1994; Moran, 1995b). Electrophoretic studies have shown that fresh clinical isolates of *H. pylori* produces SF-LPS with O-chains (Moran et al., 1992; Moran, 1995a) but serial sub-culturing on solid media leads to loss of the O-chain and production of RF-LPS (Moran et al., 1992; Walsh and Moran, 1997). However, the duration at which loss of O-chain expression is induced as a result of continuous sub-culturing on solid media, is strain-dependent (Moran, 1996).

The O-chain of *H. pylori* LPS expresses Lewis (Le) and related blood group antigens (Monteiro, 2001; Moran, 2001b). Screening of *H. pylori* strains, from various geographic region worldwide, using anti-Le antibodies as probes has shown that the expression of Le^x and Le^y determinant is present in 80 – 90 % of strains screened (Wirth et al., 1996; Heneghan et al., 2000). The expression of Le antigens in *H. pylori* has been implicated in the evasion of host immune response upon initial infection and in influencing bacterial colonisation and adhesion (Moran, 1999; Moran, 2001a). As

chronic infection progresses, Le expression is suggested to contribute to gastric auto-immunity which leads to gastric atrophy, a precursor state of gastric cancer (Heneghan et al., 2001; Hynes et al., 2005). With the expression of Le antigens on the surface of the gastric mucosa (Kobayashi et al., 1993), Wirth et al (1997) and Appelmelk et al (2000) proposed that this provides an escape for *H. pylori* from host immune response by preventing the formation of antibodies to antigens shared by the host and bacterium. Wirth et al (2006) has shown that the expression of Le^x or Le^y by *H. pylori* strains corresponds respectively to Le^a and Le^b blood group phenotypes of the host from which the individual strains were isolated. This observation, therefore, suggests that the expression of Le antigens by *H. pylori* is a mode of adapting to the gastric mucosa of the host (Moran, 2008). Also, the expression of Le^x in the O-chain has been shown to partly mediate the adhesion of *H. pylori* to the human antral gastric mucosa (Edwards et al., 2000), with the gastric receptor identified as the galactose-binding lectin, galectin-3 (Fowler et al., 2006). *H. pylori* LPS has the ability to bind laminin, an important extracellular glycoprotein located in the basement membrane, which plays an important role in cell adhesion (Valkonen et al., 1994). Slomiany et al (1991) showed that LPS-laminin binding inhibits the recognition of laminin by its receptor on the gastric epithelial cells; this interaction may play a role in the loss of gastric mucosal integrity (Moran, 1996).

1.3.4 Cytotoxic-associated antigen, CagA

CagA is a 120 – 145 kDa immunodominant protein synthesized in most *H. pylori* strains (Covacci et al., 1993; Tummuru et al., 1993). The gene, *cagA*, encoding this protein is localized at one end of the *cag* pathogenicity island (*cag* PAI), a 40 kb DNA segment containing 31 genes many of which

encodes a type IV secretion system (T4SS) (Censini et al., 1996; Tomb et al., 1997). The secretion system serves as a syringe for delivering bacterial proteins, CagA and peptidoglycan components, into the host cells (Covacci et al., 1999; Viala et al., 2004). The *cag* PAI is not expressed in all *H. pylori* strains, as only about 60 % of strains isolated in Western countries carry the DNA and almost all strains isolated in East Asia are *cag* PAI-positive (Mizushima et al., 2001). *cagA*-positive (*cag*$^+$) *H. pylori* strains are mostly associated with atrophic gastritis, peptic ulcer and gastric adenocarcinoma; and are said to be more virulent than *cagA*-negative (*cag*$^-$) strains (Blaser et al., 1995; Parsonnet et al., 1997; Hamlet et al., 1999). Using gerbil as a model, Ogura *et al* (2000) and Franco *et al* (2008) showed that less gastric inflammation and fewer gastric ulcer was observed when gerbils were infected with partially or completely disrupted *cag* PAI *H. pylori* strains as compared to the wild-type strains. Also, the deletion of CagA protein prevented gastric carcinogenesis associated with *H. pylori* in the gerbil model. The presence of cytokines, such as interleukin (IL)-1β, IL-2, IL-6, IL-8, IL-10 and Tumor necrosis factor-α (TNF-α), in the gastric mucosa of *H. pylori* infected persons is said to be at higher concentration than that of *H. pylori*-negative persons (Crabtree et al., 1991a; Crabtree et al., 1994). These cytokines are mostly pro-inflammatory, especially IL-8 that has potent stimulatory and chemotactic properties for neutrophils (as reviewed in: Cover *et al*, 2001). The gastric mucosa of patients infected with *cag*$^+$ strains is characterized by higher levels of IL-8 than that of those infected with *cag*$^-$ strains (Peek et al., 1995). This difference, partly, accounts for the intense neutrophilic mucosal inflammatory response in patients infected with *cag*$^+$*H. pylori* strains (Crabtree et al., 1991b). Tummuru *et al* (1995), Censini *et al* (1996) and Segal *et al* (1997) have shown that the direct contact of *H. pylori* with gastric epithelial cells in vitro results in synthesis of

pro-inflammatory cytokines, including IL-8. They also demonstrated that *H. pylori* strains containing the *cag* PAI stimulated the expression of epithelial IL-8 more efficiently than those lacking *cag* PAI.

The adherence of *cagA*-positive *H. pylori* strains to gastric epithelial cells results in external manifestation of the host plasma membrane phosphatidylserine, the inner leaflet-enriched phospholipid, at the site of bacterial attachment (Murata-Kamiya et al., 2010). Thus, indicating that CagA utilizes host plasma membrane phosphatidylserine as a receptor (Murata-Kamiya et al., 2010). Though the mechanism by which CagA is incorporated into the host cell is unknown, it has been reported that the interaction of CagL, a component of the T4SS, with $\alpha_5\beta_1$ integrin, a cell adhesion receptor on gastric epithelial cells, plays an essential role in the translocation of CagA (Kwok et al., 2007) (Figure 1.4). Also, the specific binding of CagY, a structural protein coating the syringe of the T4SS, to host cell β_1 integrin has been reported to be required for CagA translocation into the host cells (Delahay et al., 2008; Jimenez-Soto et al., 2009). The translocated CagA protein, once inside the host cell, is localized on the inner surface of the plasma membrane and thereafter undergoes tyrosine phosphorylation (Segal et al., 1999; Asahi et al., 2000; Higashi et al., 2002b). Tyrosine phosphorylation occurs at the Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs present in the C-terminal of the CagA protein (Backert et al., 2001; Higashi et al., 2002b); and it is mediated by Src family kinases (SFKs) and Abl kinase expressed in gastric epithelial cells (Stein et al., 2002; Backert et al., 2008). The SFKs are normally involved in controlling basic cytoskeletal processes, cell proliferation and differentiation; however, they are also key players in carcinogenesis (Amieva and El-Omar, 2008).

EPIYA motifs are present in multiple numbers in a single CagA protein and hence, termed the EPIYA-repeat region; however, the number varies from

strain to strain (Figure 1.3). For instance, CagA protein of Western standard *H. pylori* strain NCTC 11637 possesses 5 EPIYA motifs (Figure 1.3): the 1st and 2nd motifs, designated EPIYA-A and EPIYA-B respectively, are conserved in all CagA proteins; while the last 3 motifs, EPIYA-C, generated by 3-time duplication of a 34-amino acid sequence within the location of the EPIYA sequence, varies among different CagA proteins with numbers ranging from 0-4 and most species possessing one EPIYA-C (Covacci et al., 1993; Higashi et al., 2002b; Higashi et al., 2002a). The CagA protein of East Asian *H. pylori* strains possesses EPIYA motifs A and B but nor C; rather it has a unique amino acid sequence containing a distinct EPIYA motif, EPIYA-D, at a region corresponding to the 34 amino acid sequence of Western CagA (Yamaoka et al., 1998; Yamaoka et al., 1999). East Asian CagA strains with multiple EPIYA-D motif has also been reported (Miura et al., 2009).

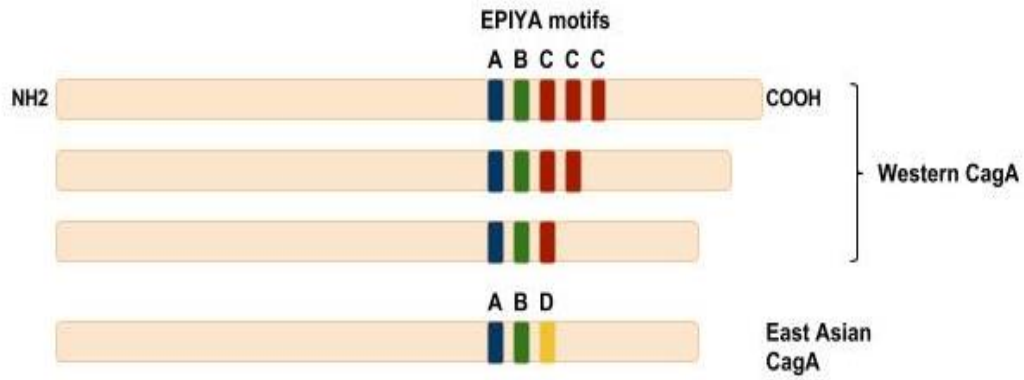


Figure 1.3 Schematic diagram showing variation in the carboxy-terminal EPIYA phosphorylation sites of *Helicobacter pylori* CagA proteins depends on their geographical origin.
(Adapted from: Hatakeyama, 2003).

Western CagA proteins contain the EPIYA-A, EPIYA-B, and EPIYA-C repeat regions in the carboxy-terminal EPIYA segment. East Asian CagA proteins contain the EPIYA-A, EPIYA-B and EPIYA-D motifs but do not possess the EPIYA-C segment. The CagA protein from the *H. pylori* NCTC 11637 Western standard strain possesses an EPIYA-A, an EPIYA-B, and three EPIYA-C motifs. These EPIYA-repeats serve as tyrosine phosphorylation sites of CagA and can be targeted by members of the Abl and Src tyrosine kinase families.

Higashi *et al* (2002a) has demonstrated that the sites of tyrosine phosphorylation for the Western and East Asian CagA are the EPIYA-C and EPIYA-D motifs, respectively. Recently, a different tyrosine phosphorylation site of CagA, TPM-C, has been reported (Puls et al., 2002). However, TPM-C is present in less than 20 % of CagA proteins (Hatakeyama, 2003).

Since tyrosine phosphorylated proteins are widely used for intracellular signalling in mammalian cells, it is thought that translocated CagA interacts and disturb signal transduction in a tyrosine phosphorylation-dependent manner (Hatakeyama, 2003). CagA forms a complex with protein tyrosine phosphatase (SHP-2) containing two Src homology 2 (SH2) domains, N-SH2 and C-SH2, in its N-terminal (Higashi et al., 2002b). This interaction depends on the tyrosine-phosphorylated EPIYA motifs of CagA and the SH2 domains of SHP-2 (Songyang et al., 1993; De Souza et al., 2002). Hof et al (1998) has shown that N-SH2 domain interacts with the catalytic cleft of the C-terminal tyrosine phosphatase domain, thereby blocking substrate access and keeping SHP-2 catalytically inactive. This interaction is reversed when a phosphotyrosine-containing peptide binds to one or both SH2 domains, thereby activating the phosphatase activity of SHP-2 as Higashi et al (2002b) has shown that formation of the CagA-SHP-2 complex strongly stimulates the SHP-2 activity (Figure 1.4). The formation of the CagA-SHP-2 complex underlies the induction of a unique morphological change, termed the hummingbird phenotype, characterized by spreading and elongation of the cell to a shape similar to the beak of a hummingbird (Segal et al., 1999). This is supported by a study showing that treatment of AGS cells with calpeptin, a SHP-2 inhibitor, prevented the induction of the hummingbird phenotype (Higashi et al., 2002b).

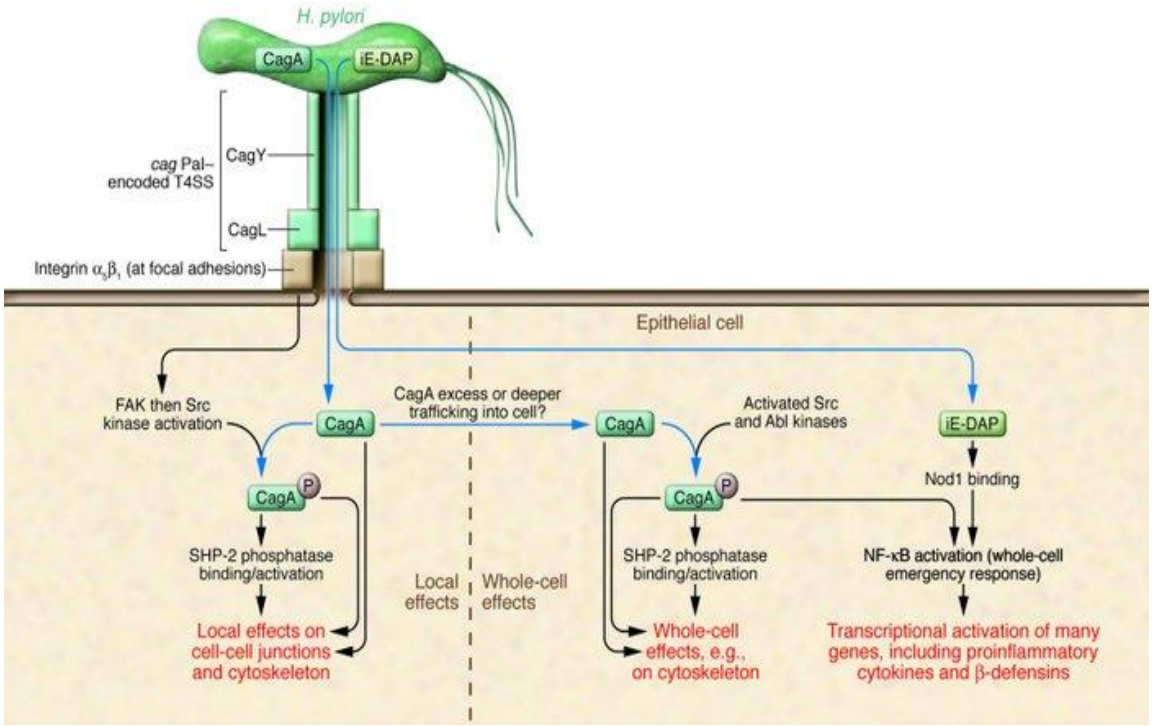


Figure 1.4 Local and whole cell effect of *H. pylori* CagA protein
(Taken from: Atherton and Blaser, 2009).

CagA is delivered into gastric epithelial cells through bacterial T4SS. The T4SS protein, CagL, binds to $\alpha_5\beta_1$ integrin, thereby activating FAK kinase and Src kinase. CagA then undergoes tyrosine phosphorylation by activated Src and Abl kinases. Tyrosine-phosphorylated CagA binds to and activates SHP2, resulting in local signalling, including formation of the hummingbird phenotype. A component of *H. pylori* peptidoglycan, γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), also enters the cell, activating NF-κB via innate Nod1 receptor recognition. The black arrows indicate epithelial cell components, blue arrows indicate *H. pylori* components, and red text indicates cellular effects.

CagA stimulates NF-κB activation and induction of potent pro-inflammatory chemokines such as IL-8 (Brandt et al., 2005); this may occur as a result of entry of γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), a soluble component of bacterial peptidoglycan, into the host cell and its recognition by the intracellular innate immune-pattern recognition receptor Nod1 (Viala et al., 2004). SHP-2 is a positive regulator of signals generated by growth factors and it is required for the activation of the Ras-Erk MAP kinase (Murata-Kamiya, 2011). CagA-activated SHP-2 stimulates Erk MAP kinase which deregulates cell proliferation, via Ras-dependent and –

independent pathways (Neel et al., 2003; Higashi et al., 2004). It also inhibits focal adhesion kinase (FAK), a tyrosine kinase that regulates focal adhesion spot turn-over, thereby inducing a growth factor-like morphological change characterized by formation of needle-like cell protusions (Tsutsumi et al., 2006). CagA has been strongly implicated in the development of *H. pylori*-associated gastric adenocarcinoma; as Churin et al (2003) has shown that CagA deregulates cell growth and motility, in a tyrosine phosphorylation-dependent manner, by binding to hepatocyte growth factor receptor, c-Met, which is involved in invasive growth of tumor cells. In the same manner, CagA also binds to growth factor receptor-bound protein 2 (Grb2) and activates Erk MAP kinase thereby stimulating cell scattering and cell growth (Mimuro et al., 2002). Higashi et al (2002a) and Fu et al (2007) have shown that East Asian CagA has a higher ability to induce the hummingbird phenotype and have more influence on Erk MAP kinase activity and cell proliferation than Western CagA, due to the fact that the consensus sequence to which SH2 domains of SHP-2 binds is much more similar to the SHP-2 binding site of East Asian CagA and differs from that of Western CagA. Though the most predominant form of CagA among Western isolates has one EPIYA-C, the level of tyrosine phosphorylation, SHP-2 bioactivity and formation of hummingbird morphology has been demonstrated to be dependent on the number of EPIYA-C motifs (Higashi et al., 2002a). Western isolates with multiple EPIYA-C motifs has also been shown to elicit an increased risk of gastric adenocarcinoma (Satomi et al., 2006). However, the East Asian CagA with EPIYA-D motif is highly associated with gastric cancer than its Western counterpart, irrespective of number of EPIYA-C repeats (Higashi et al., 2002a; Hatakeyama, 2004). Therefore, the pathological activity of CagA may be influenced by the degree of its SHP-2 binding activity.

1.3.5 Vacuolating cytotoxin, VacA

VacA is a vacuolating pore-forming cytotoxin usually synthesized by *H. pylori* and delivered to the host cell (Leunk et al., 1988). It is encoded by the *vacA* gene which, unlike *cagA*, is present in all *H. pylori* strains (as reviewed in: Atherton, 2006). VacA is initially synthesized as a 140 kDa polypeptide which undergoes cleavage at its N- and C-terminal during secretion (Telford et al., 1994). The C-terminal of pro-VacA, involved in autotransport of the toxin outside the bacterial outer membrane, is removed (Nguyen et al., 2001) to produce an 88 kDa mature toxin secreted as soluble proteins (Cover and Blaser, 1992) (Figure 1.5). The mature VacA toxin is made up of 2 subunits; p33 and p55. Though both subunits are required for toxin activity, p55 mediates the binding of the toxin to epithelial cells (Atherton, 2006).

VacA shows polymorphic variations in its signal (s) region and mid (m) region (Cover et al., 1994), resulting in differences in cytotoxicity levels (Atherton et al., 1995) (Figure 1.5). The s region types (s1 and s2) determine the vacuolating activity of VacA (McClain et al., 2001) while the m region (m1 and m2) determines cell specificity for vacuolation by influencing toxin binding to host cells (Pagliaccia et al., 1998; Ji et al., 2000). Recently identified is the intermediate (i) region *vacA* alleles with types i1 and i2 (Rhead et al., 2007), and deletion region (d) linked to disease progression (Ogiwara et al., 2009). Being recently characterised, not much is known about the role of these regions. However, the i1 VacA type has been shown to be a significant risk factor for peptic ulcer and gastric adenocarcinoma, being strongly linked to s1-type *vacA* allele which is associated with peptic ulcer and gastric cancer (Rhead et al., 2007; Jones et al., 2011; Bridge and Merrell, 2013). Of the well characterised s and m

regions, possible combinations are: s1/m1, in which vacuolating activity is highest; s1m2, with less activity; and s2m2 which has no vacuolating activity (Atherton et al., 1995). Studies in humans have shown that the s2-type VacA rarely cause *H. pylori* associated diseases (Atherton et al., 1995; van Doorn et al., 1998).

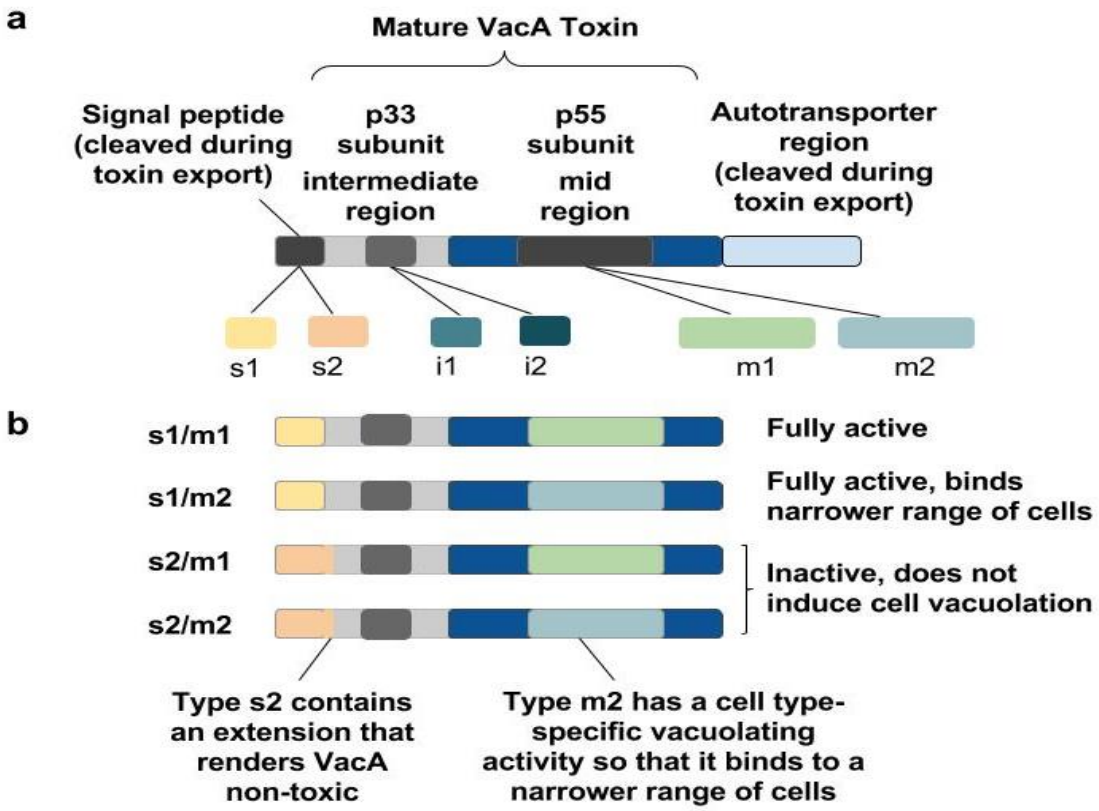


Figure 1.5 Polymorphism in vacA gene and VacA toxin in *H. pylori* strains
(Adapted from: Atherton, 2006; Polk and Peek, 2010)

a) The *vacA* gene varies most markedly in the signal region (encoding the signal peptide), and may be type s1 or s2;and in the mid-region (encoding part of the p55 binding subunit), which is either type m1 or m2. It also varies in the intermediate region (i1 or i2), with i1 and i2 being associated to s1/m1 and s2/m2 strains, respectively; and the s1/m2 strains are said to vary in i-type polymorphism. **b)** Commonly secreted toxins are: s1/m1, s1/m2, and s2/m2. The s2/m1 combination occurs but israre.

Correlations between VacA polymorphism and gastric diseases have been made, with s1 type being associated with increased risk of peptic ulcer disease and gastric cancer compared to the s2 type (Atherton et al., 1995; van Doorn et al., 1998). This observation is supported by previous studies showing that the s2 type *vacA* alleles failed to induce intracellular vacuolation and cytotoxicity (McClain et al., 2001; Atherton et al., 1995). s1/m1 VacA-type is strongly linked to duodenal ulcer, gastric ulcer and gastric adenocarcinoma (Atherton et al., 1997; Kidd et al., 1999; Miehlke et al., 2000; Figueiredo et al., 2001). The s1/m1 type strain is highly prevalent in Japan, and may contribute to the high incidence of gastric adenocarcinoma in the region (Atherton, 2006).

Transportation of VacA into host epithelial cells is either via secretion or contact-dependent transfer (Ilver et al., 2004). VacA has the ability to bind several receptors of the epithelial cell, such as receptor protein tyrosine phosphatise (RPTP)-β and –α (Padilla et al., 2000; Yahiro et al., 2003); the epidermal growth factor receptor (EGFR) (Seto et al., 1998); and glucosylphosphatidylinositol (GPI)-anchored protein (Schraw et al., 2002; Kuo and Wang, 2003). Incorporation and adaptation of VacA to the gastric environment is dependent on acid activation (McClain et al., 2000; Ricci et al., 2002). VacA is believed to mediate the formation of large vacuoles in cells by entering the membrane of late endosomal vesicles where it forms pores with chloride channel activity, alters the anion composition within endosomes, and subsequently cause osmotic swelling (Papini et al., 1994; Kuo and Wang, 2003). VacA induces cell death via apoptosis (Kuck et al., 2001; Cover et al., 2003); partly, due to release of cytochrome c, triggered by VacA-induced pore formation in mitochondrial membranes (Galmiche et al., 2000; Willhite et al., 2003). Nakayama et al (2004) has shown that VacA stimulates p38 and ERK1/2 MAP kinases, thereby resulting in interference

with cell signalling. VacA has been reported to cause leakage of ions and small molecules, such as iron, nickel, sugars and amino acids, by disrupting the barrier function of epithelial cell tight junctions; a mechanism by which *H. pylori* is thought to acquire nutrients across an intact epithelial barrier (Papini et al., 1998). VacA has also been found to interfere with immune response. It inhibits T-cell activation, in vitro, by inhibiting the activation of nuclear factor of activated T cells (NFAT), an essential transcription factor required for expression of genes involved in T-cell activation (Gebert et al., 2003). VacA interferes with phagocytosis by mononuclear phagocytes (Allen et al., 2000; Zheng and Jones, 2003), and antigen presentation by inhibiting the processing of B cell antigenic peptides and their presentation to $CD4^+$ T-cells (Molinari et al., 1998). Using Mongolian gerbils has a model, the effect of VacA has been shown in vivo; *vacA*-deficient *H. pylori* strains as well as the wild-type cause similar levels of inflammation, however, gerbils infected with *vacA*-mutant strains showed significantly less gastric ulceration (Ogura et al., 2000).

1.4 Pathogenesis

Following infection, *H. pylori* evades the bactericidal contents of the stomach and enters into the gastric mucosa (as reviewed in: Suerbaum and Michetti, 2002) where it alters and brings about changes to the gastric epithelial cells and mucosa (Figure 1.6). Urease production allows its survival in the acidic environment of the stomach by catalysing the hydrolysis of urea to ammonia, which buffers the periplasmic and cytosolic pH (Mobley, 2001). With the aid of the flagella, *H. pylori* moves towards a neutral pH into the gastric mucosa (Josenhans and Suerbaum, 2001) where it reaches the gastric epithelial cells to which it adheres using specialised

adhesins and outer membrane proteins (Falk et al., 1994). Upon attachment to the gastric epithelial cells, *H. pylori* injects bacterial proteins, CagA and peptidoglycan components, into the host cell via a type IV secretion system (Covacci et al., 1999) and also release toxic proteins, VacA and *H. pylori* neutrophil activating protein (HP-NAP). The presence of CagA in the host cells lead to alterations in cytoskeletal arrangement; and activation of NF-κB and early response transcription factor, activator protein 1 (AP-1) which induce pro-inflammatory cytokines, especially IL-8 (Naumann et al., 1999; Brandt et al., 2005) (Figure 1.6). IL-8, a neutrophil activating chemokine, induces the recruitment of polymorphonuclear leukocytes (PMN) into the lamina propria (Evans et al., 1995), thereby initiating a series of pro-inflammatory events that results in chronic inflammation. The toxic activity of VacA induces formation of large vacuoles (Kuo and Wang, 2003), alterations of tight junctions (Papini et al., 1998), and apoptosis of epithelial cells (Kuck et al., 2001). *H. pylori* also stimulates apoptosis by inducing the expression of cell surface receptor, Fas and Fas ligand (Fas-L) (D'Elios et al., 1999). The VacA- and Fas-mediated apoptosis results in the disruption of epithelial barrier promoting translocation of bacterial antigens into host cells, thereby leading to further activation of macrophages (as reviewed in: Suerbaum and Michetti, 2002).

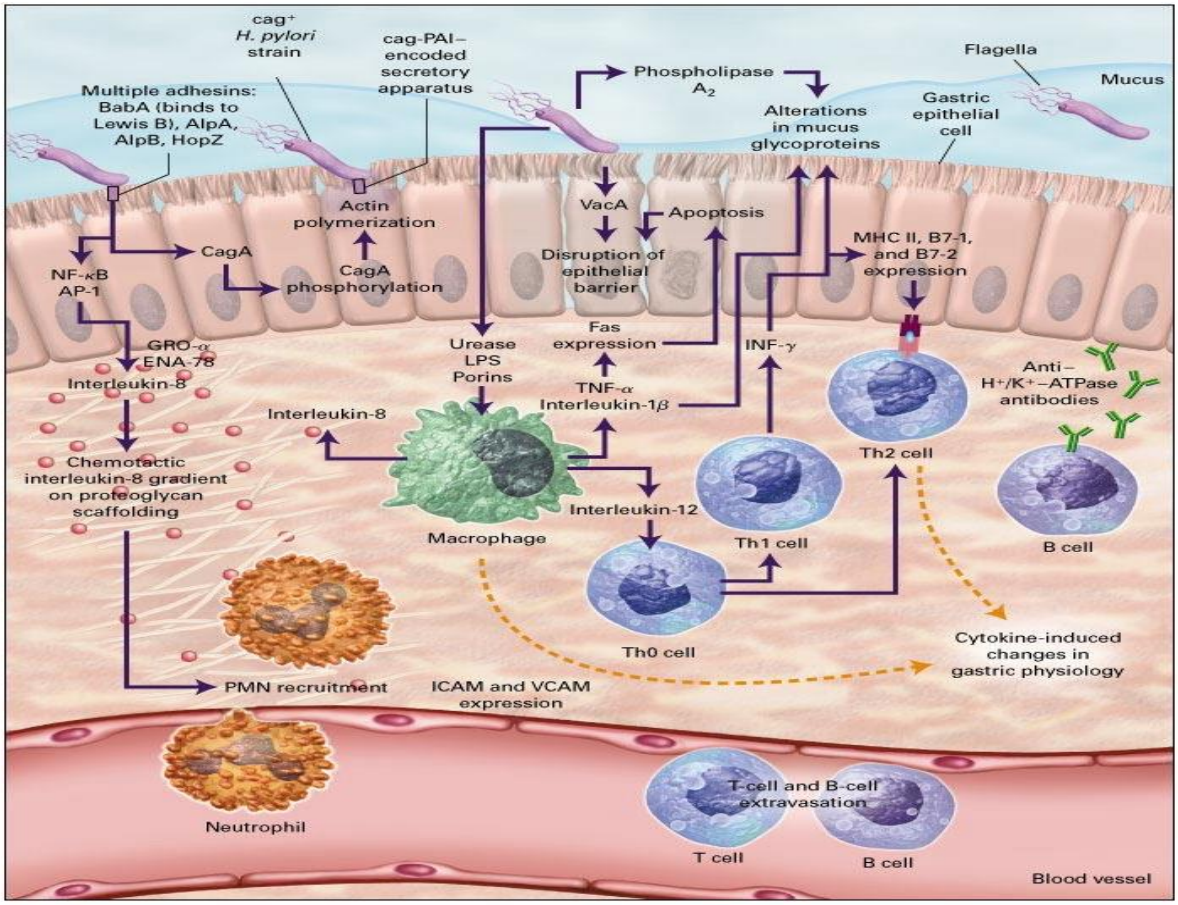


Figure 1.6 Host pathogen interactions in the pathogenesis of *H. pylori* infection.
(Taken from: Suerbaum and Michetti, 2002).

H. pylori binds to gastric epithelial cells through BabA and other adhesins. In strains that carry the Cag pathogenicity island (Cag-PAI), a type IV secretory apparatus allows translocation of effector molecules such as CagA into the host cell, resulting in the production of interleukin (IL)-8 and other chemokines, such as epithelial-cell–derived neutrophil-activating peptide 78 (ENA-78) and growth-related oncogene *a* (GRO-*a*), by epithelial cells; with the nuclear factor-*k*B (NF-*k*B) and the early response transcription-factor activator protein 1 (AP-1) playing essential roles in this process. The secreted chemokines lead to the recruitment of polymorphonuclear cells (PMNs), resulting in chronic inflammation. Lymphocyte recruitment is facilitated by chemokine-mediated expression of vascular addressins such as vascular-cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) that are required for lymphocyte extravasation. Injected CagA also associates with tight junctions and targets *H. pylori* to them. In the long term, CagA might cause disruption of the epithelial barrier and dysplastic alterations in epithelial-cell morphology. Disruption of junctions by CagA might also cause leakage of nutrients into the mucous layer and entry of bacterial VacA into the submucosa. VacA induces apoptosis in epithelial cells by reducing the mitochondrial transmembrane potential and inducing cytochrome *c* release, which might also contribute to the disruption of the epithelial barrier. Tumour-necrosis factor-α (TNF-α)-mediated apoptosis may also lead to disruption of the epithelial barrier. The chronic phase of *H. pylori* gastritis links an adaptive lymphocyte response with the initial innate response. Cytokines produced by macrophages, particularly IL-12, activate recruited cells — such as T helper cells (Th0, Th1 and Th2), which respond with a biased Th1 response. In turn, Th1-type cytokines such as interferon-*γ* (INF-*γ*) induce the expression of class II major histocompatibility complexes (MHC) and accessory molecules B7-1 and B7-2 by epithelial cells, making them competent for antigen presentation. Cytokines also alter the secretion of mucus, which contributes to *H. pylori*-induced disruption of the mucous layer, as they induce changes in gastric-acid secretion and homeostasis (dashed lines).

The macrophages that bring about IL-8 production produce pro-inflammatory cytokines, such as IL-12, involved in the activation of recruited cells, particularly T helper cells and B cells (D'Elios et al., 2003). The immature T helper (Th) 0 cells differentiate into two functional subtypes: Th1, secreting IL-2 and interferon (INF); and Th2, secreting IL-4, IL-5 and IL-10, stimulated in response to intracellular and extracellular pathogens, respectively (Suerbaum and Michetti, 2002). Since *H. pylori* is non-invasive, a Th2 response is expected, however, *H. pylori* specific gastric mucosa T cells generate a biased Th1 response (Harris et al., 2000). The secretion of TNF-α, IL-1β, INF-γ and other Th1 cytokines increase gastrin release, alters mucus glycoprotein and stimulates acid secretion (Weigert et al., 1996). TNF-α also induces a decrease in antral D cells, resulting in decreased somastatin production and indirectly enhancing acid production (Weigert et al., 1996). Gastrin release and acid production facilitates the development of peptic ulcer (D'Elios et al., 2005). In a minority of infected patients, gastric *H. pylori*-specific T helper cells show deficient cytotoxic control. This deficiency, the production of cytokines with B-cell growth factor activity and the chronic delivery of co-stimulatory signals by T helper cells, together with chronic exposure to *H. pylori* antigens result in overgrowth of B cells (D'Elios et al., 1999). Thus, facilitating the neoplastic B-cell transformation and the onset of gastric low-grade mucosa associated lymphoid tissue (MALT) B-cell lymphoma (D'Elios et al., 1999). In susceptible individuals, *H. pylori* induces autoimmune gastritis via the expansion of *H. pylori*-specific T cells that cross-react with H^+K^+-ATPase epitopes (Amedei et al., 2003). The activation of *H. pylori* H^+K^+-ATPase cross-reactive T cells would result in destruction of gastric mucosa, via Fas-ligand (FasL)-induced apoptosis and perforin-mediated cytotoxicity (Amedei et al., 2003).

1.5 Clinical outcome of *H. pylori* infection

The gastric phenotype resulting from chronic *H. pylori* infection varies, depending on microbial and host factors (Figure 1.7). The pattern and distribution of gastritis determines the risk of gastric diseases, namely; duodenal or gastric ulcer, mucosal atrophy, gastric carcinoma or gastric lymphoma (Dixon, 2001). The most common gastric phenotype is the mild pangastritis that does not alter the gastric physiology greatly, and is not associated with significant gastric disease (as reviewed in: Lochhead and El-Omar, 2007). The disease-associated gastritis are: the antral-predominant gastritis associated with high gastric acid secretion and increased risk of duodenal ulcer disease (Elomar et al., 1995); and the corpus-predominant gastritis associated with gastric ulcer, gastric atrophy, hypochlorhydria, and gastric cancer (ElOmar et al., 1997).

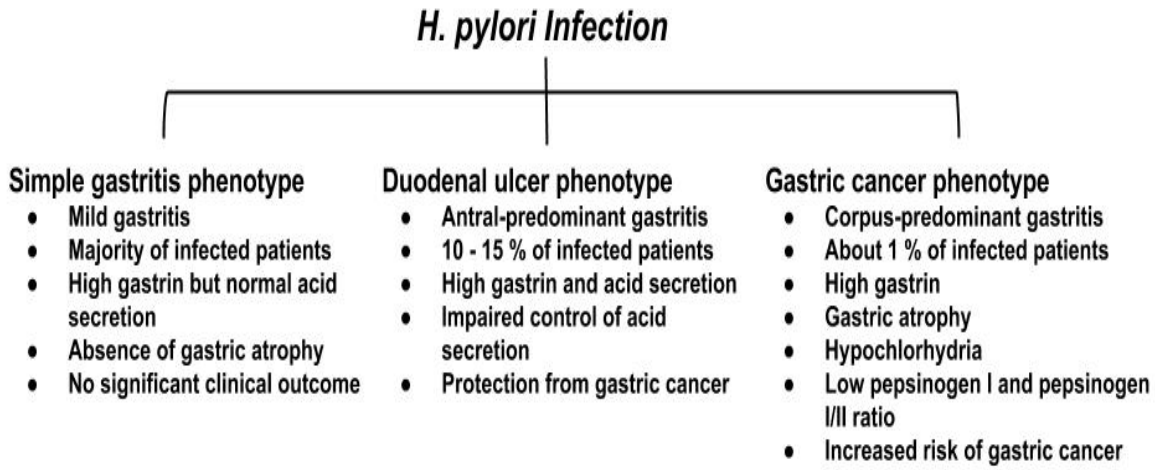


Figure 1.7 Clinical outcomes of chronic *H. pylori* infection
(Adapted from: Amieva and El-Omar, 2008)

The physiologic, histologic and clinical characteristics of the 3 potential outcomes are shown.

H. pylori is responsible for most peptic ulcer disease, as eradication of the bacteria drastically reduced the rate of *H. pylori* associated peptic ulcer (Marshall et al., 1988). In *H. pylori* infected patients, the lifetime risk of developing peptic ulcer ranges from 3 % in the United States to 25 % in Japan (Schlemper et al., 1996; Feldman, 2001). With the classification of *H. pylori* as a type 1 carcinogen (Forman et al., 1991; Nomura et al., 1991; Parsonnet et al., 1991), *H. pylori* increases the risk of gastric cancer which is currently the second most frequent cause of cancer-related deaths (Suerbaum and Michetti, 2002). In a Japanese study, gastric cancer developed in 2.9 % of *H. pylori* infected patients while no gastric cancer was observed in the non-infected control subjects (Uemura et al., 2001). Most importantly, in the study, gastric cancer was not detected in a subgroup of infected patients who received eradication therapy early on. *H. pylori* infection significantly increases the risk of gastric MALT lymphoma, and 72 – 98 % of gastric MALT lymphoma patients are infected with *H. pylori* (Parsonnet et al., 1994; Wotherspoon, 1998). Also, the eradication of *H. pylori* induces the regression of gastric MALT lymphoma in 70 – 80 % cases (Bayerdorffer et al., 1995).

1.6 Treatment of *H. pylori* infection

The eradication of *H. pylori* has been shown to heal ulcers, prevent recurrence of peptic ulcers and reduce the prevalence of gastric cancer in susceptible populations (Sepulveda and Coelho, 2002).

1.6.1 Clinical treatment

H. pylori infection is treated with a combination of antisecretory (proton pump inhibitors) and antimicrobial agents such as clarithromycin,

amoxicillin, metronidazole, tetracycline, levofloxacin, rifabutin and bismuth compounds (Yang et al., 2014). The most recommended treatment regimen for eradication of *H. pylori*, in all international guidelines, is the proton pump inhibitor (PPI)-based triple therapy that is the combination of a proton pump inhibitor with 2 antibiotics, clarithromycin and metronidazole or amoxicillin, for 7 days (Gisbert et al., 2005; Malfertheiner et al., 2007). However, the eradication rate of *H. pylori* using this therapy has fallen from >90 % to 75 – 80 % (Gisbert et al., 2000a; Gisbert and Pajares, 2005; Egan et al., 2008; Graham and Fischbach, 2010). This decrease in eradication rate is most attributed to increasing resistance of *H. pylori* to antibiotics, particularly clarithromycin which has been identified as a major factor affecting eradication rates due to its increasing *H. pylori* resistance in many regions (Megraud, 2004). Primary resistance of *H. pylori* to clarithromycin and metronidazole decreases cure rates by 50 % and 37 %, respectively (Dore et al., 2000). As a result of decreased eradication rate associated with the PPI-based triple therapy, new strategies to improve this therapy are required (Graham and Yamaoka, 2007).

The use of ranitidine bismuth citrate in dual therapy with clarithromycin for 2 weeks has been approved by the FDA (Peterson et al., 1996). Meta-analyses show that the performance of ranitidine bismuth citrate in combination with clarithromycin and amoxicillin or a nitroimidazole corresponds to that of the PPI-based triple therapy (Laheij et al., 1999; Gisbert et al., 2000b). However, this ranitidine bismuth citrate-based triple therapy has not been FDA approved (Suerbaum and Michetti, 2002). Bismuth-based triple therapy involving the combination of bismuth with metronidazole and tetracycline has also been shown to be effective (Houben et al., 1999; Laheij et al., 1999); however, its efficacy is negatively affected by metronidazole resistance (Houben et al., 1999). Although,

these alternative therapies are effective at varying duration of use, the recommended regimen by the European Maastricht 2-2000 conference is PPI (or ranitidine bismuth citrate), clarithromycin, and amoxicillin or metronidazole for 7 days (Bazzoli, 2001).

As agreed at major consensus conferences, when the recommended first treatment regimen fails, mainly due to either poor patient compliance or antibiotic resistance, a second-line therapy is recommended (Lam and Talley, 1998; Bazzoli, 2001; Malfertheiner et al., 2012). Clarithromycin resistance has been identified as a major player in the failure of the first-line regimen to eradicate *H. pylori*, with the global clarithromycin resistance rate in Europe increasing by 8.6 % between 1998 and 2009 (Malfertheiner et al., 2012). Clarithromycin resistance rates is different in various regions of Europe, with northern European countries having <10 % resistance rate and other European regions having >20 % prevalence which is considered a high resistance rate (Megraud et al., 2013). According to the most recent European *Helicobacter* Study Group (EHSG)Maastricht IV/ Florence consensus report, in regions with low clarithromycin resistance, the second-line therapy is a bismuth-quadruple therapy in which PPI or H_2-receptor antagonist is added to a bismuth-based triple regimen, with high dose of metronidazole for 10 – 14 days (Suerbaum and Michetti, 2002; Malfertheiner et al., 2012). Although in some cases, this second-line therapy fails as well due to failure of regimens containing metronidazole (Hojo et al., 2001). In such cases, a second course of PPI-based triple therapy with levofloxacin and amoxicillin, avoiding previously used antibiotics and less effective combinations, is employed (Malfertheiner et al., 2012; Yang et al., 2014). In regions with high clarithromycin resistance, the first-line treatment is the bismuth-quadruple regimen, while the PPI triple-based therapy with levofloxacin and amoxicillin is the second-line

treatment (Malfertheiner et al., 2012). In some cases where none of the above therapies is effective, a rescue therapy involving the use of rifabutin in combination with amoxicillin and pantoprazole for 10 days is recommended. This has achieved 86 % eradication rate even in patients with resistant strains (Perri et al., 2001). Where all therapies fail, treatment should be based on antimicrobial susceptibility testing results (Malfertheiner et al., 2012).

In addition to antibiotic resistance, unpleasant side effects (nausea, vomiting, epigastric pain, abdominal discomfort and diarrhoea) associated with the therapies (Galan et al., 2004), low patient compliance, high cost (Manyi-Loh et al., 2010), bacterial factors, and geographic differences can further affect eradication rate of *H. pylori*(Lee et al., 1999). Susceptibility to other bacterial infections, such as *Clostridium difficile*, as a result of continuous antibiotic use is also a problem associated with use of these therapies (Kelly et al., 1994).

The eradication of *H. pylori* is considered poor due to the fact that failure rate remains as high as 5 – 20 % and also as a result of frequent peptic ulcer relapses (Li et al., 2005). In order to manage patients with treatment failure better, it is important to understand the mechanisms by which *H. pylori* develops resistance to currently used antibiotics.

1.6.2 *H. pylori* antibiotic resistance

H. pylori acquires resistance to antibiotics used in treatment regimens by mutation, particularly point mutation which is transmitted vertically (as reviewed in: Megraud and Lehours, 2007). Antibiotic resistance generally increases the minimum inhibitory concentration and reduces the susceptibility of *H. pylori* to these antibiotics (Table 1.1).

The current most effective antibiotic used in the treatment of *H. pylori* infection is clarithromycin due to its high efficacy in vitro, with minimum inhibitory concentration being the lowest as compared to other molecules (Megraud, 1998). *H. pylori* resistance to clarithromycin is the most widely studied, with 10 – 25 % prevalence found in the United States based on strains isolated during clinical trials (Wang et al., 1998; Osato et al., 2001). Clarithromycin inhibits protein synthesis by binding to ribosomes at the peptidyl-transferase loop of 23s rRNA (Versalovic et al., 1996). Resistance to this antibiotic is characterized by a single base substitution (point mutation) at 2 nucleotide positions, 2142 (A2142C and A2142G) and 2143 (A2143G), within the peptidyl-transferase loop of 23s rRNA (van Doorn et al., 1999). This leads to a conformational change and decrease in binding of clarithromycin to the ribosomal subunit dedicated to the specific antibiotic related protein synthesis (Versalovic et al., 1996; Occhialini et al., 1997; van Doorn et al., 1999). The presence of the A2143G mutation, rather than the A2142C or A2142G mutation, has been shown to reduce *H. pylori* eradication rate (De Francesco et al., 2006).

Approximately >90 % prevalence of metronidazole resistance has been reported in developing countries (Alarcon et al., 1999; Aboderin et al., 2007; Ndip et al., 2008). This is thought to be the result of previous consumption and drug abuse of metronidazole in these regions as it is also used in the treatment of parasitic infections which commonly plagues developing countries (Alarcon et al., 1999). Metronidazole resistance reduces the efficacy of metronidazole-based regimens rather than render them completely ineffective (Houben et al., 1999; Dore et al., 2000). Metronidazole interacts with redox systems that can bring about reduction in the 5-nitro group of the imidazole ring, resulting in its activation, production of cytotoxic radicals and reactive species (Smith and Edwards,

1995). An important gene in this respect is *rdxA*, an oxygen-insensitive NADPH nitroreductase; mutation of which renders the protein inactive (Hoffman et al., 1996). However, the precise point mutation in this gene to explain resistance phenomenon is yet to be identified (Megraud, 2004). Another gene, *frxA* encoding NAD(P)H flavin oxidoreductase, is believed to be involved in the reduction process of metronidazole (Kwon et al., 2000; Matteo et al., 2006). Gerrits et al (2004) have reported that mutation in the *rdxA* and/or *frxA* gene may not be solely responsible for metronidazole resistance, but other environmental factors such as oxygen tension. They showed that *rdxA* and *frxA* mutants conferring metronidazole-resistance became susceptible to *H. pylori* under low O_2 conditions.

The prevalence of *H. pylori* resistance to amoxicillin is rare and relatively low in Europe, North America and the Middle East, at <1 % (van Zwet et al., 1998; Megraud and Lehours, 2007). Amoxicillin is a β-lactam antibiotic that interferes with peptidoglycan synthesis by blocking transporters called penicillin binding proteins (PBP) (Megraud and Lehours, 2007). PBPs are enzymes involved in the synthesis of the peptidoglycan layer of the bacterial cell wall by a glycosyl transferase-acyl transpeptidase activity (as reviewed in: Francesco et al, 2011). Resistance to amoxicillin is as a result of multiple point mutations on the *pbp1a* gene (Okamoto et al., 2002; Co and Schiller, 2006); a Ser_{414}-Arg substitution and Asn_{562}-Tyr substitution, which leads to loss of affinity between amoxicillin and PBP transpeptidase (Gerrits et al., 2006). Recent findings have shown that point mutation in *hopB* or deletion in *hopC* genes, encoding porin channels that regulate the penetration of solutes, also confer resistance to amoxicillin (Co and Schiller, 2006).

Prevalence of *H. pylori* resistance to tetracycline is minimal but appears to be increasing (Osato et al., 2001; Francesco et al., 2011). Tetracycline is a bacteriostatic which interferes with protein synthesis at the ribosomal level by inhibiting codon-anticodon link in the 30s ribosomal subunit and blocking the attachment of aminoacyl-tRNA to the acceptor site (Francesco et al., 2011). *H. pylori* resistance to tetracycline is mainly as a result of substitution of nucleotide triplet AGA with TTC at position 926-928 or 965-967 in the acceptor site of the 16s rRNA genes which affects the binding of tetracycline (Gerrits et al., 2003; Nonaka et al., 2005).

Prevalence of *H. pylori* resistance to rifampin is virtually absent due to its limited use (Megraud and Lehours, 2007). Rifampins inhibit the B subunit of the DNA-dependent RNA polymerase encoded by the *rpoB* gene. Resistance is conferred by mutation to the *rpoB* gene at positions 524, 525 and 585 (Heep et al., 1999).

Table 1.1 Minimum inhibitory concentration of antibiotics used in *H. pylori* treatment regimens

Antibiotics	MIC in susceptible strains	MIC in resistant strains
Clarithromycin	0.016 - 0.50 mg/L	≥ 1 mg/L (2 - 256 mg/L)
Metronidazole	0.5 - 2 mg/L	≥ 8 mg/L (16 - 128 mg/L)
Amoxicillin	0.06 - 0.25 mg/L	≥ 1.0 mg/L (1 - 8 mg/L)
Tetracycline	0.25 - 2 mg/L	≥ 4 mg/L (2 - 256 mg/L)
Rifampin	0.032 - 2 mg/L	≥ 32 mg/L (32 - 256 mg/L)

MIC: Minimum inhibitory concentration Adapted from: Francesco et al, 2011

1.6.3 Alternative therapies

According to intention-to-treat analysis, clinically relevant *H. pylori* eradication regimen must have a cure rate of at least 80 %, with minimal

induction of bacterial resistance and without major side effects (Suerbaum and Michetti, 2002). With over 20 years of treating *H. pylori* infection, the ideal regimen to completely eradicate this bacterium is yet to be found (Gisbert et al., 2007), mainly as a result of the increasing drug resistance problems associated with widespread use of antibiotics in combination therapies (Cameron et al., 2004). There is also a delay in developing vaccines due to a search for the best route of delivery and antigens/adjuvants that induce protective immunity (Ayala et al., 2014). Therefore, it is of utmost importance to seek new alternative therapies, preferably from natural sources, as the effective life span of any antibiotic is limited. New treatments from natural sources include those from nutraceuticals, probiotics and plants. Ayala et al (2014) has a detailed review on several alternative therapies such as probiotics, fungi, antimicrobial peptides and phototherapy; and though these therapies do not effectively eradicate *H. pylori*, low bacterial levels have been reported in their use/application (Ayala et al., 2014). However, plants are promising sources of new drugs, and they have been shown to be effective in the treatment of gastric ulcers and other microbial infections (Afolayan and Meyer, 1997; O'Gara et al., 2000; Vattem et al., 2005; Afolayan and Lewu, 2009).

1.7 Plants as a source of antimicrobial agents

Medicinal plants are the foundation for the existence of traditional medicine that has been providing mankind with novel remedies to a range of ailments and diseases. The use of medicinal plants as therapeutics dates back to time immemorial, with the first record being the use of oils from cedar, myrrh, cypress and licorice in the treatment of cough and cold in

Mesopotamia (Gurib-Fakim, 2006). These oils are still in use till date for treatment of even more severe ailments such as inflammation and parasitic infections. The use of plants as medicine has been associated with early traditional medicine systems which exist in virtually all parts of the world, that is, Africa, America, Australia and South-east Asia, India, China, Europe, Arab and North Africa, with the African traditional medicine being the oldest and most diverse of all medicine systems (Good et al., 1979).

Medicinal plants contain bioactive compounds that exhibit pharmacological activities valuable in the development of novel pharmaceutical products such as dietary supplements, nutraceuticals and therapeutic drugs (Iwu, 2002). Some important plants used in traditional medicine that has been developed into drugs for modern medicine are described in Table 1.2. Medicinal plants contribute to the advancement of modern medicine as they can be used directly as therapeutic agents, lead precursors for semi-synthetic compounds, models for development of novel synthetic compounds and as a taxonomic marker for the discovery of new biochemical compounds (Gurib-Fakim, 2006). Biologically active compounds present in plants are mainly produced as secondary metabolites, which are not involved in the primary metabolism and daily functioning of the plant (Bernhoft, 2010; Daniel, 2006). Some major secondary metabolites of plants exhibiting pharmacological activities include: alkaloids which are produced as a defence mechanism for the plant against predators; terpenoids which help attract pollinators; and flavonoids which protect the plant against free radicals generated during photosynthesis (Bernhoft, 2010). These metabolites perform certain pharmacological actions in man and they are used as analgesics and narcotics, anti-tumour agents, cardiac repressants, skin stimulants, laxative, sedatives, antiseptics, anti-inflammatory and aromatherapy

agents (Iwu, 2002). It is believed that when an herbal medicine is ingested by humans, the activities carried out by the bioactive compounds in the plant are also exhibited in the human system. For instance, cardiac glycoside used by plants to stop the heartbeat of an herbivore can be used as cardiotonics in humans. In the same way, volatile oils of plant containing antimicrobials can be used to repel microbial attack in the human system (Daniel, 2006).

Table 1.2 Botanical drugs used in traditional medicine and which have given useful modern drugs

Botanical names	English names	Indigenous use	Origin	Uses in biomedicine	Biologically active compounds
Adhatoda vasica	–	Antispasmodic, antiseptic, insecticide, fish poison	India, Sri Lanka	Antispasmodic, oxytocic, cough suppressant	Vasicin (lead molecule for Bromhexin and Ambroxol)
Catharanthus roseus	Periwinkle	Diabetes, fever	Madagascar	Cancer chemotherapy	Vincristine, Vinblastine
Condrodendron tomentosum	–	Arrow poison	Brazil, Peru	Muscular relaxation	D-Tubocurarine
Gingko biloba	Gingko	Asthma, anthelmintic (fruit)	Eastern China	Dementia, cerebral deficiencies	Ginkgolides
Harpagophytum procumbens	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, rheumatism	Harpagoside, Caffeic acid
Piper methysticum	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic, mild stimulant	Kava pyrones
Podophyllum peltatum	May apple	Laxative, skin infections	North America	Cancer chemotherapy, warts	Podophyllotoxin and lignans
Prunus africana	African plum	Laxative, 'Old man's disease'	Tropical Africa	Prostate hyperplasia	Sitosterol

Source: Gurib-Fakim, 2006

According to the World Health Organisation (WHO), 80 % of the population in developing countries rely on plant derived medicine for their healthcare (Sasidharan et al., 2011). Though some of the therapeutic activities associated with plants have proven erroneous, plants continue to be the basis for the discovery of new sources and classes of biochemical

compounds. For instance, bioactive compounds present in *Ammi majus* (bishop's weed) used in Egypt for the treatment of vitiligo, a skin condition characterised by loss of skin pigment, has been developed into a drug, β-methoxypsoralen, for the treatment of psoriasis and a range of other skin disorders (Gurib-Fakim, 2006). In addition to this, the anti-malaria drug, quinine, derived from bioactive compounds in the bark of *Cinchona calisaya* and *Cinchona succirubra*, has been used for the treatment of fevers for over 350 years by the indigenous people in the Amazon region (Andrade-Neto et al., 2003).

Plant products and their derivatives represent about 25 % of drugs in clinical use in the world today with one-quarter of best-selling drugs produced in the past 10 years being formulations based on plant derived substances or plant-derived synthetic analogues (Balunas and Kinghorn, 2005; Mukhtar et al., 2008). The therapeutic benefits of plants has therefore led to susceptibility testing of different plant extracts, particularly those used in traditional medicine, against infectious microbes in order to discover plants that harbour antimicrobial agents.

1.7.1 Mechanism of action

Since plants and their secondary metabolites offer a new alternate approach to eradicating infectious microbes, it is of great importance to understand their mode of action. The mechanism of action of plant extracts is said to be dependent on the chemical composition of its active compounds and the quantity of these compounds (Nazzaro et al., 2013). Plant extracts are able to affect bacterial cells in several processes, which include disruption of cell membrane and cell morphology, efflux of intracellular components, coagulation of cytoplasmic content, and interference with DNA/RNA synthesis, cellular metabolism and intercellular

communication (Rabinkov et al., 1998; Williams, 2007; Di Pasqua et al., 2006; Zhang et al., 2008; Garvey et al., 2011). Based on several studies investigating the antibacterial activity of plant extracts against microbes, membrane damage seems to be the primary target for antimicrobial activity (Radulovic et al., 2013). Consequently, the effect of plant extracts on cell membrane is mainly used to describe their antibacterial activity (Radulovic et al., 2013). The cell membrane provides a permeability barrier important for many cellular functions such as solute transport, sustenance of energy levels and regulation of cell metabolic activities (Poolman et al., 1987; Trumpower and Gennis, 1994). Plant extracts, particularly essential oils, are able to penetrate the lipid bilayer and bind to membrane proteins, thereby interfering with normal function (Juven et al., 1994). This shows that plant extracts may target the cell membrane before other cell components/function. Plant extracts are thought to induce antimicrobial activity by following sequential activities as follows; interaction with cell membrane, diffusion into the cytoplasm, and consequently, interaction with intracellular components and processes (Radulovic et al., 2013).

Mechanism of action associated with the interaction of plant extracts with bacterial cell membrane include cell wall degradation (Helander et al., 1998), cytoplasmic membrane damage (Ultee et al., 2002), damage to membrane proteins, leakage of cellular content as a result of increased cell membrane permeability (Juven et al., 1994; Lambert et al., 2001), reduction in proton motive force, intracellular ATP pool and membrane potential (Burt, 2004). Interaction with bacterial cell membrane also interferes with membrane proteins involved in active transport of essential molecules (Nazzaro et al., 2013). Thus, disruption of the cell membrane will ultimately lead to cell lysis and cell death.

Plant extracts contain a variety of bioactive compounds, thus it is difficult to attribute their mechanism of action to a single action or target. It is thought that many different structural and biochemical processes are affected at multiple locations in the cell so as to elicit antimicrobial action (Nazzaro et al., 2013).

1.7.2 Plants exhibiting anti-*H. pylori* activity

Several studies and publications have described the antibacterial activity of plant extracts against *H. pylori* (Galan et al., 2004; Ayala et al., 2014). Extracts of cranberry, blueberry, grape seed, oregano (Vattem et al., 2005), cinnamon, green tea, parsley, nutmeg and chilli (O'Mahony et al., 2005) have been found to have anti-*H. pylori* activity. Green tea has been shown to inhibit the growth of *H. felis* and *H. pylori in vitro*, with promising *in vivo* results (Stoicov et al., 2009). Catethins, which are antioxidants found in green tea have also shown significant antibacterial activity against *H. pylori*, with decreased mucosal haemorrhage observed in infected Mongolian gerbils treated for 2 weeks (Mabe et al., 1999). The antibacterial activity of garlic and its compounds against *H. pylori in vitro* is one that has been widely studied (Cellini et al., 1996; Sivam et al., 1997; Mahady et al., 2001; Canizares et al., 2004). However, most *in vivo* studies have presented negative results (Graham et al., 1999; McNulty et al., 2001). Broccoli which has antiviral, anticancer and antibacterial properties have been shown to exhibit antibacterial activity against *H. pylori* (Galan et al., 2004), though some *in vivo* studies have shown negative results (Sato et al., 2004; Opekun et al., 2005). Isothiocyanate sulforaphane, present in broccoli sprouts, have been found to possess significant bactericidal and bacteriostatic activity against both reference and clinical *H. pylori* isolates (Fahey et al., 2002). It has also been shown to reduce gastric colonisation by *H. pylori*, and lower

the risk of developing gastritis in infected humans and mice (Yanaka et al., 2009). Another compound that has shown significant antibacterial activity against *H. pylori* is resveratrol, found in red wine and grapes. Red wine and resveratrol inhibited the activity of urease which is essential for colonisation and infection, and also inhibited the growth of *H. pylori*(Paulo et al., 2011). In addition to this, red wine was also found to inhibit cellular vacuolisation by VacA (Tombola et al., 2003), thereby suggesting the usefulness of polyphenols in the prevention and eradication of *H. pylori* and its associated diseases.

In the search for novel antimicrobials from plant sources, extract of potato tubers has been found to exhibit antibacterial activity selective towards *H. pylori* (Bennett and Roberts, unpublished data). Bennett and Roberts showed that potato extract has bactericidal effect towards *H. pylori* while having little or no effect on other common bacteria such as *E. coli*, *S. typhimurium*, *L. monocytogenes*, *S. aureus*, *S. epidermidis* as well as probiotic, *Lactobacillus acidophilus*. They also showed that this bactericidal activity is sensitive to heat, resistant to proteinase K, pH 5.0 and a simulated gastric fluid. The discovery of potato extract anti-*H. pylori* activity calls for further research focussed on understanding its mechanism of action.

1.8 Potato Plant (*Solanum tuberosum*)

Potato plant is an herbaceous vegetable crop of the plant family, *Solanaceae*. It is a native plant of the Andes mountain of South America, with stem tubers suitable for human consumption. Potato is the third most important food crop, after rice and wheat, used as a staple food worldwide and cultivated due to its high starch content. World production of potato

reached about 368 million tonnes in 2013, with most production in developing countries and China being the largest potato producer. There are over 4,000 native cultivated varieties of potatoes, most of which are found in the Andes. Of this, there are about 80 varieties available in the United Kingdom. Some of the most available cultivars include King Edward, Maris piper, Rooster, Charlotte, Desiree, Cultra, Estima, and Lady Christi. There are also several wild potato species, which though unsuitable for consumption due to its bitter taste possesses unique qualities such as resistance to diseases, pests and unfavourable climate conditions.

In the history of microbiology, potato has been found to be of significance. Robert Koch used potato slices as solid nutrient for the isolation and growth of pathogens in pure laboratory culture(Madigan et al., 2012). Also, in 1845, the Irish potato famine that led to death by starvation and hunger-based diseases was caused by the infestation of potatoes by a fungus, *Phytophthora infestans* (late blight), which caused them to rot.

Potato tubers constitute mainly: starch, phenolic compounds, and glycoalkaloids (Friedman et al., 2003). Potato glycoalkaloids are toxic, and therefore serve as a defence mechanism for the crop against pests such as insects and fungi (Osman, 1983; Knuthsen et al., 2009). There are 2 major glycoalkaloids found in potatoes, these are alpha-chaconine and alpha-solanine, with the latter being the most toxic (Friedman et al., 2003). Though the concentration of glycoalkaloids in potato tubers is said to increase under certain environmental conditions such as light exposure, physical injury and storage, it is usually at a reduced concentration when compared to that of the stem, fruits, sprouts and leaves (Friedman et al., 2003). Glycoalkaloids are also toxic in humans; however, as a result of the low concentrations of glycoalkaloids in edible potatoes, potato poisoning

rarely occur (Bacigalupo et al., 2004). Potato glycoalkaloids, apart from causing unfavourable conditions, are beneficial. They have been shown to prevent mice *Salmonella typhimurium* infection, lower cholesterol levels (Friedman et al., 2003) and inhibit human cancer cell growth (Lee et al., 2004). Aspartic proteases and a trypsin-chemotrypsin protease inhibitor obtained from potato tubers have also been shown to possess antimicrobial activity against pathogenic fungi and bacteria (Kim et al., 2005; Mendieta et al., 2006). In addition to this, the use of potato juice as a traditional medicine in Germany and Switzerland, for the treatment of non-ulcer dyspepsia cannot be overemphasized (Chrubasik et al., 2006). The discovery of the antibacterial activity of potato extract against *H. pylori* (Bennett and Roberts, unpublished data) has stimulated further interest in understanding its mode of action. With the identification of the mechanism by which potato extract exhibit anti-*H. pylori* activity, synthetic agents that can mimic its anti-*H. pylori* activity can be synthesized and used as a treatment therapy for eradication of *H. pylori* infection. Potato extract can also serve as a lead precursor in the synthesis of anti-*H. pylori* agents; or it can be developed into orally administrable forms and used as a treatment therapy.

1.9 Aims and Objectives

This study focuses on identifying the mechanism by which potato extract kills *H. pylori*. The aims are;

- To determine the sensitivity of other *Helicobacter* and *Campylobacter* species to potato extract.
- To study the effect of potato extract on *H. pylori* cells, using transmission electron microscopy (TEM) and other bio-imaging tools.
- To investigate the mode of action of potato extract against *H. pylori*. This will involve the isolation of *H. pylori* mutants resistant to potato extract as a means to identify the targets of potato extract activity.

These investigations will provide some insights into understanding the antibacterial activity of potato extract.

Chapter 2

Materials and Methods

2.1 Bacterial Strains, Media and Growth Conditions

A list of bacterial strains used in this study is given in Table 2.1 and 2.2. Unless otherwise stated, all media and solutions were sterilized by autoclaving at 121°C for 20 min at 15 psi.

2.1.1 Chemicals, Reagents and Antimicrobial drugs

Unless otherwise stated, analytical grade chemicals and antibiotics were sourced from Sigma, Oxoid or VWR International. All media were sourced from Oxoid. Restriction endonucleases and other DNA modifying enzymes were obtained from Roche. Antibiotics, ampicillin, metronidazole, tetracycline and chloramphenicol, with concentrations ranging from 0.015 – 256 µg were used to assess the overall susceptibility of *H. pylori* strain 26695 and to compare their anti-*H. pylori* activity with that of potato extracts. Ampicillin and chloramphenicol were dissolved in water and ethanol respectively, to obtain a stock concentration of 1 mg/ml.

2.1.2 Strain Maintenance and Growth conditions

Helicobacter, *Campylobacter* and *Wolinella* species used in this study were obtained from laboratory glycerol stock. The cultures were passaged into fresh media every 2 – 4 days. Long-term storage of strains was achieved by suspending an overnight culture of the required strain in Mueller Hinton broth with 15 % (v/v) glycerol, followed by storage at -80°C. *H. pylori* was suspended in Brain heart infusion (BHI) broth with 40 % (v/v) glycerol. Over

short periods, *E. coli* was maintained on Luria-Berthani (LB) agar medium and stored at 4°C. A comprehensive list of bacterial strains and culture conditions is provided in Table 2.3.

Table 2.1 Bacterial strains used during the course of this study

Bacteria Strain	Reference/Source
Helicobacter pylori 26695	Lab strain
Helicobacter pylori 26695 *galE* mutant	Nicky High
Helicobacter pylori 26695 potato extract-resistant strains	This study
Helicobacter pylori NCTC 11637	Nicky High
Helicobacter pylori J99	Dennis Linton
Helicobacter pylori clinical antibiotic-resistant strains (Table 2.2)	Dennis Linton
Helicobacter pullorum NCTC 12824	Dennis Linton
Helicobacter canadensis 13241	Dennis Linton
Helicobacter acinonychis NCTC 12686T	Dennis Linton
Helicobacter felis NCTC 12436T	Dennis Linton
Helicobacter winghamensis NCTC 13220T	Dennis Linton
Campylobacter jejuni NCTC 11168	Dennis Linton
Campylobacter coli RM 2228	Dennis Linton
Wolinella succinogenes DSM 11484	Dennis Linton
Escherichia coli DH5α	Corbett D

Table 2.2 Clinical antibiotic resistant *H. pylori* strains and resistant phenotype

Clinical *Helicobacter pylori* isolate	Phenotype
H1 2344 0327	R to Cla & Rif: S to Met, Tet, Amx & Lev
H1 2318 0581	R to Cla, Met, Lev & Rif: S to Tet & Amx
H1 2286 0620	R to Cla & Lev: S to Met, Tet, Amx & Rif
H1 2206 0181	R to Cla, Met, Lev, Rif & Amx: S to Tet

R- resistant, S- susceptible, Cla- clarithromycin, Rif- rifampin, Met- metronidazole, Tet- tetracycline, Amx- amoxicillin, Lev- levofloxacin

Table 2.3 Bacteria strains and culture conditions

Bacteria strain	Media	Temperature		Gas requirement			
		37°C	42°C	N_2 (85%)	H_2 (5%)	O_2^m (5%)	CO_2 (10%)
Helicobacter pylori 26695*	Columbia agar with 10% defibrinated Horse Blood (supplemented with 0.25% v/v *H. pylori* selective supplement)	+	-	+	±	+	+
Helicobacter pylori 26695 *galE* mutant	Same as above	+	-	+	±	+	+
Helicobacter pylori 26695potato extract resistant mutants	Same as above	+	-	+	±	+	+
*Helicobacter pylori*NCTC 11637	Same as above	+	-	+	±	+	+
*Helicobacter pylori*J99	Same as above	+	-	+	±	+	+
Helicobacter acinonychis	Columbia agar with 10% defibrinated Horse Blood	+	-	-	±	+	+
Helicobacter felis	Columbia agar with 5% defibrinated Horse Blood	+	-	-	+	+	+
Helicobacter pullorum NCTC 12824	Columbia agar with 5% defibrinated Horse Blood	-	+	-	±	+	+
Helicobacter canadensis 13241	Columbia agar with 5% defibrinated Horse Blood	-	+	-	±	+	+
Helicobacter winghamensis NCTC 13220T	Columbia agar with 5% defibrinated Horse Blood	+	-	-	+	+	+
Campylobacter jejuni NCTC 11168	Columbia agar with 5% defibrinated Horse Blood	+	+	-	±	+	+
Campylobacter coli RM 2228	Columbia agar with 5% defibrinated Horse Blood	+	+	-	±	+	+
Wolinella succinogenes[b] DSM 11484	Columbia agar with 5% defibrinated Horse Blood (supplemented in 3g/L Sodium formate and 3g/L Sodium fumarate, added before autoclaving)	+	-	+	-	-	+
Escherichia coli[a] DH5α	Luria-Berthani (LB) agar	+	-	+	+	aero be	+

(+)- essential, (±)- nonessential, (-)- unfavourable, ([a])- aerobe, ([b])- strict anaerobe, ([m])- microaerophilic, (*)- *H. pylori* strain 26695 was used in all *H. pylori*-related assays, except where otherwise stated.

2.2 DNA Manipulation

2.2.1 Extraction of Chromosomal DNA

DNA samples were prepared from bacteria culture grown on solid media using the ArchivePure DNA Purification kits according to the manufacturer's instructions.

2.2.2 Restriction Endonuclease digestion of DNA

Restriction endonuclease digestion was performed according to the restriction endonuclease manufacturer's instructions. Typically, 1 unit of the required restriction endonuclease (NEB or Roche) was added to a solution containing the DNA and 10 x digestion buffer. Then the DNA was digested in a final reaction volume of 20 µl with sterile distilled water. The digestion mixture was thoroughly mixed and incubated at 37°C for 3 h. Enzyme was then heat inactivated according to the manufacturer's instructions. Analysis of the digested DNA was carried out by agarose gel electrophoresis.

2.2.3 Agarose Gel Electrophoresis

DNA fragments were separated by agarose gel electrophoresis with 1% (w/v) agarose, carried out at 6 Vcm^{-1} in Tris-acetate-EDTA buffer (TAE: 0.5 M Tris acetate, 5.7 % acetic acid, 10 mM EDTA pH 8.0) containing 5 µg ml^{-1} ethidium bromide. DNA solutions were mixed with 6 x loading buffer (0.25 % bromophenol blue, 15 % ficoll-400 dissolved in sterile distilled water) prior to loading into agarose submarine slab gel wells. DNA fragment sizes were calibrated with 1 kb or 100 bp markers (Hyperladder I and IV, Bioline). Gels were then visualised under UV light.

2.2.4 Natural Transformation of *H. pylori*

H. pylori 26695 was naturally transformed using the method previously described by (Wang et al., 1993). *H. pylori* was cultured on Columbia blood agar plates and allowed to grow for 24 h. This was then harvested and transferred as thick patches to fresh agar plates and incubated. After 5 h, 4 µg donor DNA was added to the patches. After 24 h of incubation, the culture was suspended in PBS and a 10-fold dilution was carried out. 100 µl of appropriate dilutions was then spread on selective agar plates. The transformation control was plated on non-selective agar plates. Colonies were counted after 5 days of incubation, and the transformation frequency was calculated.

2.3 Protein Purification and Analysis

2.3.1 Cellular fractionation

2.3.1.1 Total membrane protein extraction

Total membrane protein samples were prepared from agar-grown *H. pylori* strains incubated for 2 days. Cultures were harvested and suspended in 50 mM Tris-HCl (pH 7.0) with 20 % sucrose, and 1 % Protease inhibitor added before use. Cells were treated with 10 mM EDTA and 10 µg/ml lysozyme, and incubated for 10 minutes at room temperature. Samples were centrifuged at 8,000 x g for 10 min at 4°C and the pellet resuspended in 10 mM Tris-HCl (pH 7.0) with 1 % protease inhibitors. Solution was pressed 3 times in French pressure cell and centrifuged at 10K rpm for 20 minutes at 4°C to remove cell debris and unbroken cells. Centrifugation was repeated until no pellet is formed. The clarified sample was ultra-centrifuged at 100,000 x g for 1 h at 4°C. The supernatant (cytoplasmic fraction) was discarded and the tight pellet (total membrane fraction) was washed with 10 mM Tris-HCl and stored at -20°C until required.

2.3.1.2 Periplasmic protein extraction

Periplasmic extract were prepared from *H. pylori* strains grown on solid media for 2 days. Cultures were harvested and suspended in 1 ml Tris-sucrose-EDTA (TSE) buffer [200 mM Tris-HCl pH 8.0, 500 mM sucrose, 1 mM EDTA], and incubated on ice for 30 min. Bacterial suspensions in TSE buffer was centrifuged at 16,000 x g for 30 min at 4°C. The supernatant was re-centrifuged until no pellet was formed and stored at -20°C until required.

2.3.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Mini-Protean 3 system (Bio-Rad) was used to set the polyacrylamide gels. The gel was composed of 12 % resolving gel and 5 % stacking gel, into which protein samples were loaded. The resolving and stacking gels were thoroughly mixed before the addition of ammonium persulphate (APS) and N,N,N',N', tetramethylethylenediamine (TEMED), which catalyse the polymerisation of the acrylamide gel. After the addition of APS and TEMED to the resolving gel solution, the solution was applied to glass plates, covered with a layer of distilled water and allowed to set for a minimum of 45 min. Once the gel is set, the water was drained and the top of the gel was washed with distilled water about 10 times. The top of the gel was dried with filter paper before the addition of the stacking gel. APS and TEMED were added to the stacking gel solution, this was then poured into the glass plate assembly and the desired comb was inserted into the stacking gel to form the wells. The gel was allowed to set for a minimum of 30 min. After setting, the comb was gently removed and the wells rinsed with SDS running buffer [192 mM glycine, 25 mM Tris, 3.5 mM SDS]. Protein samples were then boiled for 10 min in 1 x SDS loading buffer [62

mM Tris pH 6.8, 1 % (v/v) SDS, 0.1 % (v/v) glycerol, 0.1 % bromophenol blue, and 10 % (v/v) 2-mercaptoethanol added immediately before use). Typically, 10 µl of the samples were loaded onto the gel along with molecular weight standards (Bio-Rad). The samples were allowed to migrate at an initial 80 V for 20 min to enable samples to stack on the resolving gel before being electrophoresed at 150 V until the samples have migrated to the end of the gel. The gels were visualised by staining in Coomassie-based staining solution (InstantBlue, Expedeon) overnight. The solution was then removed and the gel was rinsed several times with distilled water and stored. Protein sizes were estimated by comparison with the molecular weight markers.

2.3.2.1 Resolving gel (12 %)

2.0 ml acrylamide solution [30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide]

1.3 ml 1.5M Tris-HCl pH 8.8

50 µl 10 % SDS

1.6 ml distilled water

50 µl 10 % ammonium persulphate (APS)

2 µl TEMED

2.3.2.2 Stacking gel (5 %)

330 µl acrylamide solution

250 µl 1M Tris-HCl pH 6.8

20 µl 10 % SDS

1.4 ml distilled water

20 µl 10 % (w/v) ammonium persulphate (APS)

2 µl TEMED

2.3.3 Protein quantification

Protein samples were quantified and standardised using the Bradford protein assay. A series of protein standards was prepared using lyophilised bovine serum albumin (BSA) (0.8, 0.6, 0.4, and 0.2 mg/ml BSA). 20 µl of each protein sample and BSA standard was pipetted into a clean cuvette and 1 ml of Bio-Rad dye reagent (diluted with 4 parts distilled deionised water) was added to each cuvette tube. The protein standards and samples were incubated at room temperature for 5 min and the absorbance (A) was measured using a spectrophotometer at wavelength 595 nm. Using the absorbance measured, a BSA standard curve was plotted and the protein concentration in each potato extract sample was calculated with respect to the absorbance.

2.4 Polyacrylamide gel electrophoresis of lipopolysaccharide

2.4.1 Preparation of whole-cell lysates for LPS analysis

LPS samples were prepared as previously described (Kimura and Hansen, 1986) with some modifications. *H. pylori* strains were grown for 3 days on solid media, harvested and suspended in PBS. Bacterial suspensions were adjusted to OD_{600} 1.0, of which 1 ml was centrifuged at 5000 x g for 10 min. Bacterial pellets were re-suspended in 300 µl PBS, to which 150 µl concentrated digestion buffer [0.1875M Tris-HCl pH 6.8, 6 % (w/v) SDS and 30 % (v/v) glycerol] was added. Samples were heated to 100°C for 5 min and the resulting lysates were stored at -20°C.

Following this, the method described by (Prendergast et al., 2001) was followed. When required, lysates were thawed at room temperature and an equal volume of phenol (preheated to 65°C) was added. Samples were mixed for 1 min using a vortex and then incubated for 10 min at 65°C.

Samples were then cooled on ice for 1 min and centrifuged at 10,000 x g for 5 min. At this stage, separate layers (water-protein-phenol) were visible in the suspension. The water phase, containing LPS, was carefully pipetted out and residual phenol was removed by extracting with diethyl ether. The diethyl ether phase was discarded and the water phase (containing LPS) was placed in a fume hood for 1 h to allow the remaining diethyl ether to evaporate. 10 µl of the sample was diluted with 35 µl LPS digestion buffer [0.0625M Tris-HCl pH 6.8, 0.1 % (w/v) SDS, 10 % (v/v) glycerol and 0.005 % (w/v) bromophenol blue] and heated to 100°C for 5 min before loading.

2.4.2 Tricine- SDS -Polyacrylamide gel electrophoresis (T-SDS-PAGE)

LPS samples were resolved by T-SDS-PAGE as previously described (Lesse et al., 1990). The gel was prepared and run using Bio-Rad Protean II xi vertical slab gel apparatus, with 20 cm plates and 1.5 mm spacers. Samples were loaded into a 4 % stacking gel and seperated in a 16.5 % resolving gel. A discontinuous buffer system with anode buffer [0.2M Tris-HCl, pH 8.9] and cathode buffer [0.1M Tris, 0.1M Tricine and 0.1 % (w/v) SDS] was used. Up to 35 µl of each sample was applied to the wells of the stacking gel and a constant voltage of 110 V was applied to the gel for 16 – 18 h or overnight. Gels were then removed from the apparatus and LPS was visualised by silver staining.

2.4.2.1 T-SDS-PAGE Resolving gel

27.6 ml acrylamide solution [30 % (w/v) acrylamide, 0.8 % (w/v) bis-acrylamide]

16.6 ml gel buffer [3M Tris-HCl pH 8.45, 0.3 % SDS]

5.2 ml glycerol

500 µl distilled water

100 µl 10 % (w/v) ammonium persulphate

10 µl TEMED

2.4.2.2 T-SDS-PAGE Stacking gel

1.6 ml acrylamide solution

3.1 ml gel buffer

7.8 ml distilled water

150 µl 10 % (w/v) ammonium persulphate

10 µl TEMED

2.5 Visualisation of LPS by silver staining

LPS silver staining was carried out according to the method of (Tsai and Frasch, 1982). Gels were incubated with agitation at room temperature for at about 45 min in fixative [25 % (v/v) isopropanol, 7 % (v/v) glacial acetic acid] and oxidised for 15 min in 2.67 % (v/v) fixative, 0.7 % (w/v) periodic acid. Gels were then washed four times in copious volumes of distilled water, for 1 hour per wash. After washing, the gels were stained with ammoniacal silver nitrate solution. This solution was prepared by adding 1.4 ml of ammonia solution (0.88 SG) to 21 ml 0.1M NaOH. Silver nitrate (20 %) was freshly prepared and added drop wise to the ammonia/ NaOH solution until saturation (when the solution did not clear, approximately 7 ml) and was then cleared with one drop of ammonia. The solution was made up to a final volume of 100 ml with distilled water. Gels were stained for 15 min and then washed four times in copious volumes of distilled water for 10 min per wash. Finally, developer [0.02 % (v/v) formaldehyde solution, 0.005 % (w/v) citric acid] was added until LPS could be visualised. This was stopped by washing the gel in copious volumes of distilled water.

2.6 Morphological Test (Gram staining)

The Gram staining reaction was used to confirm the identity of *H. pylori* in culture. A smear of each isolate was made on a glass slide and heat fixed. The heat fixed smear is then flooded with crystal violet for 1 min and washed with water. The film is then covered with iodine and a contact time of 1 min is allowed before washing with water. This is followed by decolourization with alcohol for 15 sec and then washed with water. Safranin is then added to the film as the counter stain and is allowed for about 1 – 2 min, and washed with water. The underneath of the slide is then cleaned up and placed in a draining rack to air dry. The smear was then examined microscopically, after a drop of immersion oil is placed on the smear. The cells are observed and recorded. Water used for washing the reagents was distilled water.

2.7 Biochemical test (Urease test)

Urease test was used to confirm the identity of *H. pylori*. The principle of the urease test is that urease, an enzyme produced by *H. pylori*, hydrolyses urea to produce carbon dioxide and ammonia as seen in the equation below.

$$\underset{\text{Urea}}{(NH_2)_2CO} + 2\,H_2O \xrightarrow{\text{Urease}} \underset{\text{Carbon dioxide}}{CO_2} + \underset{\text{Water}}{H_2O} + \underset{\text{Ammonia}}{2\,NH_3}$$

Single colonies of *H. pylori* were streaked on Christensen urea agar slants, and incubated at 37°C in the CO_2 incubator for 24 h. A colour change from orange to bright pink, caused by the production of ammonia and

consequent increase in pH, is indicative for urease-positive and colour change for urease-negative.

2.8 Antibacterial assays

2.8.1 Viable count assay

Quantitative assessment of anti-*H. pylori* activity of potato extracts was carried out by viable count using the method described by (O'Mahony et al., 2005) as follows: With a sterile loop, a plate of *H. pylori* confluent that had been incubated for 2 days was suspended in 1 ml phosphate buffer saline (PBS) at pH 7.2 and the density adjusted to an OD_{600nm} of 1.0 in an Ultraspec 2100 Pro (Amersham Biosciences). This is equivalent to 1.0×10^8 CFU/ml and was verified by viable cell counting. The bacterial suspension was again adjusted to the desired cell density of 1.0×10^5 CFU/ml, required for the assay, by serial dilution. 100 µl of this inoculum was then added to potato extracts in a 1:1 ratio and then incubated under appropriate growth conditions for 90 min. A positive control made up of the bacterial suspension and PBS was prepared. The samples (100 µl) were then spread on agar assay plates, allowed to dry and processed for viable counts using standard procedures. Assays were carried out in duplicate and repeated three times.

2.8.2 Agar dilution method

The agar dilution method was used to determine the minimum inhibitory concentration (MIC) of potato extract against *H. pylori* strains. Briefly, Columbia blood agar was cooled to 50°C and supplemented with different concentrations of potato extract (typically a two-fold dilution series) in a 1:10 ratio. The desired inoculum was then spread on the agar plate and allowed to dry. The MIC was defined as the lowest concentration of potato

extract that prevented visible growth of viable colonies under defined conditions.

2.8.3 Oxoid MIC Evaluator Method

The Oxoid M.I.C.Evaluator (M.I.C.E.™) strips impregnated with increasing concentration of antibiotics (from 0.015 – 256 µg) were used to carry out MIC assays according to the manufacturer's instructions.

2.9 Plant Material

The variety of potato tuber used in this study is the British Maris Piper potatoes. All potatoes used were purchased from Sainsbury supermarket, the "best before" date was recorded and the potato tubers were stored at $4^{o}C$ until required.

2.10 Standardized extraction of potato extracts

The potato tubers were peeled, washed and weighed. After which the tubers were macerated in their own volume using a domestic blender containing 10 ml of 100 mM sodium metabisulfite per 300 g of potatoes, so as to prevent oxidation of potato polyphenols and degradation of ascorbic acid which subsequently leads to darkening of the potato juice. The potato juice was then sieve filtered and centrifuged twice at 9,000 rpm for 20 min. The resultant juice was frozen overnight at $-80^{o}C$ and then freeze-dried for 26 h to reduce the water content and concentrate the juice. The resulting crude juice was then precipitated with ethanol in a ratio 1:6; followed by filtration to obtain the ethanol-soluble fraction. This fraction was then evaporated to dryness using nitrogen N_2 gas; re-suspended in sterile water; frozen at $-80^{o}C$ and freeze dried to obtain dry weight. The resultant extract obtained divided into portions of 250 mg each; each 250 mg portion was suspended in 1 ml sterile water, sterile filtered through a 0.22 µm

membrane; and was used in the antibacterial assays. When not in use immediately, the lyophilised dry weight was stored at -80°C.

2.11 Determination of the protein and carbohydrate concentration in potato extract

2.11.1 Determination of carbohydrate concentration

Carbohydrate content of potato extract was estimated using the phenol sulphuric acid method (Dubois et al., 1956). The principle of this method is that in hot acidic medium, glucose is dehydrated to hydroxymethyl furfural, which forms a yellow-brown coloured product in the presence of phenol, which has a maximum absorption at 490 nm.

A series of glucose standards was prepared in distilled water (1.0, 0.8, 0.6, 0.4, 0.2, 0.1, 0.05, and 0.02 mg/ml glucose). 200 µl of each extract sample and glucose standard was pipetted into a clean test tube, and 200 µl of 5 % phenol was added. 1 ml of concentrated sulphuric acid was then added to each cuvette and mixed by vortexing. After 10 min, the samples were vortexed again and placed in a water bath set at 30°C for 20 min. Following this, absorbance (OD) was measured using a spectrophotometer at wavelength 490 nm. Using the absorbance measured, a glucose standard curve was plotted and the carbohydrate concentration in each potato extract sample was calculated with respect to the absorbance.

2.11.2 Determination of protein concentration

Protein concentration in potato extract was quantified using the Bradford protein assay as previously described in section 2.3.3.

2.12 Preparation of concentrations of potato extract

Various concentrations of potato extract used in this study were obtained by diluting 250 mg/ml potato extracts with sterile water. The concentrations prepared are as follows: 125, 62.5, 31.2, 15.6, 7.8, 3.7, 1.95, 0.97, 0.48, 0.24 and 0.12 mg/ml of potato extract.

2.13 Antibacterial activity of potato extract

This was determined using the viable count assay as described above. Known cell densities of *H. pylori* and *E. coli* were incubated with 250 mg/ml potato extracts in a 1:1 ratio for 90 min. The samples were vortexed and processed for viable count against a PBS control. *E. coli* was used as a negative control.

2.14 Determination of minimum inhibitory concentration

Using the viable count method, different concentrations of potato extract were tested to determine the minimum concentration at which potato extracts show anti-*H. pylori* activity. Dilutions of 0.97, 1.95, 3.7, 7.8, 15.6, 31.2, 62.5, 125 and 250mg/ml potato extract were tested for growth inhibition on agar plates against a PBS control. The lowest concentration at which potato extract showed 100% growth inhibition was recorded.

2.15 Bactericidal activity of potato extract (Time-kill assay)

Potato extract concentrations that killed 100 % of *H. pylori* (that is, no colonies) within 90 min were further evaluated at 0, 5, 15, 30, 45, 60 and 90 min interval and the killing curve was plotted. Approximately 0.1 ml of 1 x 10^5 CFU/ml of test bacterial strain was introduced into eppendorfs containing 0.1 ml each of 250, 125, 62.5, 31.2 and 15.6 mg/ml potato extract. The control consisted of a duplicate culture without potato extract

(negative control). All experiments were incubated in a CO_2 incubator at 37°C. At different time points (5, 15, 30, 45, 60 and 90 min), 0.1 ml of sample was withdrawn, diluted in 3 ten-fold series of PBS. 0.1 ml of each dilution was spread in duplicate on Columbia blood agar plates. After 4-5 days incubation at 37°C, emergent bacterial colonies were counted and compared with the count of the culture control without extract. Viable counts were calculated to give the CFU/ml, and kill curves were plotted with time against the calculated CFU/ml. The experiment was performed three times.

2.16 Stability of potato extracts over a specified period under different storage conditions

Freshly prepared potato extract was stored at room temperature, 2 – 8°C, and -80°C for 3 months. The antibacterial activity of each stored extract was evaluated against *H. pylori* using the viable count method as previously described. The lowest concentration at which freshly prepared extract killed 100 % of the bacteria cells was recorded and compared to the value for the extracts stored for 3 months at different storage conditions. The experiment was carried out in duplicates and repeated three times.

2.17 Toxicity of potato extract towards closely-related species to *H. pylori*

Known density (10^5 CFU/ml) of bacterial suspensions of *H. felis, H. acinonychis,H. canadensis*, *H. pullorum*, *H. winghamensis*, *C. jejuni*, *C. coli* and *W. succinogenes* were assayed against known concentrations of potato extract. This was carried out using the viable count method described above. The concentration at which potato extract inhibited growth of each

of these bacteria was recorded. Appropriate controls, *H. pylori* treated with potato extract and PBS controls for each test organism, were used.

2.18 Toxicity of potato extract towards clinical antibiotic resistant strains

Clinical antibiotic resistant *H. pylori* strains with resistance to clarithromycin, metronidazole, levofloxacin, rifampin and amoxicillin were tested for susceptibility to potato extract. 1 x 10^5 CFU/ml of bacteria suspensions were treated with known concentrations of potato extract and incubated under suitable conditions. The assay was carried out using the viable count method. Wild-type *H. pylori* strain and PBS controls of each antibiotic resistant strain were used as negative controls. The lowest concentration at with potato extract killed 100 % of the cells was recorded. Assay was carried out in triplicate three times.

2.19 Synergy between potato extract and antibiotics

Synergy assay was carried out using a combination of the agar dilution method with the Oxoid MIC Evaluator method. Firstly, the lowest concentration of potato extract that completely inhibited the growth of *H. pylori* was determined using the agar dilution method. 1 x 10^8 CFU/ml of bacteria suspension was then spread on Columbia blood agar plates supplemented with concentrations of potato extract lower than the MIC, which allowed the growth of *H. pylori* as a lawn culture. Oxoid M.I.C.Evaluator (M.I.C.E™) strips impregnated with increasing concentrations of antibiotics (from 0.015 – 256 µg) was then applied to the plates and incubated under suitable conditions for 3 – 5 days. Appropriate controls, with Columbia blood agar plates not supplemented with potato extract were included. The MIC of antibiotic on the strip, determined by

the zones of inhibition formed, was recorded and compared to that of the control agar plates. The difference in MIC was recorded and used to determine the presence or absence of synergy between the antibiotics and potato extract.

2.20 Effect of potato extract on the morphology of *H. pylori*

Transmission electron microscopic observations were carried out on *H. pylori* 26695 cells treated with potato extract. 500 µl of 10^8 CFU/ml of *H. pylori* suspension was incubated with 250 mg/ml of potato extract at different time points; 5, 15, 30, 45, 60 and 90 min. PBS-treated cultures of the different time points were used as controls. The controls and the extract-treated cells were fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde for 1 h and later post-fixed in 1 % osmium tetraoxide and 1.5 % potassium ferrocyanide for 1 h. The cells were then washed twice with 0.1 M sodium cacodylate buffer, pH 7.4; after which 1 % tannic acid was added for 1 h and washed three times with distilled water. Cells were then treated with 1 % uranyl acetate overnight. After eliminating the uranyl acetate, the samples were washed twice with distilled water and dehydrated in 50, 75, 90 and 100 % ethanol at 5 min interval. Following dehydration, the cells were infiltrated twice in propylene oxide at 5 min interval; and then in equal parts of propylene oxide and epoxy resin for 1 h in a rotor, after which it was removed completely from the cells. Fresh 100 % resin was added to the cells and left in a rotor for about 3 – 4 h; then it was transferred to an oven at 60°C to polymerise overnight or longer. Ultra-thin sections were then cut from polymerized cell blocks using an ultramicrotome; and analysed in the Transmission Electron Microscope (Philip Tecnai 12 Biotwin).

2.21 Effect of potato extract on the membrane integrity of *H. pylori*

The effect of potato extract was assessed using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes) with minor modification to the manufacturer's protocol. *H. pylori* cells cultured for 2 days on Columbia blood agar was suspended in 1 ml 0.85 % NaCl and adjusted to a density of 10^8 CFU/ml. Known concentrations of potato extract, filtered through a 0.22 µm membrane, was added to the bacteria suspension in a 1:1 ratio and incubated for 15 min under microaerophilic conditions. Cells were then harvested by centrifugation (8,000 x g, 5 min), washed twice with 0.85 % NaCl and resuspended in 1 ml of 0.85 % NaCl. The dyes from the BacLight kit, Syto 9 and Propidium iodide (PI), were then mixed in a 1:1 ratio to make the staining mix. 3 µl of this staining mix was the added to 1 ml of bacteria suspension and incubated in the dark for 15 min. 8 µl of the stained bacterial suspension was then trapped between a slide and coverslip, and viewed under a fluorescence microscope. Appropriate controls, treated in the same way as the sample, include cells treated with ampicillin (100 µg/ml), chloramphenicol (25 µg/ml), propanol (100 %) and 0.85 % NaCl respectively. Images were collected on an Olympus BX51 upright microscope using a 100x objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Specific band pass filter sets for Syto 9 and PI were used to prevent bleed through from one channel to the next. Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij).

2.22 Effect of potato extract on the membrane potential of *H. pylori*

Relative changes in plasma membrane potential, caused by potato extract treatment in *H. pylori* were measured using the method previously described by (Pag et al., 2004), with some modifications. The

measurements were carried out in a black opaque 96-well microplate (BD Falcon). *H. pylori* cells were grown on Columbia blood agar plates for 2 days at 37°C. The cells were suspended in PBS and adjusted to a density of 1 x 10^{7} CFU/ml. Then 1 µl of 1 mg/ml membrane potential-sensitive slow-response fluorescent probe, bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC4[3]; Molecular Probes) was added to 1 ml of the bacteria suspension. 100 µl of this was then dispensed into wells. Fluorescence was then measured at 5 min interval from the addition of DiBAC4 [3], at 37°C. After 25 min, when the dye uptake was maximal as indicated by a steady decrease in fluorescence, 100 µl of potato extract (62.5, 125, and 250 mg/ml), filtered through a 0.22 µm membrane was added. Changes in fluorescence were then measured for 60 min at 5 min interval. Fluorescence was measured at the excitation and emission wavelength of 485 nm and 528 nm, respectively using a BioTek™ Synergy™ HT Multi-detection Microplate Reader. Background fluorescence resulting from potato extract being added to the bacteria suspension was determined and the results were corrected.

2.23 Effect of potato extract on the ATP concentration of *H. pylori*

The available energy in *H. pylori* cells treated with potato extract was assessed by measuring the level of cellular ATP in the cells. This was measured using the ENLITEN® ATP Assay System Bioluminescence Detection Kit (Promega), according to manufacturer's protocol. Cultures of *H. pylori* (10^{8} CFU/ml) were treated with different concentrations of potato extract (62.5, 125, and 250 mg/ml), filtered through a 0.22 µm membrane, in a 1:1 ratio and incubated at 37°C for 15 min under microaerophilic conditions. Cells were then harvested by centrifugation (10,000 x g, 5 min), washed twice with PBS and re-suspended in 500 µl PBS.

To extract ATP from the cell suspensions, the method described by (Sanchez et al., 2010) was followed. 250 µl of ice-cold 24 % (v/v) perchloric acid was added to 500 µl of each previously treated cell suspensions, and placed on ice for 20 min and centrifuged at 10, 000 x g for 5 min. The supernatants were then neutralised with 125 µl 4 M KOH, and placed on ice for 30 min and centrifuged as described above. The boiling deionised water (DW) method previously described (Yang et al., 2002) was also used to extract ATP, and compared to the perchloric acid method. The boiling DW method involves the addition of boiling DW to previously pelleted bacteria cells in order to extract ATP. The cell suspension was then vortexed and centrifuged at 12,000 g for 5 min at 4°C. The resulting supernatant is the ATP sample

In order to quantify ATP, 50 µl of each sample supernatant and ATP standards, provided in the ATP assay kit, was transferred to a white opaque 96-well plate (BD Falcon) and 100 µl of the ENLITEN® rLuciferase/Luciferin reagent from the ATP assay kit (Promega) was added. Bioluminescence was measured using a BioTek™ Synergy™ HT Multi-detection Microplate Reader. Using the bioluminescence values obtained, an ATP standard curve was plotted and the ATP concentration in each potato extract-treated sample was calculated with respect to bioluminescence.

2.24 Chemical mutagenesis of *H. pylori*

The chemical mutagen, ethyl methanesulfonate (EMS), was used as previously described (Segal et al., 1992). *H. pylori* 26695 was grown on 10 % Columbia blood agar plates overnight at 37°C. Cells were then harvested, suspended in PBS and spun down at 13K rpm for 1 min. The cells were re-suspended in 2 ml PBS in a conical flask and 7.6 ml of 1 M Tris-HCl (pH 7.5) was added to the suspension. 100 µl of EMS (1.17 g/ml) was added, and

the culture was placed in the CO_2 incubator at 37°C for 5 – 15 min with constant shaking. 90 ml of Brucella broth, supplemented with 5 % fetal bovine serum (FBS), was added to the culture and grown overnight at 37°C with constant shaking. The cells were spun down following overnight incubation, and a 10-fold serial dilution was carried out in PBS. Appropriate dilutions were plated on Columbia blood agar and placed in the CO_2 incubator for 3 – 5 days when individual colonies appear. The mutagenized *H. pylori* stock was suspended in 2 x Brain heart infusion (BHI) media with 80 % glycerol and stored at -80°C.

2.25 Generation *H. pylori* strains resistant to potato extract

In order to isolate *H. pylori* mutants resistant to potato extract, the agar dilution method described above was employed. In this instance, the density of the mutagenized *H. pylori* stock was determined from serial dilution series by viable colony count. Appropriate dilutions were then spread on Columbia blood agar plates supplemented with potato extract, with final concentrations of 25, 12.5 and 6.25 mg/ml. Controls included mutagenized *H. pylori* cells spread on plates without potato extract, and the wild-type strain, of equal density (10^6 CFU/ml) with the mutagenized sample, spread on plates with and without potato extract. Plates were incubated for 4 – 8 days in a CO_2 incubator at 37°C. Colonies from the mutagenized cell suspension that appeared on agar plates supplemented with potato extract were then re-streaked on agar plates supplemented with extract and incubated for 3 – 4 days. This process was repeated twice; after which the mutant colonies were verified as *H. pylori* by Gram staining and a positive urease test.

2.26 Statistical Analysis

Results are reported as mean ± standard error of the mean. Student's paired *t*-test was used for statistical analysis of all viable cell count as well as diameter of inhibition zones. Results were considered significant at $P \leq 0.05$.

Chapter 3

Evaluating the Antibacterial Activity of Potato Extract

3.1 Introduction

Crude potato extract has previously been shown to exhibit antibacterial activity against *H. pylori* (Bennett and Roberts, unpublished data); and this discovery has stimulated further interest in understanding its mode of action. In order to do this, the antibacterial activity of potato extract was further investigated. In this chapter, a number of antibacterial assays were carried out to evaluate the activity of potato extract against *H. pylori*, clinical antibiotic resistant *H. pylori* strains and a range of other bacteria so as to determine its range/spectrum of activity.

3.2 Potato extract exhibit antibacterial activity against *H. pylori*

At the start of this project, a new extraction method for obtaining potato extract was devised as described in the Materials and methods section. Therefore, it was important to determine if the extraction procedure retained the anti-*H. pylori* activity of potato extract.

H. pylori 26695 was treated with different concentrations of potato extract and anti-*H. pylori* activity was assessed quantitatively using the viable count method as described. *E. coli* has previously been shown to be resistant to potato extract (Bennett and Roberts, unpublished data) so it was used as a positive control and also as a model to test the effect of the extract on other Gram-negative bacteria. For the negative control, *H. pylori* cell suspension was mixed with PBS in a 1:1 ratio. Potato extract showed significant antibacterial activity against *H. pylori* (P= 0.009) as no viable cell was observed after *H. pylori* was incubated with potato extract (62.5, 125

and 250 mg/ml) for 90 min and processed for viable count (Figure 3.1). When potato extract was tested for activity against *E. coli*, antibacterial activity was observed in the treatment with 250 mg/ml potato extract as compared to the PBS control; however, the observed activity is not significant (P= 0.12). Lower concentrations of potato extract, 62.5 and 125 mg/ml, used showed no significant activity against *E. coli* (P > 0.05) (Figure 3.1).

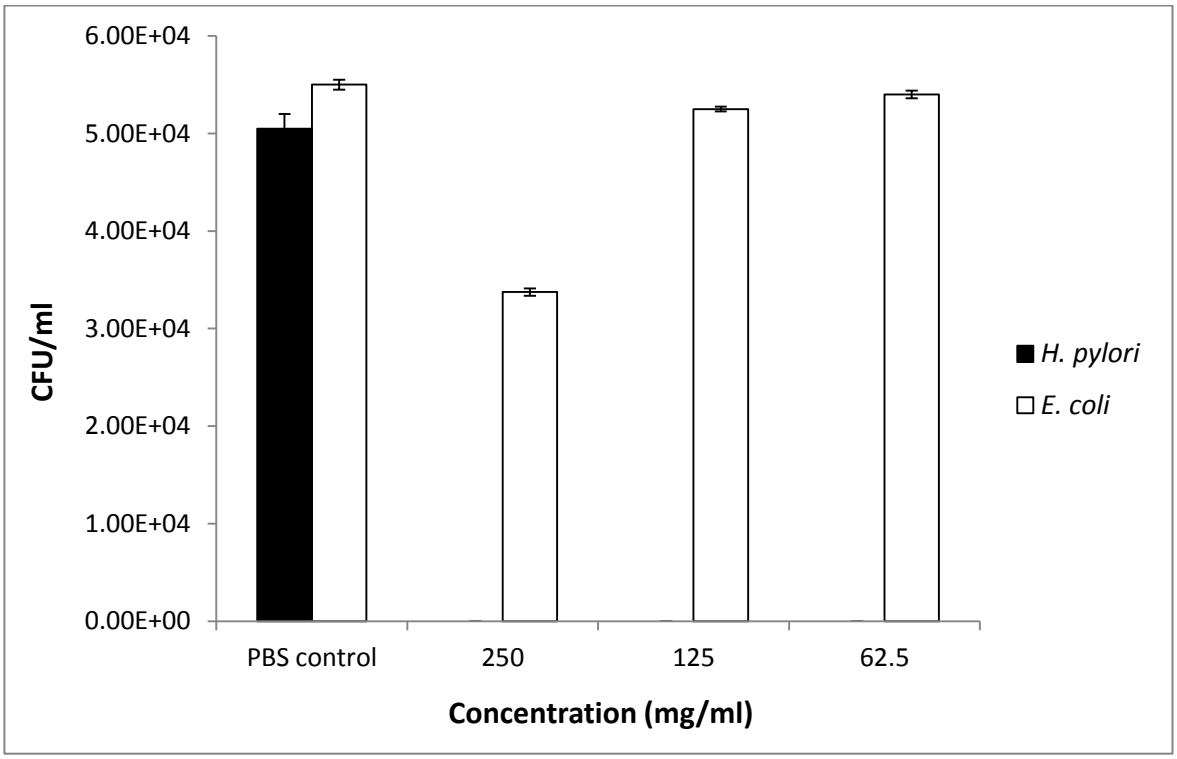


Figure 3.1 Antibacterial activity of potato extract against *H. pylori* and *E. coli*

Viable cell count of *H. pylori* and *E. coli* after incubation with known concentrations of potato extract for 90 min. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained. The mean difference is considered significant at P ≤ 0.05.

3.3 Minimum inhibitory concentration of potato extract against *H. pylori*

The antibacterial assay described above, against *H. pylori*, demonstrated that 62.5 mg/ml of potato extract totally killed *H. pylori* (10^5 CFU/ml). It is therefore important to determine the lowest concentration at which potato extract completely kills *H. pylori* (10^5 CFU/ml), that is, 100 % killing. This was evaluated quantitatively by viable count assay using decreasing concentrations of potato extract (Figure 3.2). It was observed that potato extract exhibited antibacterial activity in all concentrations tested (0.97, 1.95, 3.9, 7.8, 15.6, 31.2, 62.5, 125 and 250 mg/ml), completely killing *H. pylori* at 250 to 15.6 mg/ml. The anti-*H. pylori* activity was also observed to decrease from 7.8 mg/ml down to 0.97 mg/ml (Figure 3.2).

By statistical analysis, anti-*H. pylori* activity of potato extract observed at concentrations, 7.8, 3.9, 1.95 and 0.97 mg/ml, were significant as P value was less than 0.05. The lowest concentration at which potato extract killed 100 % of *H. pylori* cells, when compared to the PBS control, is 15.6 mg/ml. Therefore, the minimum inhibitory concentration (MIC) is recorded as < 15.6 mg/ml.

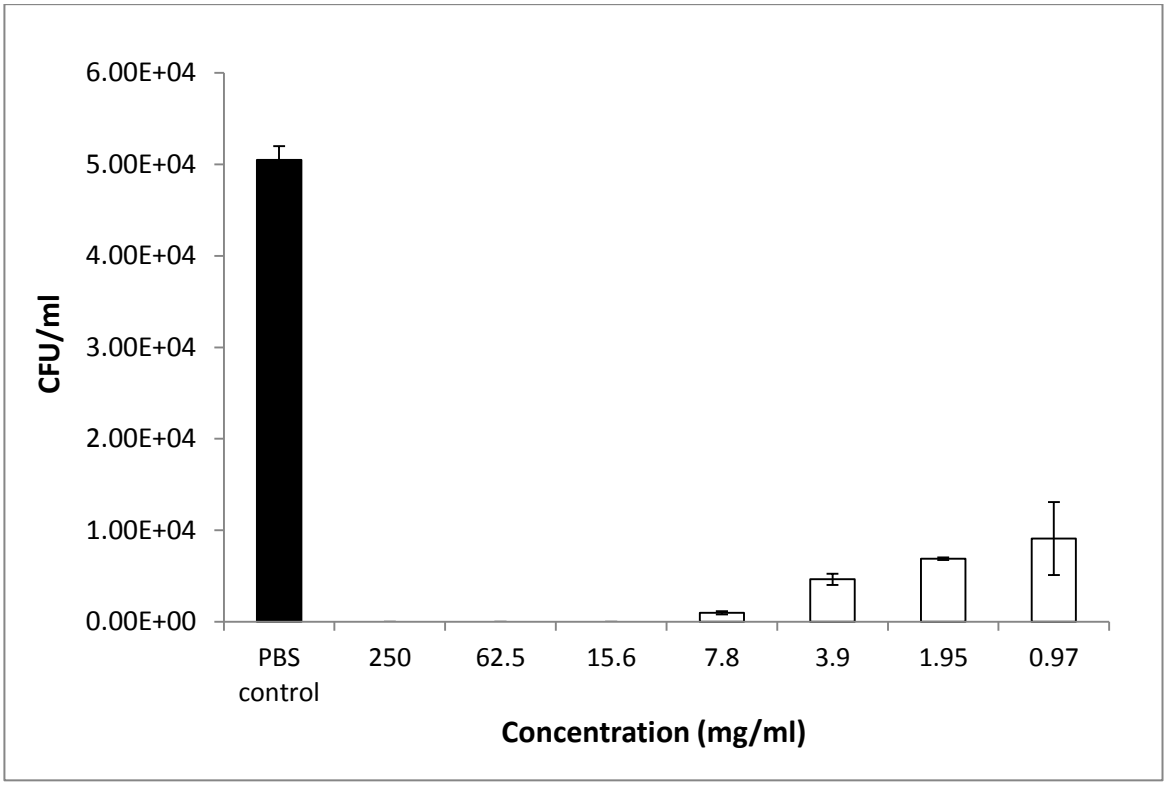


Figure 3.2 Antibacterial activity of potato extract against *H. pylori* at different concentrations

Viable cell count of *H. pylori* after incubation with known concentrations of potato extract for 90 min was performed as described in the Materials and Methods section. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained. The mean difference is considered significant at $P \leq 0.05$.

The effectiveness of potato extract at killing *H pylori* was expressed as percentage of bacteria killed, in comparison to the PBS control (Table 3.1). 100 % killing of *H. pylori* was achieved at 15.6 mg/ml to 250 mg/ml; and the percentage-kill is seen to decrease with decreasing concentration, that is, from 7.8 mg/ml to 0.97 mg/ml (Table 3.1). A significant 82 % kill was observed at the lowest concentration of potato extract tested. This observation indicates that potato extract shows significant anti-*H. pylori* activity at a concentration of 0.97 mg/ml within a short time frame.

Table 3.1 Percentage-kill of potato extract at decreasing concentration

Concentration of potato extract (mg/ml)	Percentage-kill (%)
250	100
62.5	100
15.6	100
7.8	98
3.9	91
1.95	86
0.97	82

Percentage-kill evaluated by calculating the percentage of viable cell colonies in relation to the PBS control.

3.4 Bactericidal activity and kill-kinetics of potato extract

With regards to mode of action, antimicrobial agents are classified based on whether they are bacteriostatic or bactericidal towards susceptible micro-organisms. As a bacteriostatic agent, bacterial growth is inhibited when the antimicrobial is in contact with the bacteria while as a bactericidal agent the antimicrobial kills the bacteria. In Figure 3.2 above, potato extract was shown to be bactericidal against *H. pylori*. Therefore, the rate at which potato extract kills *H. pylori* was evaluated. This was determined by incubating *H. pylori* cells with potato extract, washing the cells free of the extract at specific time points and then culturing the *H. pylori* cells back on antimicrobial free media. *H. pylori* cells were treated with potato extract concentrations (250, 125, 62.5, 31.2 and 15.6 mg/ml) that completely killed *H. pylori* as seen in Figure 3.2, and washed free of the extract at time points (T in min): T_5, T_{15}, T_{30}, T_{45}, T_{60} and T_{90}. The kill-kinetics was quantitatively assessed by viable count and the results are shown in Figure 3.3.

The results showed that at T_5, *H. pylori* cells treated with 250 and 125 mg/ml potato extract were completely killed (100 % killing). Other concentrations tested gradually killed the cells. At T_{30}, *H. pylori* cells treated with 62.5 mg/ml potato extract were completely killed, while 31.2 and 15.6 mg/ml potato extract achieved 100 % killing of *H. pylori* at T_{90} (Table 3.2). The PBS control showed gradual cell death which is attributed to lack of nutrients as the treated and un-treated cells were initially incubated in the absence of nutrient media before being transferred to agar plates.

It can be observed from the result presented, that potato extract exhibit a definite bactericidal action against *H. pylori* within 5 – 90 min as no viable colony was observed after incubating cells previously treated with potato extract on antibiotic free media.

Table 3.2 Percentage-kill of potato extract at different time points (in min)

Time point (min) / Concentration (mg/ml)	5	15	30	45	60	90
250	100	100	100	100	100	100
125	100	100	100	100	100	100
62.5	64	89	99	100	100	100
31.25	62	76	84	91	94	100
15.6	53	63	73	83	93	100
PBS control	0.3	8	12	21	27	34

Percentage-kill evaluated by calculating the percentage of viable cell colonies in relation to number of colonies at starting time point, T_0.

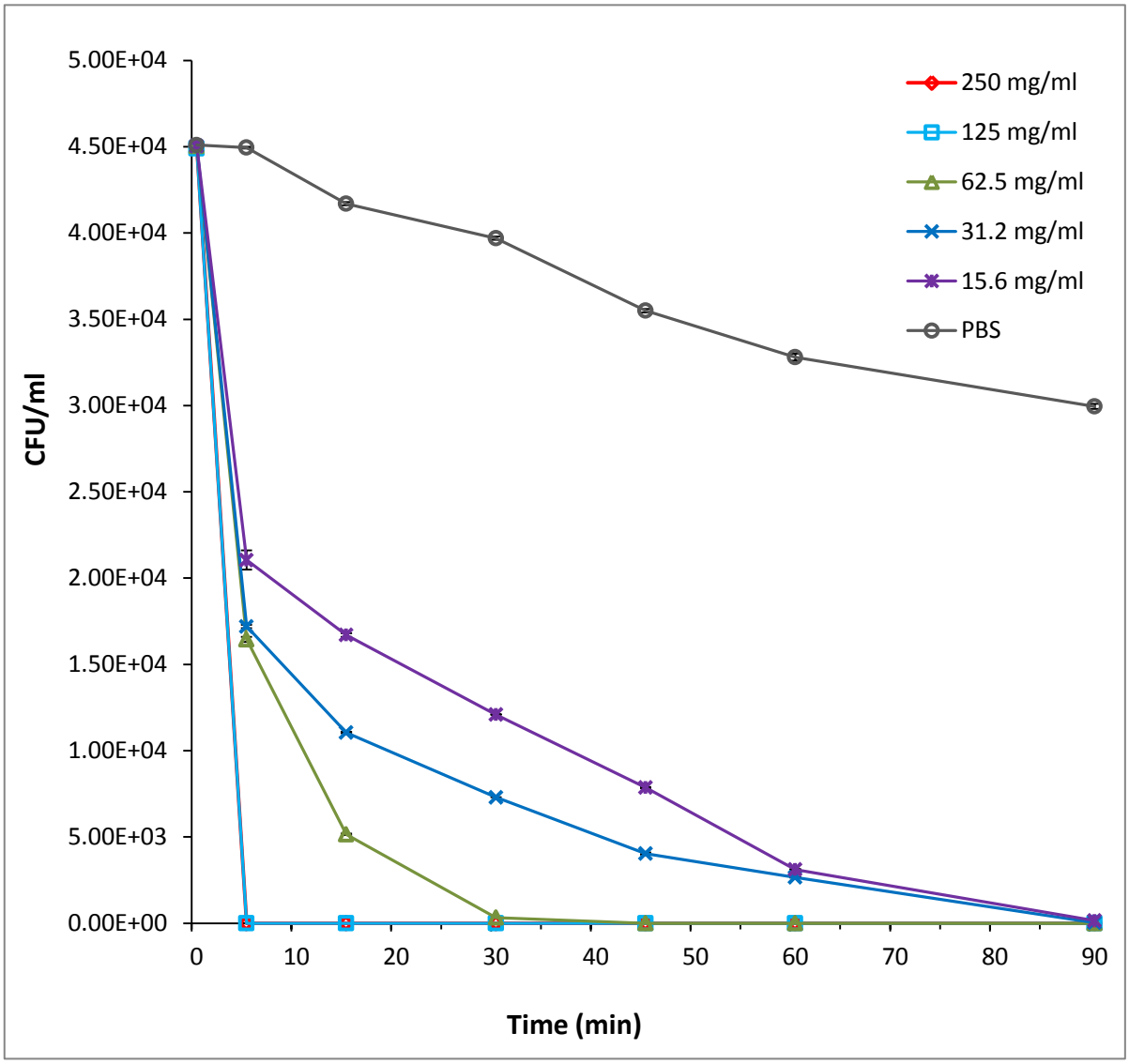


Figure 3.3 Time-kill studies of potato extract against *H. pylori*

Viable cell count of *H. pylori* 26695 after incubation with known concentrations of potato extract for 5 – 90 min. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained.

3.5 Potato extract retains anti-*H. pylori* activity after long-term storage

As a potential anti-*H. pylori* agent, it is important to determine whether potato extract loses activity after a period of time. In order to do this, freshly prepared lyophilised potato extract was stored at -80°C, 2 – 8°C and room temperature (RT) for 3 months; and then evaluated for anti-*H. pylori* activity by viable count.

The result obtained was compared to that of freshly prepared extract (Figure 3.4). The freshly prepared extract, extract stored at 2-8°C and at room temperature achieved 100 % killing at 15.6 mg/ml. Although extract stored at -80°C completely killed *H. pylori* at 31.2 mg/ml, it achieved 98.5 % killing at 15.6 mg/ml. This result indicates that potato extract stored at -80°C slightly loses activity in comparison to the freshly prepared extract and extract stored at 2-8°C and room temperature. However, in general, potato extract retained its activity when stored in a lyophilised state for 3 months at the above conditions, with the best storage condition in the fridge at 2 – 8°C.

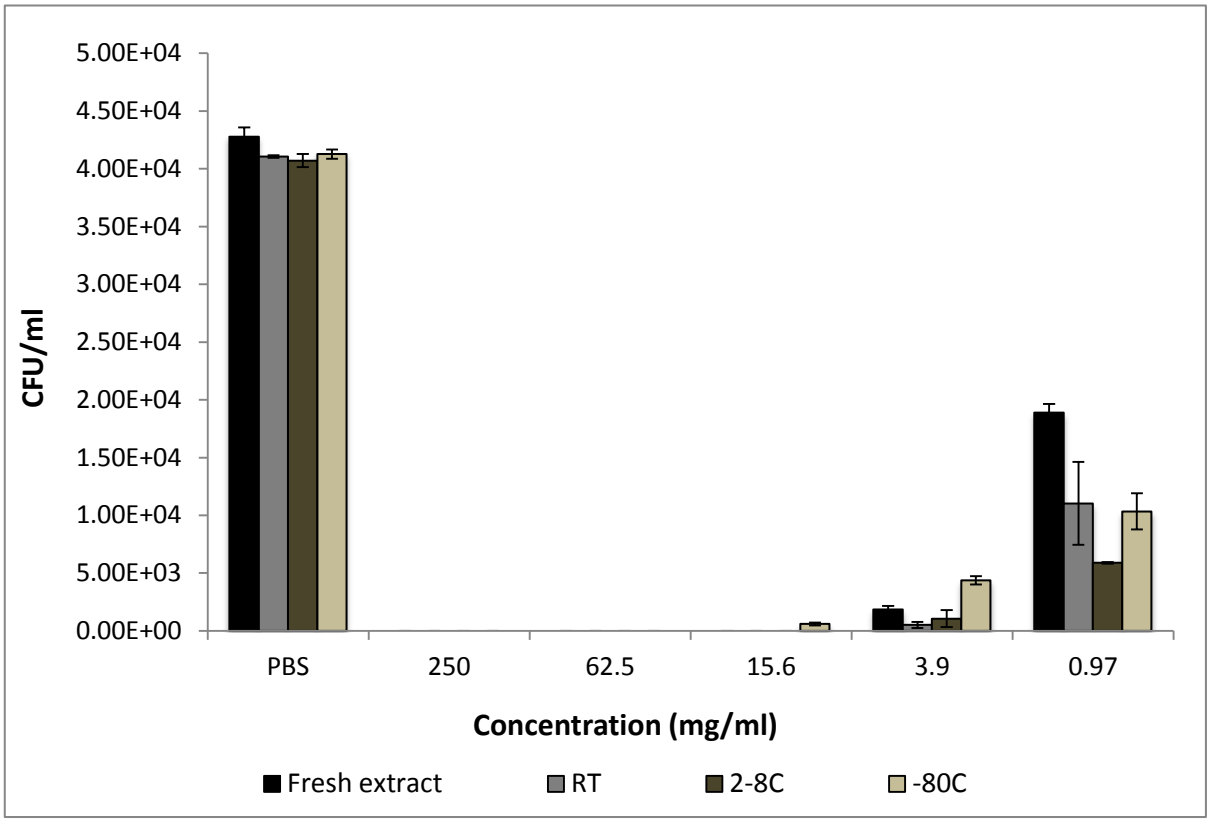


Figure 3.4 Stability of potato extract after storage for 3 months under different conditions

Viable cell count of *H. pylori* after incubation with known concentrations of potato extract for 90 minutes. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained. The mean difference is considered significant at $P \leq 0.05$.

3.6 *H. pylori* 26695 *galE* mutant is more susceptible to potato extract than the wild-type strain

In an attempt to determine the mode of action of potato extract, its effect was evaluated against a *H. pylori* 26695 *galE* mutant strain available in the laboratory. The *galE* mutant produces a truncated lipopolysaccharide structure that lacks the O-antigen side chain (Edwards et al., 2000). It has been shown that the presence or absence of the O-antigen side chain determines the degree of susceptibility of bacteria to antimicrobial agents (Tsujimoto et al., 1999). This experiment was, thus, carried out to

determine whether differences in the LPS structure, i.e. O-antigen side chain, between the *H. pylori* 26695 wild-type strain and the *galE* mutant affect the antibacterial activity of potato extract.

Results show that the lowest concentration at which potato extract killed approx. 100 % of *galE* mutant cells is 3.9 mg/ml (P= 0.0006) (Figure 3.5). This is a lower concentration as compared to that of the wild-type at 15.6 mg/ml (Figure 3.5). This result demonstrates that *H. pylori galE* mutant is more susceptible to potato extract than the wild-type strain; and suggests a possible role of the lipopolysaccharide or cell wall/cell membrane in the susceptibility of *H. pylori*.

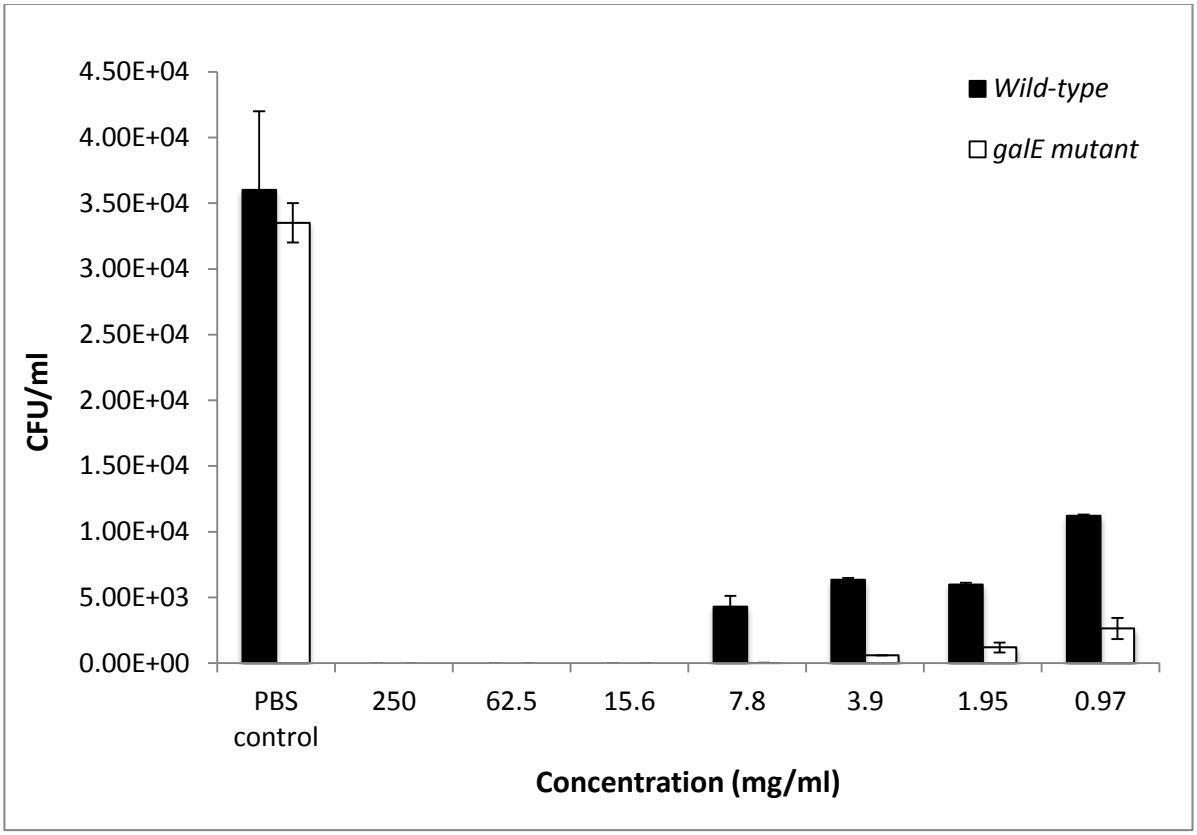


Figure 3.5 Comparison of anti-*H. pylori* activity of potato extract between *H. pylori* 26695 wild-type strain and *galE* mutant

Viable cell count of *H. pylori* 26695 and *galE* mutant after incubation with known concentrations of potato extract for 90 min. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained. The mean difference is considered significant at P ≤ 0.05.

3.7 *H. pylori* is more susceptible to potato extract than other bacteria of *Helicobacter* and *Campylobacter* genus

Following verification of the antibacterial activity of potato extract against *H. pylori*, potato extract was tested against some bacteria strains of the *Helicobacter* and *Campylobacter* species, which has close phylogeny to *H. pylori*. This is to determine the specificity of the antibacterial activity exhibited by potato extract; whether this activity is conserved to *H. pylori* or *Helicobacter* type of species. Using the viable count assay, potato extract at concentrations ranging from 0.97 mg/ml to 250 mg/ml, was tested against *H. pylori* 11637, *H. pylori* J99, *H. acinonychis*, *H. felis*, *H. winghamensis*, *H. canadensis*, *H. pullorum*, *C. jejuni*, *C. coli* and *W. succinogenes* (all strains kindly provided by Dr Dennis Linton). Cells treated with PBS were used as a negative control while *H. pylori*26695 cells treated with potato extract was used as a positive control.

Results showed that *H. pylori* strains 11637 and J99 are as susceptible to potato extract as the strain 26695. Among other bacteria strains tested, *H. acinonychis* is the most susceptible to potato extract with the concentration at which 100 % killing was achieved at <15.6 mg/ml (Figure 3.6). This is the same for *H. pylori*26695 except that at 0.97 mg/ml, potato extract kills 82 % of *H. pylori* cells while killing only 27 % of *H. acinonychis* cells ($P < 0.05$). *H. felis* and *H. winghamensis* were also susceptible to potato extract, with 100 % killing achieved at 62.5 mg/ml (Figure 3.6). The other bacteria tested, with the exception of *W. succinogenes*, were killed at 250 mg/ml while all other concentrations of potato extract showed no significant antibacterial activity ($P > 0.05$) (Figure 3.6). *W. succinogenes* was the least susceptible to potato extract, with potato extract killing 90 % of the cells at 250 mg/ml ($P= 0.0001$) (Figure 3.6). From the result obtained, 250 mg/ml of potato extract had significant antibacterial effect on all the

bacteria tested (P < 0.05). However, *H. pylori* is more susceptible to potato extract than the other bacteria tested.

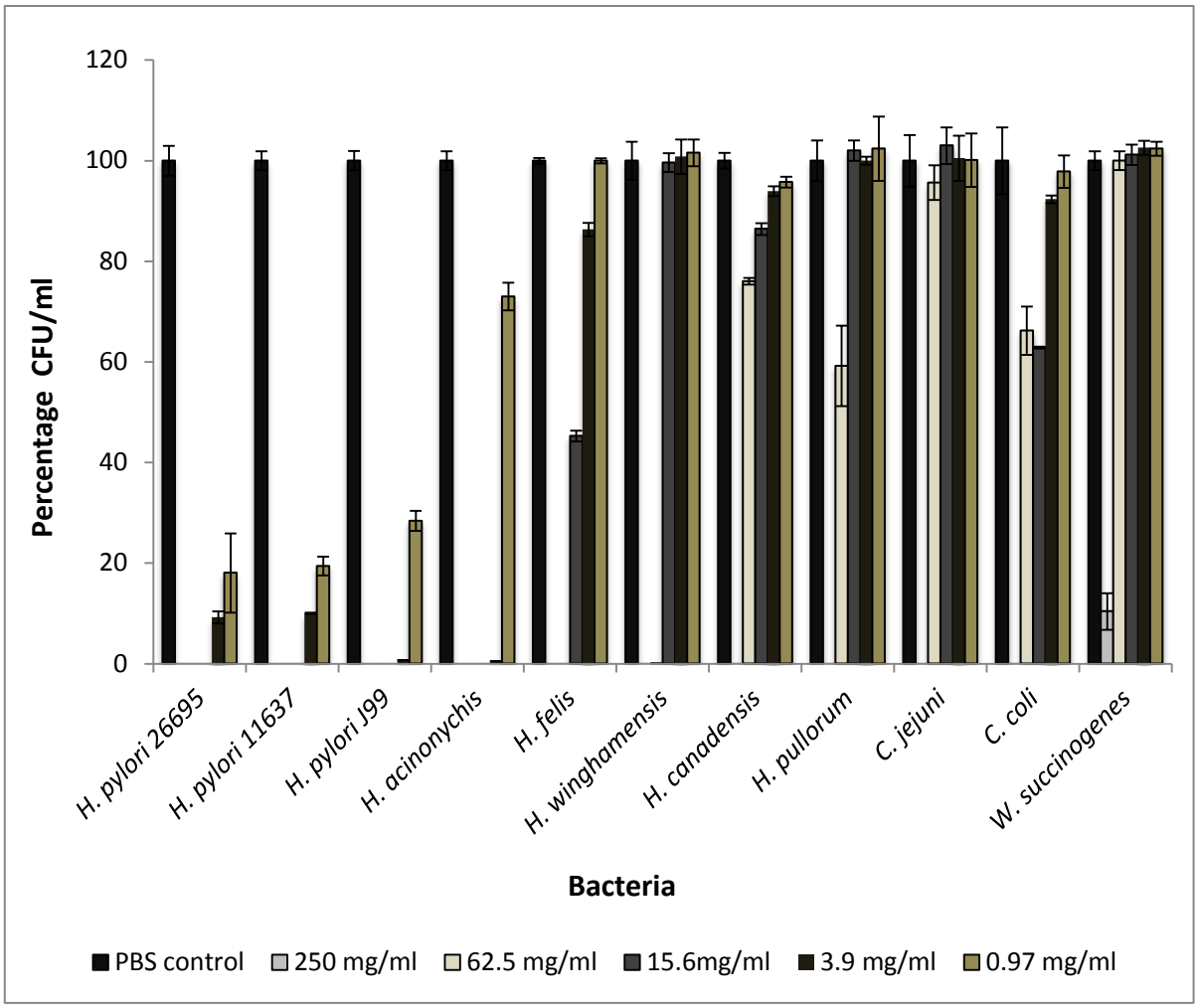


Figure 3.6 Toxicity of potato extract to other bacteria in comparison to that of *H. pylori*

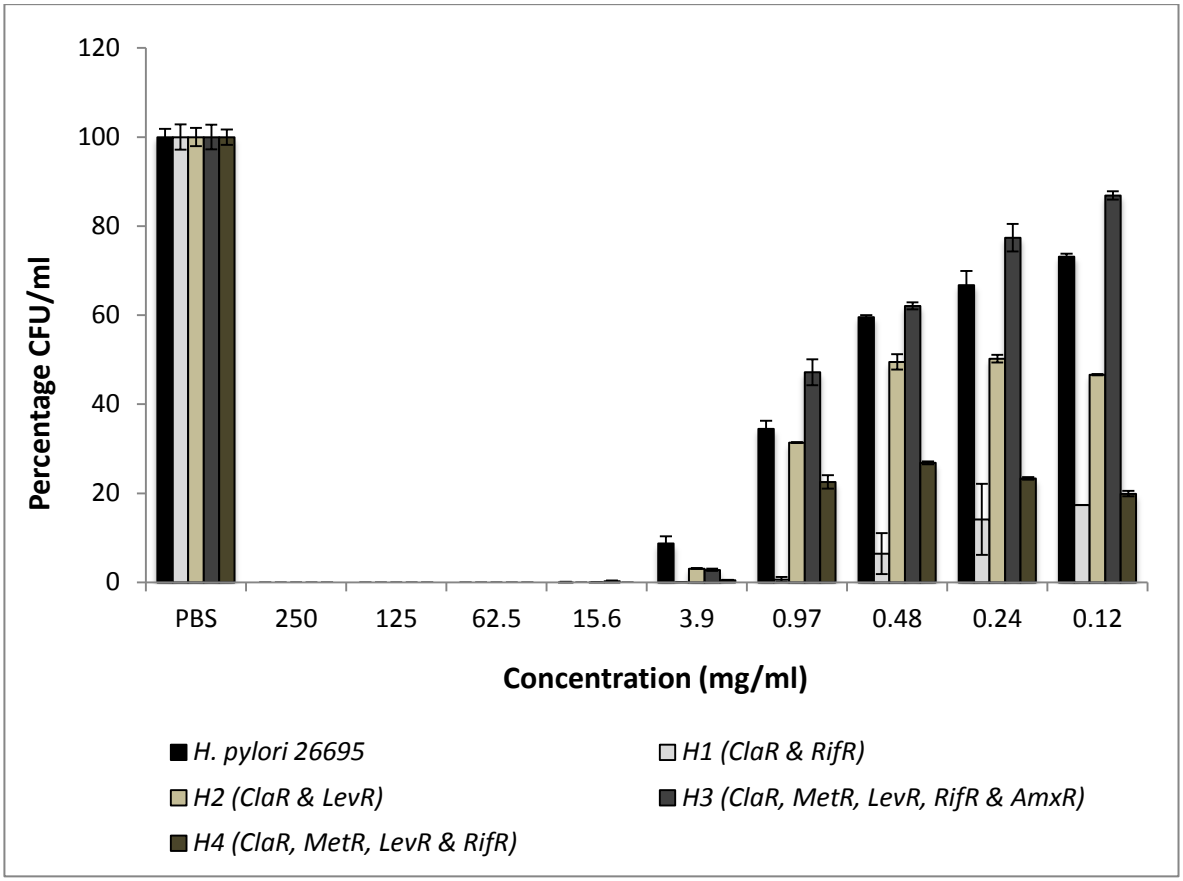
Viable cell count of *H. pylori* 26695, *H. pylori* 11637, *H. pylori* J99, *H. acinonychis*, *H. felis*, *H. winghamensis*, *H. canadensis*, *H. pullorum*, *C. jejuni*, *C. coli* and *W. succinogenes*, after incubation with known concentrations of potato extract for 90 min. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained in percentage. The mean difference is considered significant at P ≤ 0.05.

3.8 Clinical antibiotic resistant strains of *H. pylori* are susceptible to potato extract

In order to further validate the potential use of potato extract as a source of anti-*H. pylori* agent, clinical *H. pylori* strains were sourced and assayed against known concentrations of potato extract using the viable count method. Four clinical antibiotic-resistant strains, designated H1, H2, H3 and H4, with multiple resistance to antibiotics including amoxicillin, clarithromycin, rifampin, levofloxacin, and metronidazole were treated with known concentrations of potato extract for 90 min. Lab strain, *H. pylori* 26695 was used as a positive control.

Results obtained showed that all the resistant strains are sensitive to potato extract; with the lowest concentration at which 50 % of the cells are killed (MIC_{50}) ranging from 0.24 to 0.97 mg/ml as compared to that of lab strain with MIC_{50} between 0.48 and 0.97 mg/ml (Figure 3.7). H1 (resistant to clarithromycin and rifampin) and H4 (resistant to clarithromycin, metronidazole, levofloxacin and rifampin) were the most susceptible to potato extract. Both strains are more susceptible than the lab strain, with MIC_{100} at approx. 3.9 mg/ml as compared to 15.6 mg/ml for the lab strain and the other clinical antibiotic-resistant strains tested (Figure 3.7). H3 (resistant to clarithromycin, metronidazole, levofloxacin, rifampin, and amoxicillin) was the least susceptible to potato extract and slightly less susceptible than the lab strain, with potato extract killing 13 % of the cells at 0.12 mg/ml as compared to 27 % of the lab strain (Figure 3.7).

This result indicates that clinical antibiotic resistant strains are as susceptible to potato extract as the lab strain, *H. pylori* 26695.



ClaR- clarithromycin-resistant, RifR- rifampin-resistant, MetR- metronidazole-resistant, TetR- tetracycline-resistant, AmxR- amoxicillin-resistant, LevR- levofloxacin-resistant

Figure 3.7 Toxicity of potato extract to clinical antibiotic resistant *H. pylori* strain

Viable cell count of *H. pylori* 26695 and clinical antibiotic resistant strains after incubation with known concentrations of potato extract for 90 min. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained in percentage. The mean difference is considered significant at $P \leq 0.05$.

3.9 Synergistic interactions between potato extract and conventional antibiotics

With the problem of emerging antibiotic-resistant microorganisms, it may become necessary to combine conventional antibiotics with other antimicrobial therapies so as to improve the efficacy of the antibiotics. This approach could also be a potential effective tool for antimicrobial therapy. As such, it is important to evaluate the interactions between medicinal

plant extracts and antibiotics. Thus, the ability of potato extract to act synergistically with antibiotics currently used in treating *H. pylori* infections was investigated using the agar dilution method in combination with Oxoid MIC Evaluator method.

Firstly, the MIC of potato extract was determined using the agar dilution method as described in the Materials and methods section. A "sterile H_2O" control, in which water was added to the agar in a 1:10 ratio, was included in the assay so as to ascertain that the addition of potato extract (dissolved in H_2O) to the agar did not interfere with the growth of *H. pylori*. The result shows that the lowest concentration of potato extract that prevented the growth of *H. pylori* on Columbia blood agar plates supplemented with potato extract is 6.25 mg/ml (Table 3.3). This was determined by the lack of appearance of viable colonies on the agar plates. At lower concentrations (3.12, 1.56 and 0.78 mg/ml), a lawn of bacteria growth was observed. These concentrations were then used in combination with antibiotics for the synergy test.

Table 3.3 MIC of potato extract against *H. pylori* by agar dilution method

Concentration of potato extract (mg/ml)	Presence or absence of bacterial lawn
Control	+
Sterile H_20 control	+
25	-
12.5	-
6.25	-
3.12	+
1.56	+
0.78	+

(+): Presence of bacterial lawn; (-): Absence of bacterial colony or lawn

3.9.1 Synergy between potato extract and antibiotics against *H. pylori* 26695

Synergy between potato extract and antibiotics, ampicillin, tetracycline and metronidazole, was evaluated against *H. pylori* 26695 strain.

Result shows the presence of synergistic activities between 3.12 mg/ml of potato extract and the antibiotics tested (Table 3.4). This is indicated by the decrease of the MIC of the antibiotics in combination with potato extract when compared to the control sample without potato extract. The MIC of ampicillin decreased from 0.015 to < 0.015 µg; tetracycline from 0.12 to 0.03 µg; and metronidazole from 8 to 4 µg. The exact MIC of ampicillin in combination with 3.12 mg/ml potato extract could not be determined because the lowest concentration on the Oxoid MIC evaluator strip is 0.015 µg. Potato extract at 1.56 and 0.78 mg/ml showed no synergy in combination with the antibiotics, with the exception of tetracycline where MIC reduced from 0.12 to 0.06 µg. A “no antibiotic” control was included in the experiment to confirm a confluent growth of bacteria on agar plates supplemented with potato extract. The result obtained indicates the presence of synergistic activity between potato extract and antibiotics used in the treatment of *H. pylori* infection.

Table 3.4 Synergy between potato extract and antibiotics against *H. pylori* 26695

Antibiotic (µg)	Control	3.12 mg/ml P.E	1.56 mg/ml P.E	0.78 mg/ml P.E
AMP	0.015	< 0.015	0.015	0.015
TET	0.12	0.03	0.06	0.06
MET	8	4	8	8
"No Antibiotic" Control	Presence of a confluent lawn of bacteria			

AMP- Ampicillin, TET- Tetracycline, MET- Metronidazole, P.E- Potato extract.

MIC of antibiotics was evaluated in combination with potato extract at the concentrations indicated. The values shown are the MIC of each antibiotic (in µg)in combination with potato extractor sterile H_2O in the control sample. Experiment was carried out in duplicates and repeated three times.

3.9.2 Synergy between potato extract and antibiotics against clinical antibiotic-resistant *H. pylori* strain

The presence of synergistic activities between potato extract and antibiotics, amoxicillin and metronidazole, was evaluated against clinical antibiotic resistant *H. pylori* strains.

A decrease in MIC of amoxycillin, from 0.015 to < 0.015 µg, was observed against *H. pylori* 26695 (Table 3.5). However, no synergistic activity was observed in the antibiotic-resistant strains as the MIC of the antibiotics in combination with potato extract remained the same in comparison to the control sample without potato extract (Table 3.5).

Table 3.5 Synergy between potato extract and antibiotics against clinical antibiotic-resistant *H. pylori* strain

Combination / Strain	Amoxycillin (µg)				Metronidazole (µg)			
	Control	**3.12 mg/ml P.E**	**1.56 mg/ml P.E**	**0.78 mg/ml P.E**	**Control**	**3.12 mg/ml P.E**	**1.56 mg/ml P.E**	**0.78 mg/ml P.E**
***H. pylori* 26695**	0.015	< 0.015	0.015	0.015	8	4	8	8
H1 2206 0181	0.25	0.25	0.25	0.25	> 256	> 256	> 256	> 256
H1 2318 0581	0.015	0.015	0.015	0.015	> 256	> 256	> 256	> 256
"No Antibiotic" Control	Presence of confluent lawn of bacteria				Presence of confluent lawn of bacteria			

H1 2206 0181 (R to Met, Cla, Lev, Rif, Amx), H1 2318 0581 (R to Met, Cla, Lev, Rif),
R- resistant, Amx- amoxycillin, Met- metronidazole, Cla- clarithromycin, Rif- rifampin, Lev- levofloxacin, P.E- potato extract.

MIC of amoxicillin and metronidazole was evaluated in combination with potato extract at the concentrations indicated. The values shown are the MIC of each antibiotic (in µg) in combination with potato extract or sterile H_2O in the control sample, against each *H. pylori* strain indicated. The "no antibiotic control" for each strain showed presence of confluent lawn of bacteria. Experiment was carried out in duplicates and repeated three times.

3.10 Discussion

Previous research have reported that potato extract exhibit antibacterial activities against *H. pylori* (Bennett and Roberts, unpublished data). In this chapter, a newly modified extraction method for potato extract was developed and the antibacterial activity of the extract was further elucidated.

3.10.1 Antibacterial activity of potato extract against *H. pylori*

Potato extract showed antibacterial activity against *H. pylori* while exhibiting no significant activity against *E. coli*, a Gram-negative bacterium (Figure 3.1). It was, therefore, deduced that potato extract actively inhibits and kills *H. pylori*. Minimum inhibitory concentration (MIC) studies indicate that potato extract exhibit 100 % killing of *H. pylori* at 15.6 mg/ml (Figure 3.2); and showed antibacterial activity even at 0.97 mg/ml, at which it kills 82 % of the bacteria ($P < 0.05$) (Table 3.1). Using the agar dilution method, the lowest concentration at which potato extract completely kills *H. pylori* was 6.25 mg/ml (Table 3.3). This is 2.5 times lower than the MIC obtained using the viable colony count method. This difference in MIC values is attributed to the difference in susceptibility testing methods used. However, MIC values of potato extract (using the same susceptibility testing method) has been reported to differ with different varieties of potatoes (O'Brien et al., 2011); and this may be as a result of variations in concentration/levels of biological compounds, mainly starch, phenolic compounds and glycoalkaloids, present in different varieties of potatoes (Friedman et al., 2003).

Incidentally, when the disc diffusion method was used to evaluate the anti-*H. pylori* activity of potato extracts in this study, no significant antibacterial

activity was observed. This observation supports previous studies, which states that the slow growth nature of *H. pylori* contributes to the inefficacy of the disc diffusion assay in anti-*H. pylori* studies (Megraud and Lehours, 2007). Another study has also suggested that it may due to the unstable release and diffusion of antimicrobial agents from the disc into the agar media (Njume et al., 2011a). It is also possible that the active components in the potato extract bind to the paper disc and hinder diffusion into the agar media; however, it is difficult to ascertain this theory since the active components are unknown.

The time-kill assay was performed to investigate the bactericidal activity of potato extract on *H. pylori*. Kill kinetics of potato extract against *H. pylori* showed that *H. pylori* cells (10^4 CFU/ml) were killed within 90 min of treatment with potato extract (Figure 3.3). At higher concentrations (62.5 – 250 mg/ml), *H. pylori* cells were killed within 5 – 30 min. The antibacterial activity observed is irreversible as the cells did not recover after washes to remove the potato extract; thus, showing that potato extract is bactericidal towards *H. pylori*. Various studies have shown kill-kinetics of plant extracts against *H. pylori* ranging from 1 – 24 h (Kawakami et al., 2006; Njume et al., 2011b). Time-kill studies of antibiotics, amoxicillin, metronidazole, clarithromycin and kanamycin, have also been demonstrated against *H. pylori* from 3 to over 20 h (Irie et al., 1997; Hassan et al., 1999). In comparison, potato extract rapidly exhibit bactericidal activities against *H. pylori* in less than 1 h.

Evaluation of the antibacterial activity of potato extract against clinical antibiotic resistant *H. pylori* strains showed that the strains tested were susceptible to potato extract (Figure 3.7). The antibiotic resistant strains are resistant to multiple antibiotics including clarithromycin,

metronidazole, rifampin, levofloxacin and amoxycillin; and the strains were as susceptible to the extract as the lab strain, with 2 of the strains been even more susceptible than the lab strain. This shows that potato extract is antibacterial against both antibiotic-susceptible and antibiotic-resistant *H. pylori* strains.

3.10.2 Increased susceptibility of *H. pylori* 26695 *galE* mutant to potato extract

The evaluation of the anti-*H. pylori* activity of potato extract against *H. pylori* 26695 *galE* mutant showed that the *galE* mutant is more susceptible to potato extract than the wild-type strain; with its MIC at 3.9 mg/ml as compared to 15.6 mg/ml of the wild-type (Figure 3.5). The *galE* gene encodes UDP-galactose-4-epimerase which is a prerequisite for the synthesis of UDP-galactose (Adhya, 1987), as *H. pylori* lacks galactokinase and is unable to utilize exogenous galactose (as reviewed in: (Edwards et al., 2000). A *galE* mutant produces a truncated lipopolysaccharide (LPS) structure lacking the O-antigen side chain (Edwards et al., 2000). Therefore, it is suggested that the increased susceptibility of the *galE* mutant to potato extract may be as a result of its truncated (rough) LPS, which may have further facilitated entry of active components of the extract into the cell cytoplasm. This has been demonstrated previously, where it was shown that bacteria lacking the O-antigen are more susceptible to antibiotics, particularly hydrophobic antibiotics (Tsujimoto et al., 1999). This is due to the fact that rough-type LPS, either lacking the O-antigen side chain or with reduced O-side chains, renders the cell surface hydrophobic (Tsujimoto et al., 1999). This may be because the LPS is made up of the hydrophobic lipid A and the hydrophilic polysaccharide chain, which includes the O-antigen side chain (Rittig et al., 2003); and with the absence of the O-antigen, the

LPS will then be made up of mainly the hydrophobic lipid A component, thereby rendering the cell surface hydrophobic and allowing hydrophobic molecules to penetrate the cell. Although, potato extract is soluble in water, the dissolved solution contains some insoluble particles that have been shown to be more potent against *H. pylori* than the soluble portion of the extract (as shown in Figure 4.2 in Chapter 4). These insoluble particles may be able to diffuse through the hydrophobic cell surface of the *galE* mutant strain since it lacks the O-antigen side chain, resulting in the higher antibacterial activity observed. It has also been suggested that hydrophobic molecules are able to penetrate the LPS especially when it is more permeable due to alterations in its structure (Nikaido and Vaara, 1985). The result, thus, suggests that the mode of action of potato extract against *H. pylori* may be cell membrane/cell wall mediated.

3.10.3 Specificity of potato extract to *H. pylori*

Various studies have shown the efficacy of certain plant extracts against *H. pylori* (O'Mahony et al., 2005; Galan et al., 2004). The activity of isothiocyanate sulforaphane, present in broccoli sprouts, have been demonstrated against *H. pylori*(Fahey et al., 2002; Yanaka et al., 2009) Other plants that have been shown to exhibit antibacterial activity against *H. pylori* include ginger, cumin, turmeric, chilli, green tea, cinnamon, parsley and nutmeg (O'Mahony et al., 2005). However, these plants have a broad spectrum of activity against several range of bacteria (O'Mahony et al., 2005); and are, therefore, not specific for eradication of *H. pylori* infection.

Due to the promising anti-*H. pylori* activity of potato extract observed, the specificity of potato extract to *H. pylori* was evaluated by testing the extract against a range of bacteria with close phylogeny to *H. pylori*. This

experiment was also carried out so as to determine whether the activity of potato extract is conserved to *Helicobacter* species. Bacteria tested include *H. acinonychis*, *H. felis*, *H. winghamensis*, *H. canadensis*, *H. pullorum*, *C. jejuni*, *C. coli* and *W. succinogenes*. Results demonstrate, in comparison to activity of potato extract against *H. pylori*, that potato extract had no significant effect on the bacteria tested; with the exception of *H. acinonychis* and *H. felis*, to which it exhibited significant toxicity (Figure 3.6). The antibacterial activity observed towards *H. acinonychis* and *H. felis* is attributed to the fact that both *Helicobacter* species are closely related to *H. pylori*. *H. acinonychis* was first isolated from cheetahs with gastritis (Eaton et al., 1993). It has been shown to be phenotypically similar to *H. pylori*(Eaton et al., 1993), being able to establish mixed infections and recombine with some *H. pylori* strains (Dailidiene et al., 2004). By phylogenetic analysis, *H. acinonychis* has a 97.4 % and 96.3 % similarity to *H. pylori* and *H. felis* respectively (Eaton et al., 1993). This similarity with *H. pylori*, thus, explains the comparable antibacterial activity of potato extract observed in *H. acinonychis* and *H. felis*.

Previous study in which the activity of potato extract was evaluated on a range of Gram-positive and Gram-negative bacteria, including probiotics, showed selective antibacterial activity of potato extract towards *H. pylori* (Bennett and Roberts, unpublished data). Therefore, since in this present study, potato extracts showed no significant antibacterial effect against *E. coli* and a range of closely related species of *H. pylori* tested, this confirms that potato extract has specificity for *H. pylori*. Being highly selective towards *H. pylori*, it is indicated that the active components in potato extract may offer some potential as anti-*H. pylori* agents and this further enhance its selection as a candidate for eradication of *H. pylori* infection.

3.10.4 Synergistic interactions between potato extract and antibiotics

Synergistic activities resulting from the combination of plant extracts that exhibit antimicrobial activity with antibiotics commonly used in the eradication of a range of bacteria have been reported in several studies (Adwan et al., 2010; Mahboubi and Bidgoli, 2010; Mandal et al., 2010; Rodrigues et al., 2009). Due to the antibacterial activity observed in potato extract, its combination with antibiotics was investigated for synergistic activities. Synergistic activity was observed when antibiotics, ampicillin, metronidazole or tetracycline, were combined with sub-lethal concentration (3.12 mg/ml) of potato extract against *H. pylori* 26695 (Table 3.4).However, when potato extract was combined with amoxicillin or metronidazole against clinical antibiotic-resistant *H. pylori* strains, no synergy was observed (Table 3.5). This absence of synergy may be due to the fact that the clinical strains have multiple resistances to a range of antibiotics or may be related to the antibiotic resistant mechanisms of the strains. However, additional testing will need to be carried out to ascertain the presence or absence of synergy between potato extract and antibiotics against antibiotic-resistant strains. This may include repeating the experiment with strains having single antibiotic resistance and/or testing with higher, lethal concentrations of potato extract.

It has been described that the combination of antimicrobial agents which contains bioactive compounds with different targets, results in the antimicrobial agents acting together to affect not only one target, but multiple targets, thereby presenting a synergistic or an agonistic response (Wagner and Ulrich-Merzenich, 2009). Furthermore, synergistic interactions may be as a result of one compound increasing the solubility

and absorption of the other bioactive compound, thus, producing an enhanced bioactivity (Wagner and Ulrich-Merzenich, 2009). The synergistic activity observed in this study, thus, suggests possible interactions between the respective antibiotics tested and the active components present in potato extract. Also, our hypothesis is that the mode of action of potato extract is cell wall/membrane related. Therefore, the synergy observed suggests the presence of agonistic compounds in potato extract that increases the diffusion of the antibiotics into the cells thereby enhancing the antibacterial activity of the antibiotics.

In summary, in this study, potato extract showed significant bactericidal activity against *H. pylori* 26695, as well as antibiotic-resistant *H. pylori* strains. Therefore, this further suggests potato extract as a promising source for the development of new anti-*H. pylori* agents.

Chapter 4

Evaluating the Mode of Action of Potato Extract against *H. pylori*

4.1 Introduction

Following the evaluation of the antibacterial activity of potato extract, it was important to investigate its mode of action against *H. pylori*. This was carried out by determining the effect of the extract on the morphology, membrane integrity, transmembrane potential and ATP concentration of *H. pylori*.

4.2 Potato extract disrupts the morphology of *H. pylori*

In order to determine the effect of potato extract on *H. pylori* cells, transmission electron microscopic (TEM) analysis was carried out on *H. pylori* cells treated with potato extract. 10^8 CFU/ml *H. pylori* treated with 250 mg/ml potato extract for 5 to 90 min was analysed. Two PBS controls were included: one that shows the morphology of *H. pylori* immediately after suspension in PBS; and another that shows the morphology of *H. pylori* after incubation with PBS for 90 min. Changes in the appearance of *H. pylori* cells was observed upon treatment with potato extract (Figure 4.1A-F).

After 5 – 30 min exposure, the cell cytoplasm begins to coagulate, and the cells begin to lose their cell wall and membrane (Figure 4.1B and 4.1C). After 45 min exposure, significant coagulation of cell cytoplasm and loss of cell wall and membrane was observed (Figure 4.1D). After 90 min of exposure to the extract, the precipitated cytoplasm began to disintegrate forming pieces of cellular material as seen in Figure 4.1E. In comparison to

the control groups, the morphology of *H. pylori* was not significantly changed after 90 min treatment in PBS (Figure 4.1A and 4.1F). However, a change in morphology of *H. pylori* cells, characterised by separation of the cell wall from the cell membrane, was observed in cells treated in PBS for 90 min in comparison to cell treated in PBS for 0 min (Figure 4.1A and 4.1F). This change is attributed to prolonged lack of nutrients. However, at this stage about 70 % of the cells remain viable and culturable as previously observed (Figure 3.3).

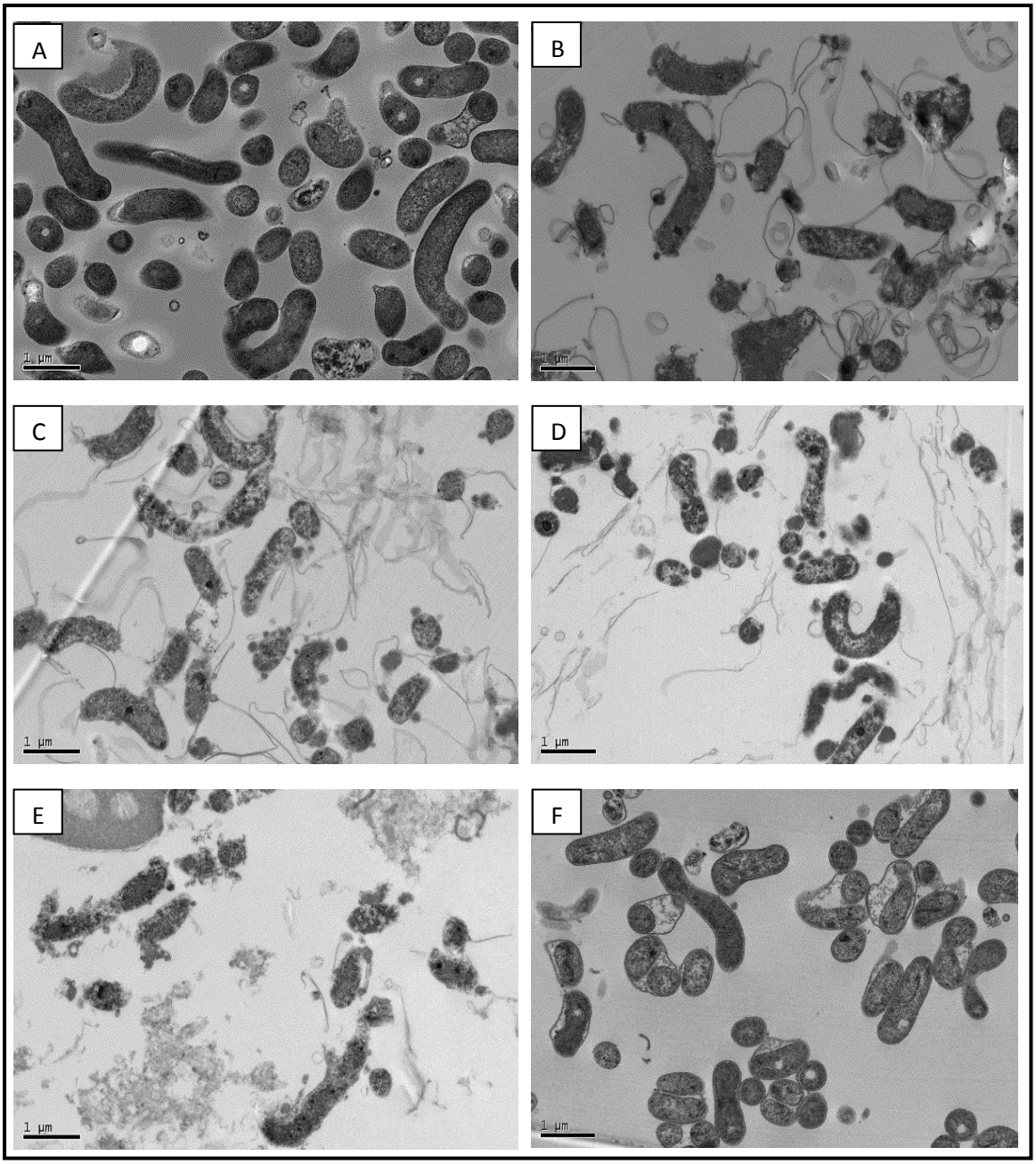


Figure 4.1 Transmission electron micrographs of *H. pylori* cells treated with potato extract for 5 - 90 min

(A), exposure to PBS at 0 minutes; **(B-E)**, exposure to 250 mg/ml of potato extract at 5, 15, 45 and 90 min respectively in alphabetical order; **(F)**, exposure to PBS for 90 minutes. In comparison to cells treated with potato extract, the morphology of *H. pylori* after 5 - 90 min in PBS remains in normal spiral/bacillary form.

H. pylori cells were treated with potato extract for 5 – 90 min and fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde for 1 h and later post-fixed in 1 % osmium tetraoxide and 1.5 % potassium ferrocyanide for 1 h. The fixed cells were then polymerised in epoxy resin for 3-4 h. The photos were taken at 2900x magnification. The scale bar is indicated in 1 µm.

In the TEM micrographs shown above, it was observed that debris was present in the samples treated with potato extract (Figure 4.1A-F). The debris was from undissolved particles in the potato extract, and this interfered with the proper observation as well as interpretation of the changes observed. In light of this, we decided to repeat the experiment using filtered potato extract so as to get rid of the undissolved particles and debris.

As such, it was important to evaluate the activity of the filtered extract in comparison to the unfiltered extract, as well as determine the time kill kinetics of the filtered extract. Potato extract was filtered through a 0.22 µm PES membrane filter and the supernatant was used in the antibacterial assay against *H. pylori*, using the viable count method. The result shows that the filtered extract is less active against *H. pylori* than the unfiltered extract (Figure 4.2). The lowest concentration at which the filtered extract completely killed *H. pylori* was 125 mg/ml compared to 15.6 mg/ml for the unfiltered extract. It was then deduced that the undissolved particles in the unfiltered extract contributed to its bioactivity. This hypothesis was confirmed by testing the antibacterial activity of the residue from the filtered potato extract against *H. pylori*. The result showed that the residue was more potent in killing *H. pylori* than the unfiltered and filtered extract, killing >99 % of *H. pylori* cells at 3.9 mg/ml (Figure 4.2).

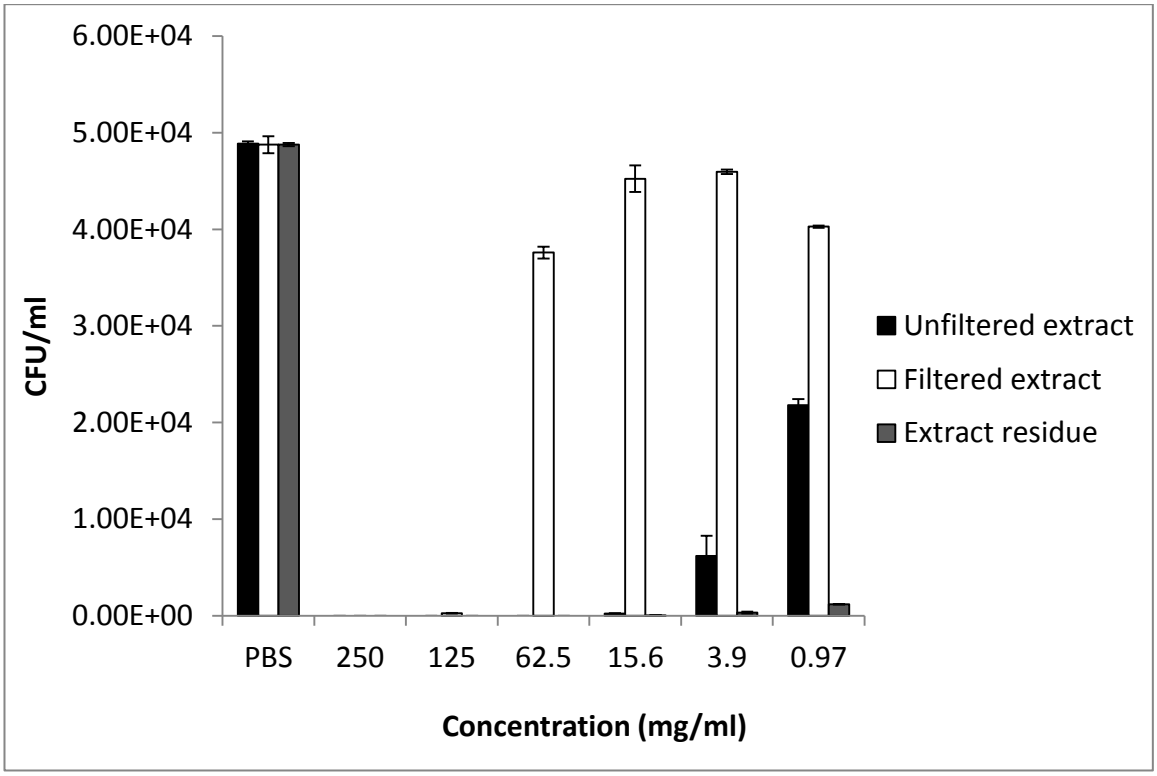


Figure 4.2 Differences in the anti-*H. pylori* activity of potato extract after filtration

Viable cell count of *H. pylori* cells after incubation with unfiltered potato extract, extract filtered through a 0.22 µm PES membrane filter, and extract residue for 90 minutes. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained.

The kill-kinetics of filtered potato extract (62.5 to 250 mg/ml) was evaluated so as to be able to correlate it to the observation of the TEM analysis to be carried out using filtered extract. This was quantitatively assessed using the viable count method as previously described. *H. pylori* cells were treated with filtered potato extract of concentrations 250, 125 and 62.5 mg/ml, and washed free of the extract at time points (T in min): T_5, T_{15}, T_{30}, T_{45}, T_{60} and T_{90}. The result showed that within 5 min (T_5) of treatment, filtered 250 mg/ml potato extract completely killed *H. pylori* cells (Figure 4.3). Filtered 125 mg/ml extract completely killed the cells at T_{90}, while 62.5 mg/ml extract only achieved 26 % killing within the 90 min assay (Figure 4.3).

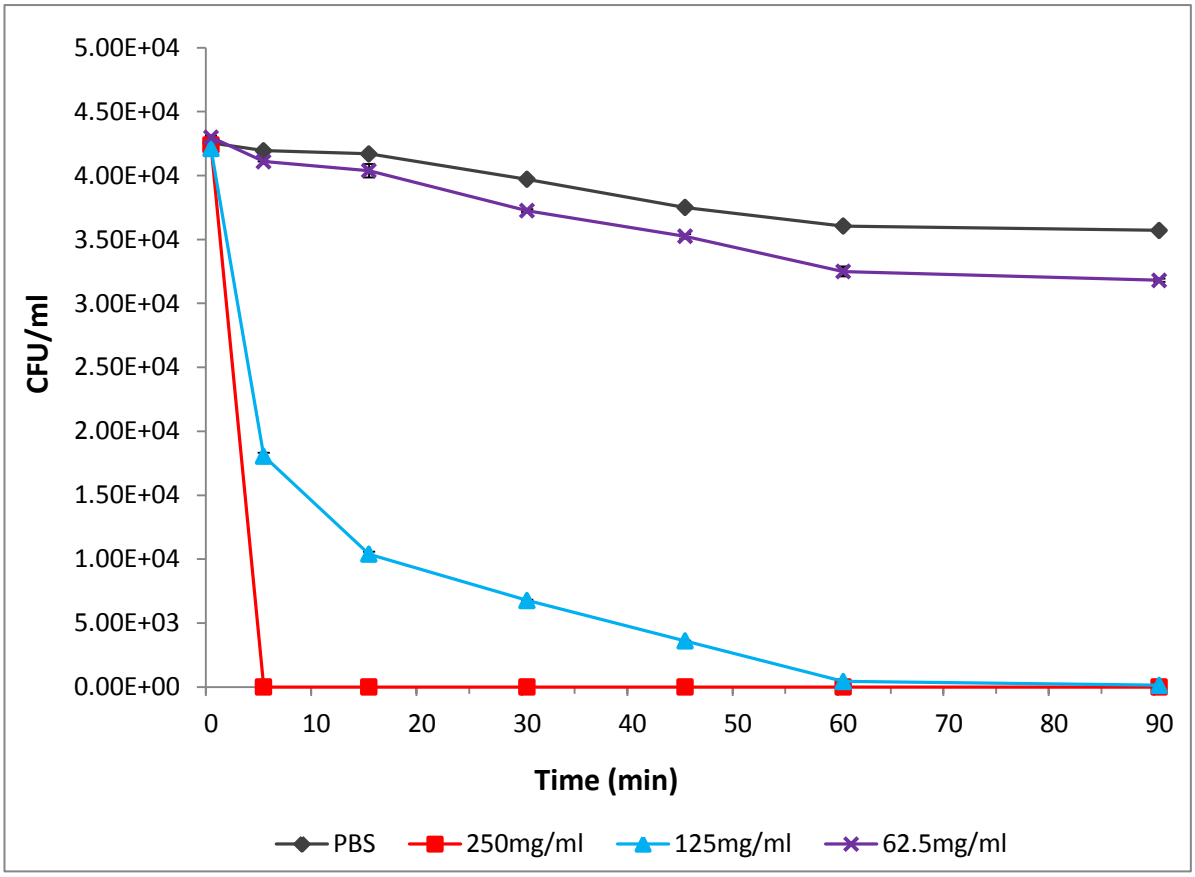


Figure 4.3 Kill-kinetics of filtered potato extract against *H. pylori* 26695

Viable cell count of *H. pylori* 26695 after incubation with known concentrations of filtered potato extract for 5 – 90 min. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained.

In light of the antibacterial assays carried out with filtered potato extract, the TEM analysis was repeated with filtered 250 mg/ml and 125 mg/ml extract.

4.2.1 TEM analysis using filtered 250 mg/ml potato extract

H. pylori cells (10^8 CFU/ml) were treated with filtered 250 mg/ml potato extract for 5 to 90 min, and prepared for TEM analysis. Two PBS controls were included in this experiment as described in the initial TEM analysis. Fixation of the cells with osmium tetraoxide preserved the cell membrane structures and enabled its clear identification in the TEM micrographs. Morphological change of *H. pylori* cells was observed upon treatment with potato extract (Figure 4.4A-F). After 5 – 15 min of exposure to potato extract, separation of the bacterial cell wall from the outer membrane was observed (Figure 4.4B and 4.4C). After 45 min of exposure, significant separation of cell wall from the outer membrane and separation of the cytoplasmic content of the cells was observed (Figure 4.4D). After 90 min of exposure, cytopathic activity was observed (Figure 4.4E). However, in comparison to the control groups, the morphology of *H. pylori* was not significantly changed after 90 min in PBS (Figure 4.4A and 4.4F).

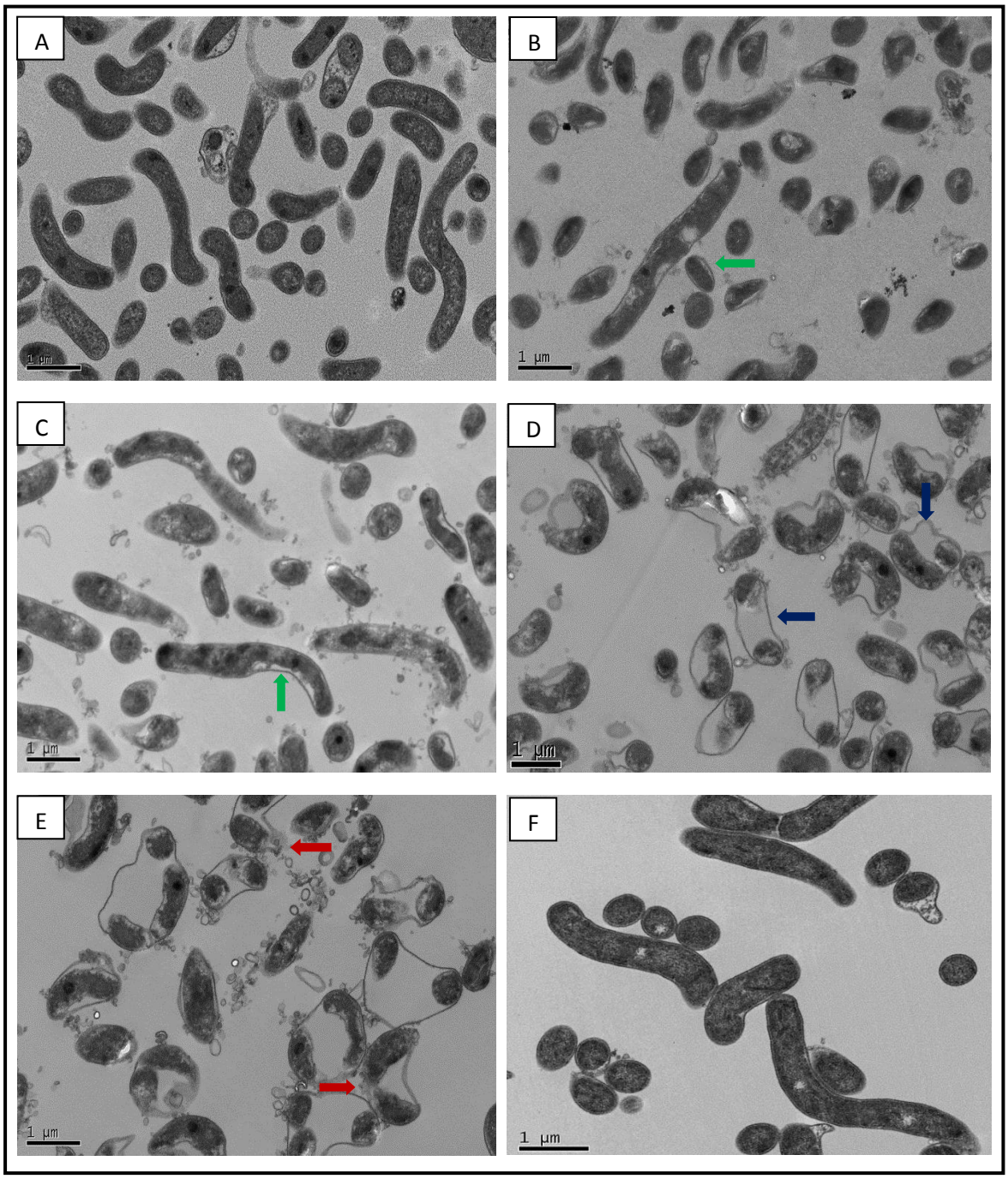


Figure 4.4 Transmission electron micrographs of *H. pylori* after exposure to potato extract

(A), exposure to PBS at 0 min; **(B-E)**, exposure to 250 mg/ml of potato extract at 5, 15, 45 and 90 min, respectively in alphabetical order. After 5 to 15 min of exposure, separation of cell cytoplasmic membrane from the outer membrane is observed (denoted by green arrow). After 45 min, cytopathic activity and separation of cell wall and cytoplasmic content is observed (denoted by blue arrow); and after 90 min of exposure, cytopathic activity is observed (denoted by red arrow). **(F)**, exposure to PBS for 90 min. In comparison, the morphology of *H. pylori* after 90 min in phosphate buffer saline (PBS) remains in normal spiral/bacillary form. The photos were taken at 2900x magnification. The scale bar is indicated in 1 µm.

4.2.2 TEM analysis using filtered 125 mg/ml potato extract

Considering the fact that high concentration of potato extract (250 mg/ml) used in the previous TEM analysis may have brought about plasmolysis of *H. pylori* cells as indicated by the observed changes in cell morphology characterised by separation of cell cytoplasmic content (Figure 4.4A-F); the TEM analysis was repeated using a lower concentration, 125 mg/ml potato extract.

In this experiment, *H. pylori* cells (10^8 CFU/ml) were treated with filtered 125 mg/ml potato extract for 15 to 90 min, and fixed for TEM analysis. A PBS control was included for each time point assessed, i.e. 15, 30, 45, 60 and 90 min. TEM micrographs of *H. pylori* cells treated with 125 mg/ml potato extract were then compared to that of the control sample, treated with PBS. The result showed cytopathic activities in cells treated with potato extract, as well as separation of the cytoplasmic membrane from the outer membrane (Figure 4.5 – 4.9). Separation of the cell membrane structure was evident due to the activity of the fixatives, which preserve the cell membrane structures and allow excellent visualisation. These changes were observed within 15 to 90 min following incubation with potato extract, and are identical to the change in morphology observed when *H. pylori* cells were treated with filtered 250 mg/ml potato extract (Figure 4.4A-F).

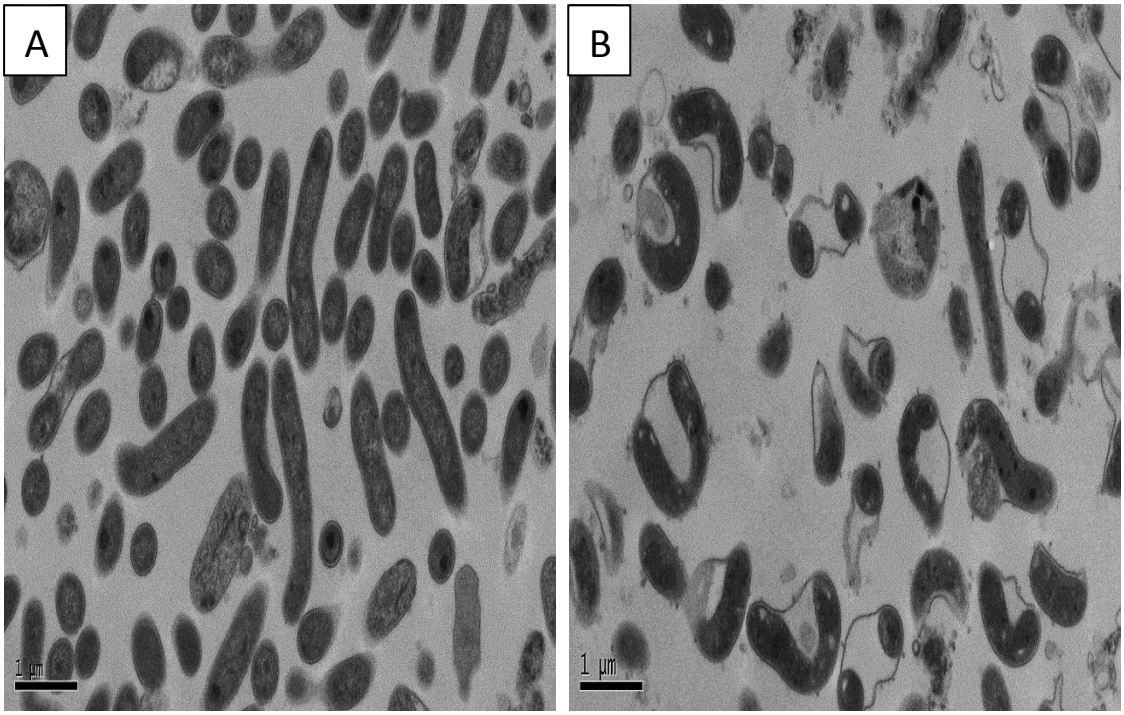


Figure 4.5 (A) *H. pylori* cells treated with PBS for 15 min; (B) *H. pylori* cells treated with filtered 125 mg/ml potato extract for 15 min

H. pylori cells were treated with PBS and potato extract respectively for 15 min; and fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde for 1 h; and later post-fixed in 1 % osmium tetraoxide and 1.5 % potassium ferrocyanide for 1 h. The fixed cells were then polymerised in epoxy resin for 3 – 4 h. The photos were taken at 2900x magnification. The scale bar is indicated in 1 µm.

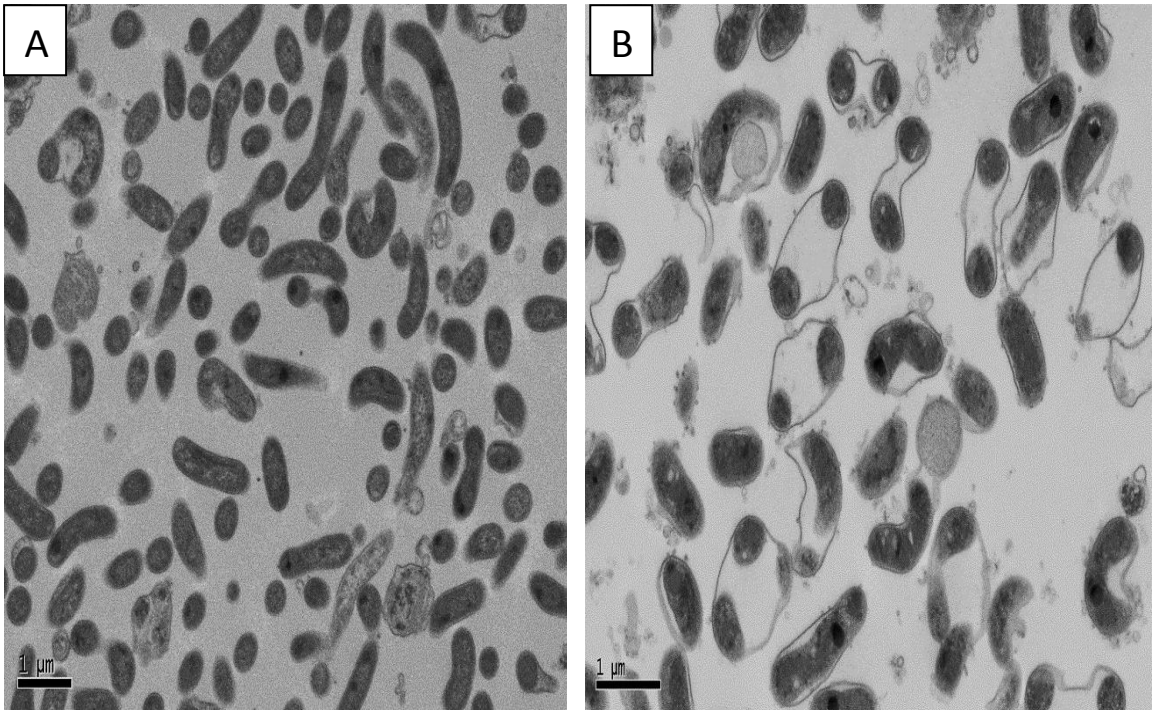


Figure 4.6 (A) *H. pylori* cells treated with PBS for 30 min; (B) *H. pylori* cells treated with filtered 125 mg/ml potato extract for 30 min

H. pylori cells were with PBS and potato extract respectively for 30 min; and fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde for 1 h; and later post-fixed in 1 % osmium tetraoxide and 1.5 % potassium ferrocyanide for 1 h. The fixed cells were then polymerised in epoxy resin for 3 – 4 h. The photos were taken at 2900x magnification. The scale bar is indicated in 1 µm.

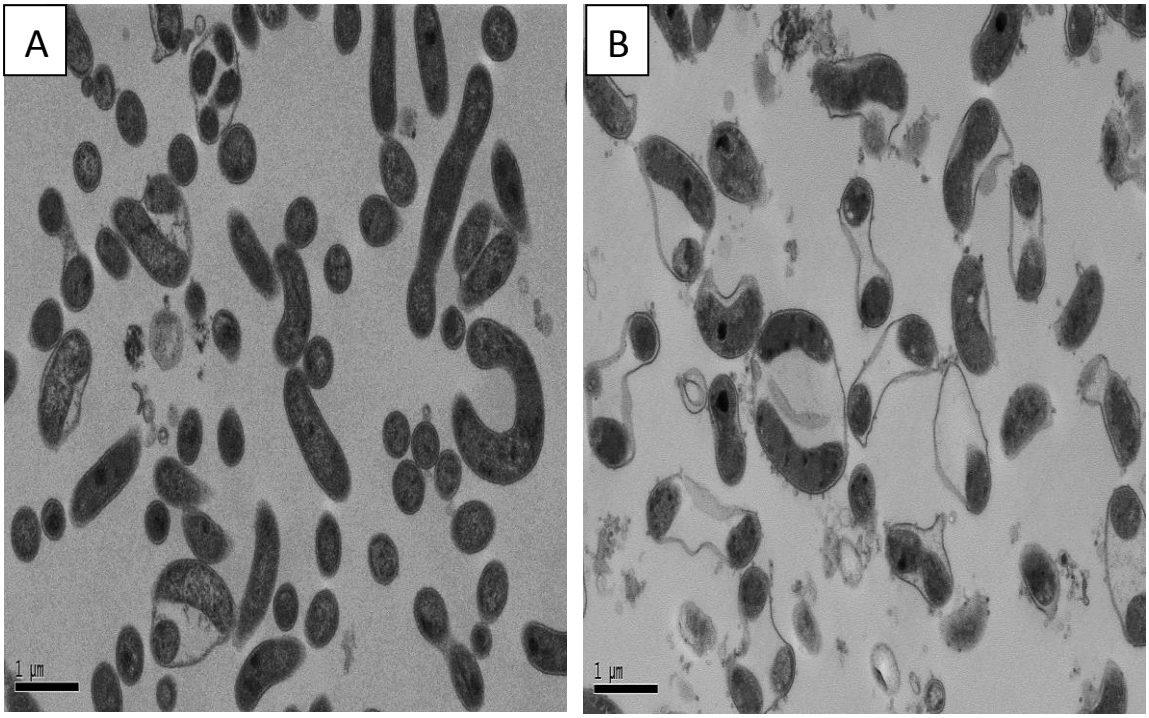


Figure 4.7 (A) *H. pylori* cells treated with PBS for 45 min; (B) *H. pylori* cells treated with filtered 125 mg/ml potato extract for 45 min

H. pylori cells were treated with PBS and potato extract respectively for 45 min; and fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde for 1 h; and later post-fixed in 1 % osmium tetraoxide and 1.5 % potassium ferrocyanide for 1 h. The fixed cells were then polymerised in epoxy resin for 3 – 4 h. The photos were taken at 2900x magnification. The scale bar is indicated in 1 µm.

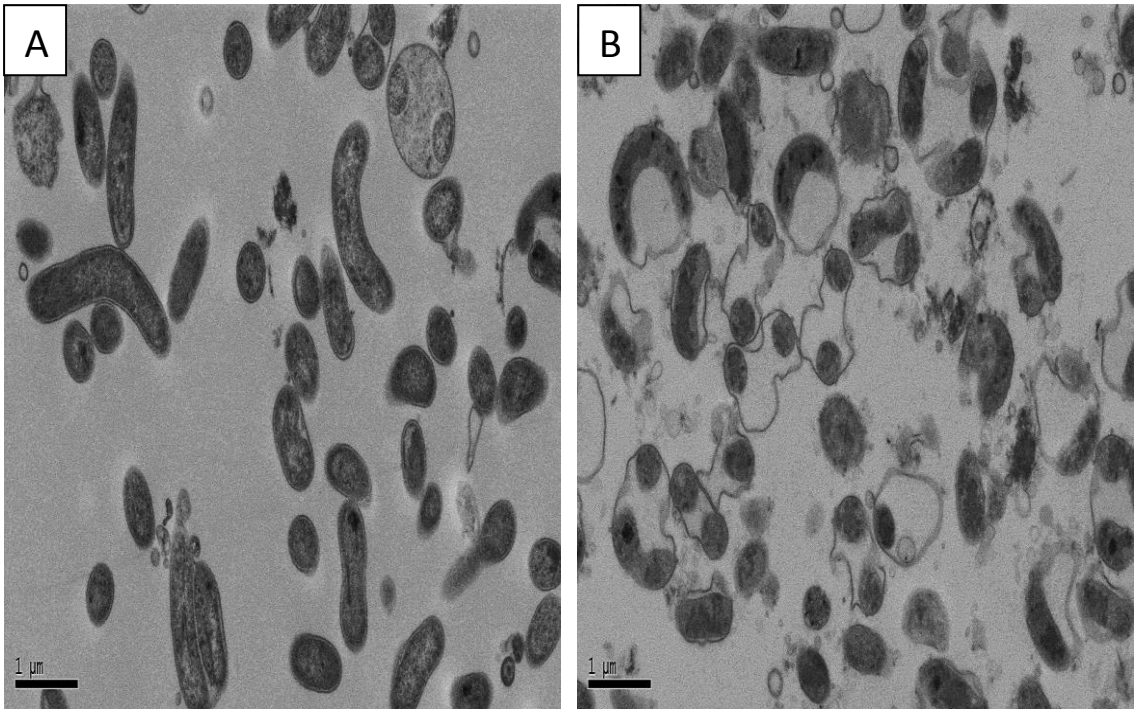


Figure 4.8 (A) *H. pylori* cells treated with PBS for 60 min; (B) *H. pylori* cells treated with filtered 125 mg/ml potato extract for 60 min

H. pylori cells were treated with PBS and potato extract respectively for 60 min; and fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde for 1 h; and later post-fixed in 1 % osmium tetraoxide and 1.5 % potassium ferrocyanide for 1 h. The fixed cells were then polymerised in epoxy resin for 3 – 4 h. The photos were taken at 2900x magnification. The scale bar is indicated in 1 µm.

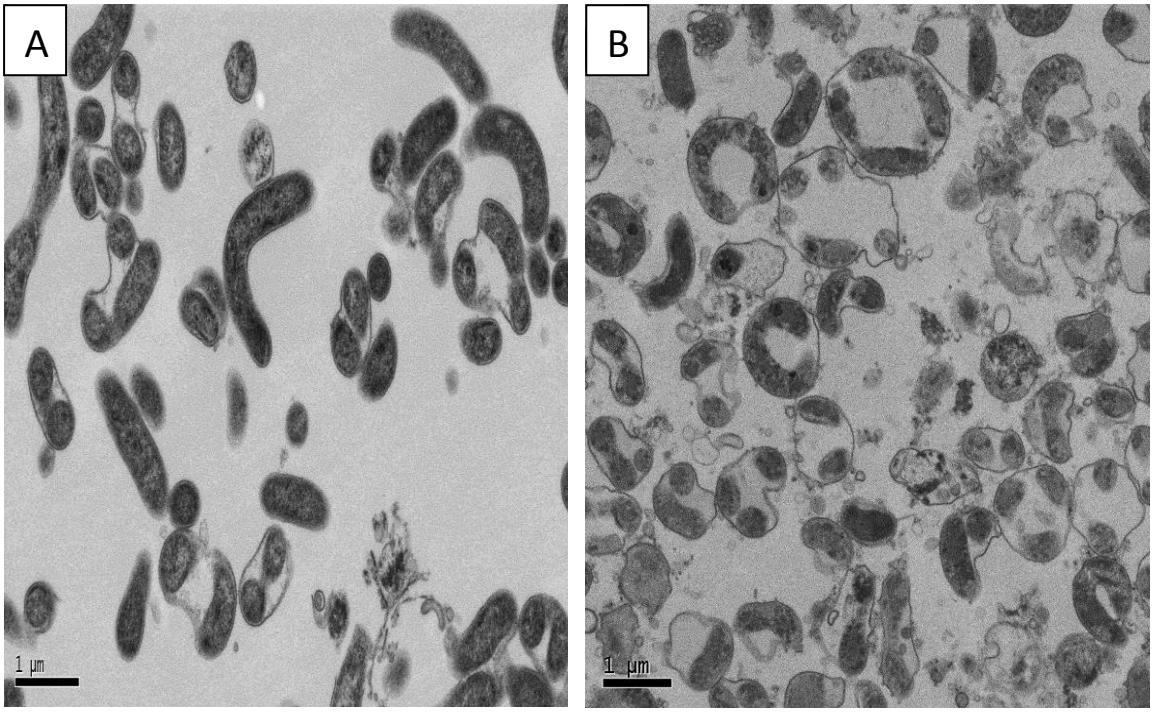


Figure 4.9 (A*) H. pylori* cells treated with PBS for 90 min; (B) *H. pylori* cells treated with filtered 125 mg/ml potato extract for 90 min

H. pylori cells were treated with PBS and potato extract respectively for 90 min; and fixed in 4 % paraformaldehyde and 2.5 % glutaraldehyde for 1 h; and later post-fixed in 1 % osmium tetraoxide and 1.5 % potassium ferrocyanide for 1 h. The fixed cells were then polymerised in epoxy resin for 3 – 4 h. The photos were taken at 2900x magnification. The scale bar is indicated in 1 µm.

4.3 Potato extract damage the membranes of *H. pylori*

It was observed from the TEM analysis that potato extract disrupts the morphology of *H. pylori* as characterised by the separation of cell wall from the outer membrane and the leakage of cellular material. From this observation, it is believed that the membrane integrity of the cell is compromised thereby allowing the passage of foreign materials, such as potato extract, into the cell. However, it was important to confirm this theory.

The LIVE/DEAD BacLight bacterial viability kit from Molecular Probes was used to assess the membrane integrity of *H. pylori* cells treated with filtered 250 and 125 mg/ml potato extract. The kit uses 2 nucleic acid stains (green-fluorescent syto9 dye and red-fluorescent propidium iodide dye) to distinguish bacteria with intact and non-intact membranes. It is expected that bacteria cells with intact cell membrane fluoresce bright green, whereas bacteria with damaged membrane exhibit significantly less green fluorescence and often fluoresce red. Cells treated with PBS and 25 µg/ml chloramphenicol were included as the negative control, and cells treated with 100 % propanol and 100 µg/ml ampicillin as the positive control. Following the treatment of *H. pylori* cells with the respective antibacterial agent for 15 min, syto9 and propidium iodide (in a ratio 1:1) were added to the treated-cell suspension and cells were viewed under a fluorescence microscope. The results are expressed as live-dead cell percentage ratio, with live cell representing cells with intact membrane (stained green) and dead cell representing cells with damaged membranes (stained red). Fluorescence microscopic observations show that cells treated with filtered 250 mg/ml and 125 mg/ml potato extract were majorly stained by the red-fluorescent propidium iodide dye thereby indicating membrane damage,

with live-dead cell percentage ratio of approx. 30:70 and 40:60 respectively (Figure 4.10). Cells treated with propanol, ampicillin, and chloramphenicol also indicated damaged membranes as cells were stained by the red-fluorescent dye, with live-dead cell percentage ratio of approx. 19:81, 35:65 and 35:65 respectively (Figure 4.10). In comparison, *H. pylori* cells treated with PBS maintained an intact cell membrane as indicated by the predominant green fluorescence of the cells, with a live-dead cell percentage ratio of 78:22 (Figure 4.10). The result show that potato extract disrupts the membrane integrity of *H. pylori* because in comparison to the PBS control, a greater percentage of cells treated with potato extract were stained with the red fluorescent dye indicating membrane damage.

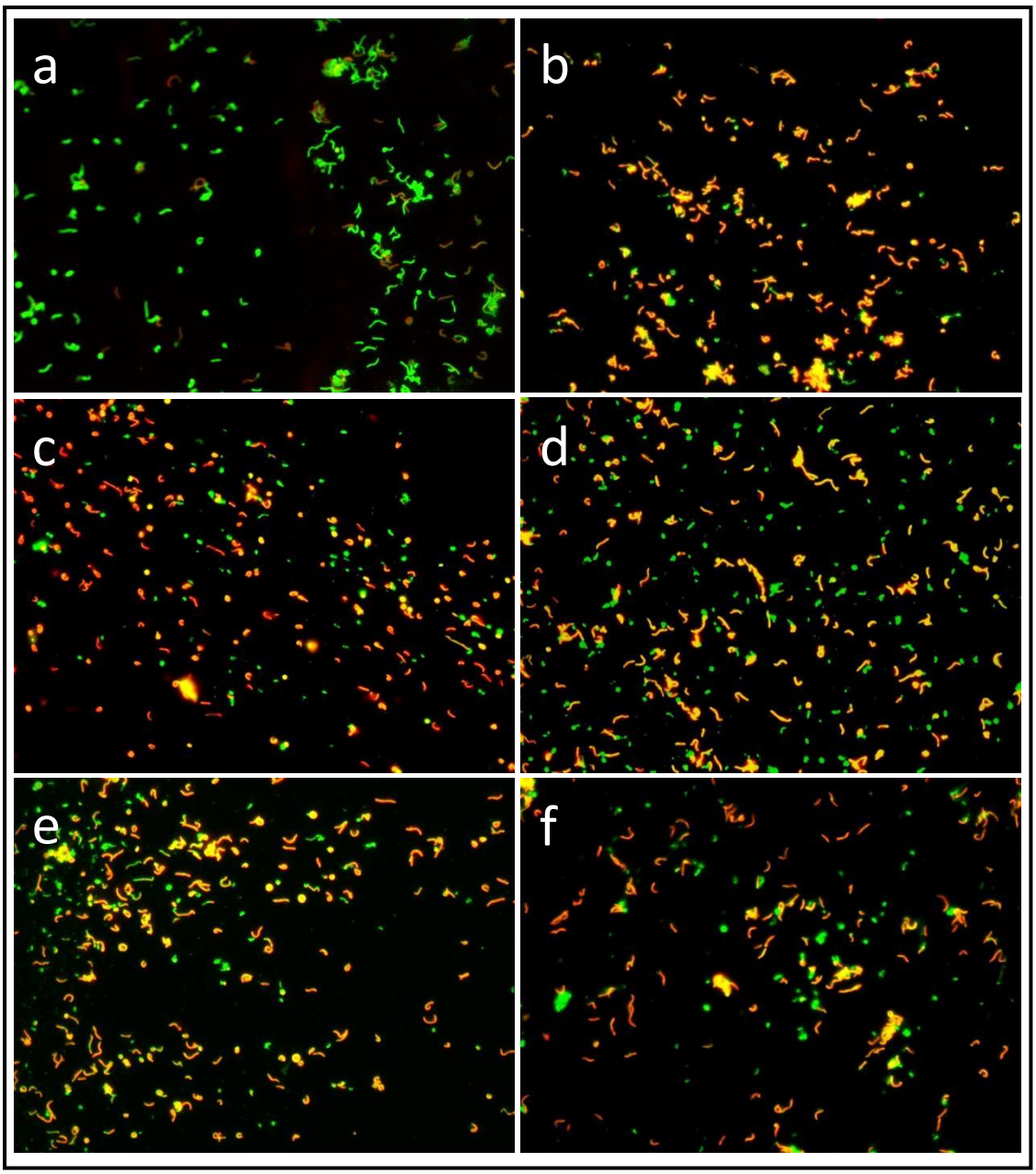


Figure 4.10 *H. pylori* cells treated with a) PBS; b) 100 % propanol; c) filtered 250 mg/ml potato extract; d) filtered 125 mg/ml potato extract; e) 25 µg/ml chloramphenicol; f) 100 µg/ml ampicillin, for 15 min

Antibiotic-treated and untreated *H. pylori* cells were stained with syto9 (which appears green in cells with intact membrane) and propidium iodide (which appears red in cells with damaged membrane) from the LIVE/DEAD Baclight kit. Cells were analysed by fluorescent microscopy. Photos were taken at 100x magnification.

4.4 Potato extract hyperpolarises the plasma membrane of *H. pylori*

Changes in the plasma membrane potential of *H. pylori* was studied by fluorescence measurement of the anionic membrane potential-sensitive slow-response fluorescent probe, bis-(1,3-dibutylbarbituric acid)-trimethine oxonol (DiBAC4[3]) (Plášek and Sigler, 1996; Bashford et al., 1985). DiBAC4[3] accumulates in depolarised cell membranes where it binds to intracellular proteins and results in an increased fluorescence (Plášek and Sigler, 1996).

H. pylori cells were treated with filtered 250 mg/ml and 125 mg/ml potato extract and changes in membrane potential was evaluated. Valinomycin, a K^+ selective ionophore which dissipates the membrane potential by facilitating the movement of K^+ across the lipid bilayer (Tosteson et al., 1967; Pressman, 1976), was used as a positive control. DiBAC4[3] was added to *H. pylori* cells 25 min before the addition of potato extract, until the dye uptake was optimal (Figure 4.11). Potato extract treatment of *H. pylori* cells loaded with DiBAC4[3] resulted in decrease in fluorescence compared to the control in which sterile water was added (Figure 4.11A). This decrease in fluorescence indicated that the dye did not accumulate in the cell due to plasma membrane hyperpolarisation. The rate of hyperpolarisation was observed to increase with increasing concentration of potato extract, with 250 mg/ml exhibiting the lowest fluorescence values (Figure 4.11 – 4.13).

In contrast, valinomycin produced a depolarisation effect as expected, indicated by a rapid increase in fluorescence intensity. At T_5 (T in min), after the addition of the antimicrobial agent to the sample, an immediate increase in fluorescence was observed in all samples followed by the respective hyperpolarisation or depolarisation effect (Figure 4.11A). In

order to clearly show the hyperpolarisation and depolarization effect detected, the data was normalised by the subtraction of the control values from the values obtained in samples where potato extract and valinomycin were added (Figure 4.11B). The kinetics of the hyperpolarisation and depolarisation effect observed in cells treated with potato extract and valinomycin respectively, varied among different experiment (compare results in Figure 4.11, 4.12 and 4.13) but showed a very similar trend.

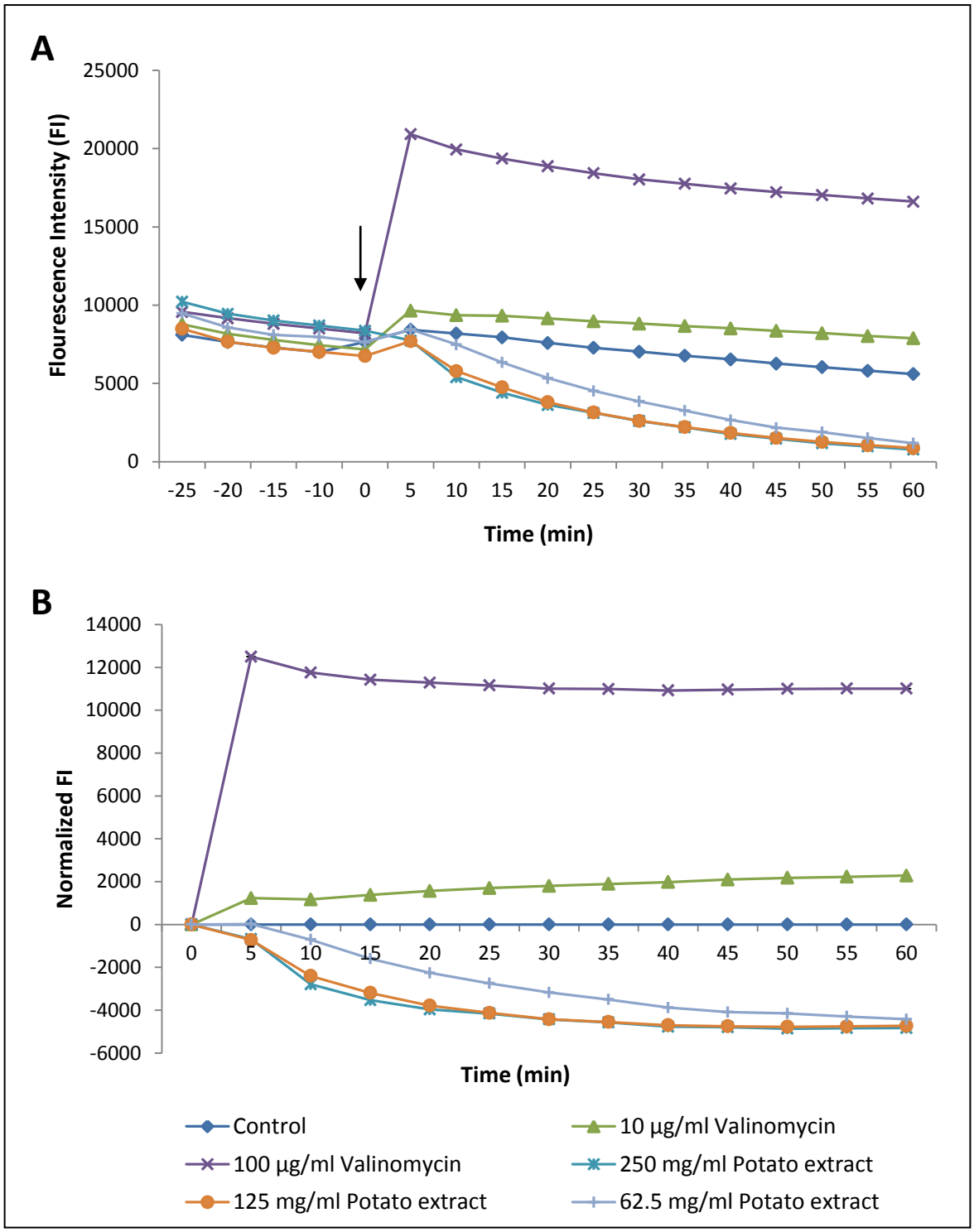


Figure 4.11 Effect of potato extract on the membrane potential of *H. pylori*

The effect of potato extract on the membrane potential of *H. pylori* was measured with the fluorescent dye DiBAC4[3].

A. Flourescence intensity (FI) values were recorded at 5 min intervals. The fluorescent dye was added 25 min before the addition of potato extract and valinomycin to the respective bacterial sample. Arrow at time 0 shows the exact moment potato extract and valinomycin were added. The control was an identical sample to which sterile water alone was added. Note that FI values are in arbitrary units.

B. Normalized values of the data presented in A, obtained by the subtraction of the control values from the time of addition of potato extract and valinomycin.

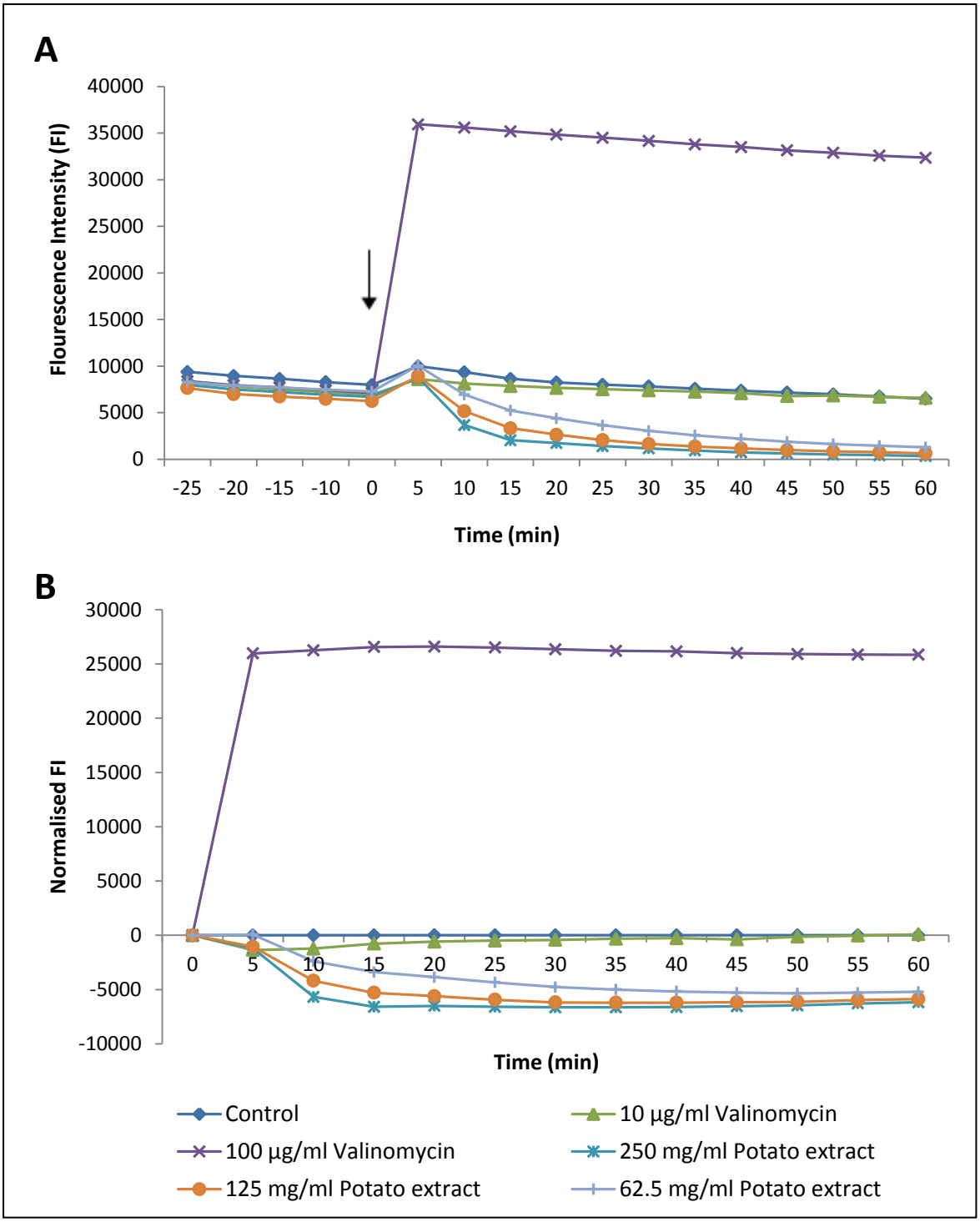


Figure 4.12 Effect of potato extract on the membrane potential of *H. pylori*

The effect of potato extract on the membrane potential of *H. pylori* was measured with the fluorescent dye DiBAC4[3].

A. Flourescence intensity (FI) values were recorded at 5 min intervals. The fluorescent dye was added 25 min before the addition of potato extract and valinomycin to the respective bacterial sample. Arrow at time 0 shows the exact moment potato extract and valinomycin were added. The control was an identical sample to which sterile water alone was added. Note that FI values are in arbitrary units.

B. Normalized values of the data presented in A, obtained by the subtraction of the control values from the time of addition of potato extract and valinomycin.

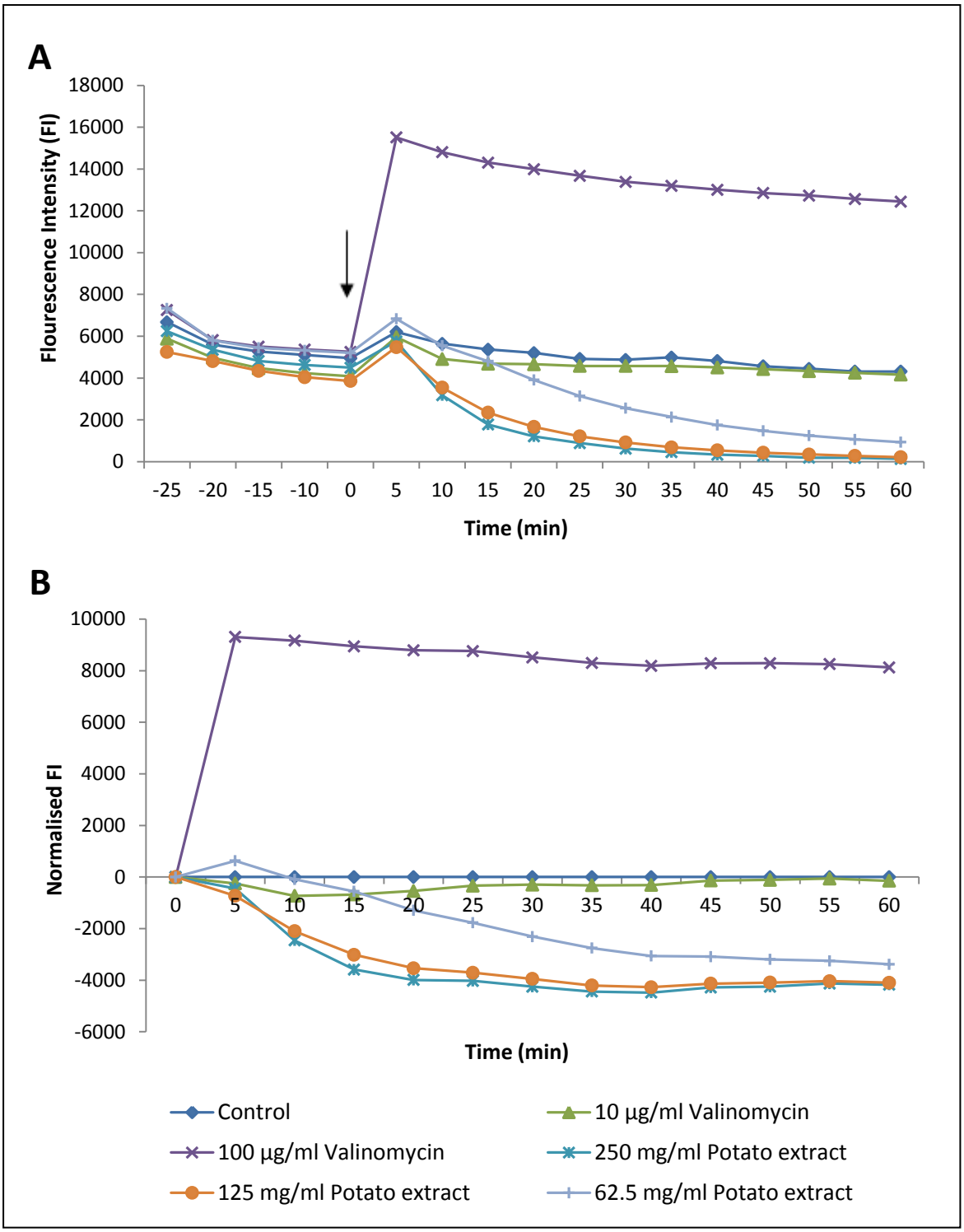


Figure 4.13 Effect of potato extract on the membrane potential of *H. pylori*

The effect of potato extract on the membrane potential of *H. pylori* was measured with the fluorescent dye DiBAC4[3].

A. Flourescence intensity (FI) values were recorded at 5 min intervals. The fluorescent dye was added 25 min before the addition of potato extract and valinomycin to the respective bacterial sample. Arrow at time 0 shows the exact moment potato extract and valinomycin were added. The control was an identical sample to which sterile water alone was added. Note that FI values are in arbitrary units.

B. Normalized values of the data presented in A, obtained by the subtraction of the control values from the time of addition of potato extract and valinomycin.

4.5 Effect of potato extract on ATP concentration of *H. pylori*

Relative changes in ATP content of *H. pylori* cells upon treatment with potato extract was evaluated using the ENLITEN® ATP Assay System Bioluminescence Detection Kit (Promega). Bioluminescence is generated by the interaction of luciferin/luciferase with ATP, where the intensity of light emitted is proportion to ATP concentration (McElroy and DeLuca, 1983; Lundin and Thore, 1975).

H. pylori cells were treated with filtered potato extract (250, 125 and 62.5 mg/ml) and ATP was extracted from the cells. The ATP sample was then treated with luciferin/luciferase reagent (provided in the ATP assay kit) and analysed for bioluminescence. An ATP standard curve was generated (Figure 4.14) by measuring the relative bioluminescence of ATP standards (provided in the ATP assay kit) with known concentration of 10^{-7} to 10^{-11} M.

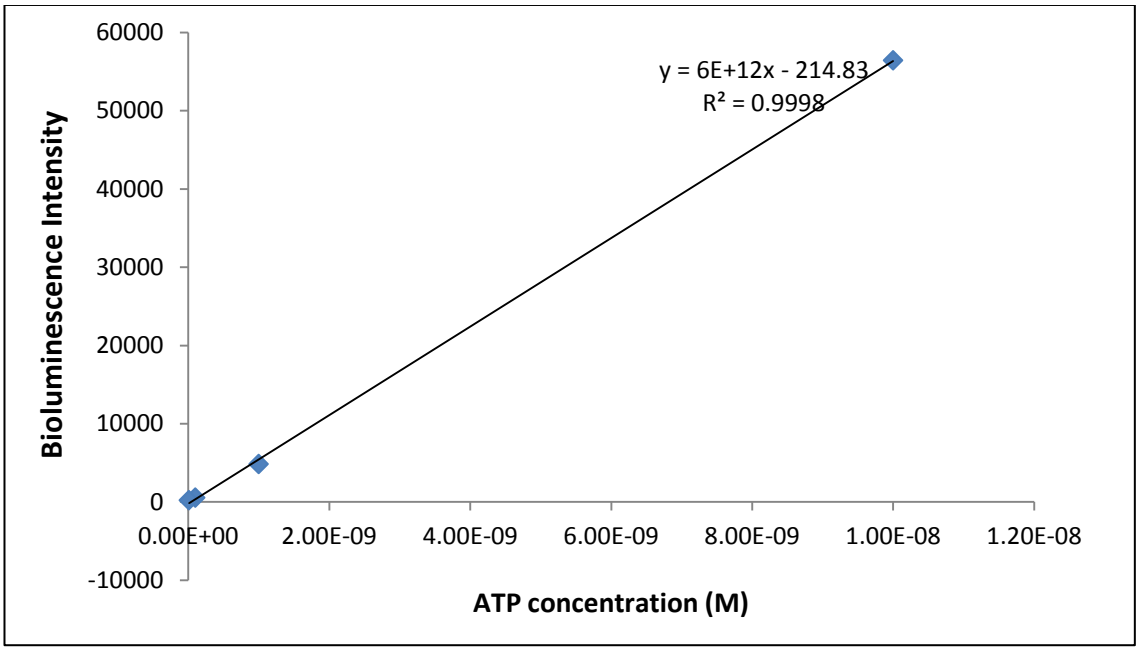


Figure 4.14 ATP standard curve obtained using the ENLITEN® ATP Assay System
Ten microliters of ATP of known concentration diluted to the proper concentrations was added to a 96-well plate and assayed using the luciferin/luciferase reagent. Relative bioluminescence was measured using a BioTek™ Synergy™ HT Multi-detection Microplate Reader.

The ATP concentration in the potato extract-treated sample was then calculated using the data generated from the ATP standard curve. A reduction in the ATP content of *H. pylori* was observed in cells treated with 250 mg/ml potato extract, in comparison to the PBS control ($P < 0.05$) (Figure 4.15). Whereas, treatment with 125 and 62.5 mg/ml potato extract had no effect on the ATP concentration of the cells (Figure 4.15).

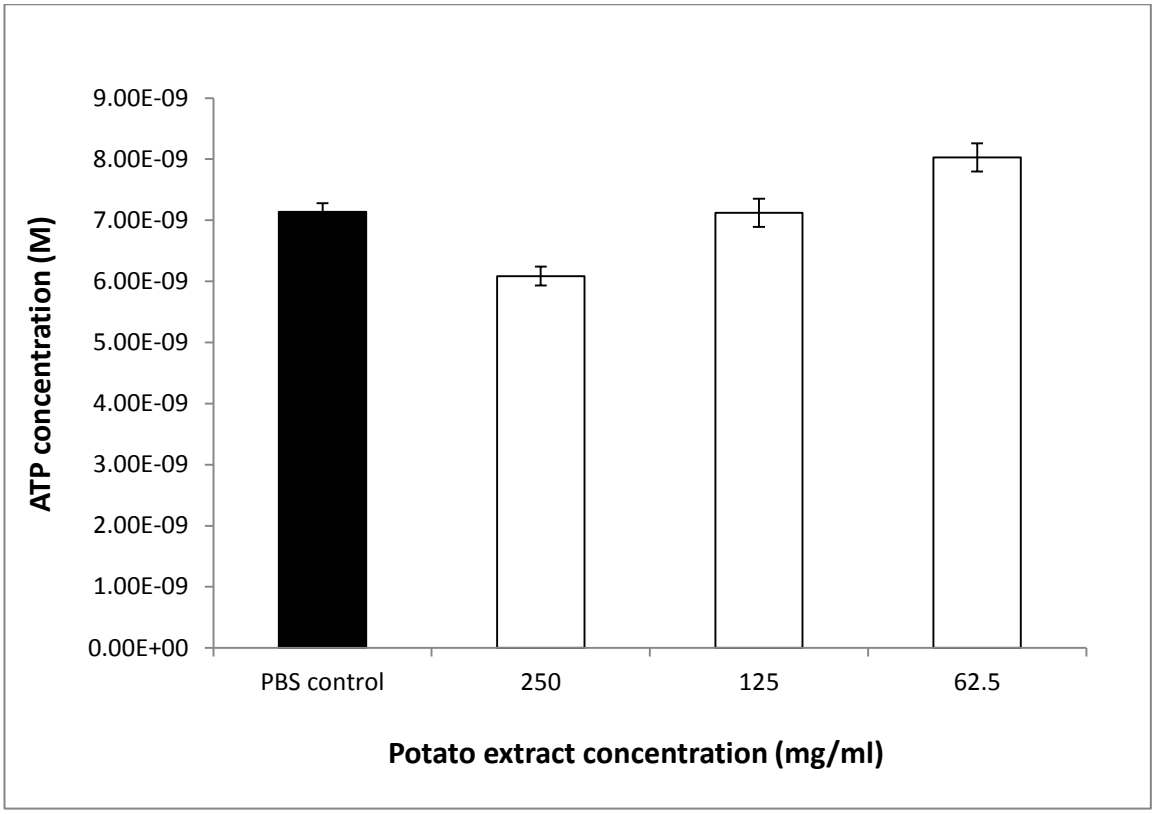


Figure 4.15 Effect of potato extract on the ATP concentration of *H. pylori*

Ten microliters of ATP extracted from *H. pylori* cells treated with potato extract was added to a 96-well plate and assayed using the luciferin/luciferase reagent. Relative bioluminescence was measured using a BioTek™ Synergy™ HT Multi-detection Microplate Reader and the ATP concentration was determined using an ATP standard curve. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained.

4.6 Discussion

4.6.1 Changes to the morphology of *H. pylori* cells following treatment with potato extract

Previous results in this study has shown that potato extract exhibit a definite bactericidal action against *H. pylori*; it was therefore important to establish its mode of action. Several studies have implicated membrane damage as the primary mode of action of many plant extracts (Sanchez et al., 2010; Nazzaro et al., 2013). Thus, the effect of potato extract on the membrane of *H. pylori* was studied using transmission electron microscopy.

The experiment was initially carried out using unfiltered potato extract which has been used in all the previous experiments. However, due to interference of undissolved particles present in the extract with the proper interpretation of changes observed in the cells, filtered extract was used in the imaging assays to get rid of the debris. Observation of the transmission electron micrographs (Figure 4.4, 4.5 – 4.9) show that potato extract disrupts the morphology of *H. pylori* by causing separation of the inner cytoplasmic membrane from the outer membrane, separation of the cell cytoplasmic content, and other cytopathic activities. These observations were detected within 5 to 90 min following incubation with potato extract. In the TEM micrographs where unfiltered potato extract was used, a different morphological change was observed (Figure 4.1). This was characterised by coagulation of cell cytoplasmic content and partial/total loss of the cell envelope. This difference in morphological change is attributed to a possible effect caused by the undissolved particles in the extract, as it has been demonstrated to be more bactericidal against *H. pylori* (Figure 4.2). Hence, this may be a case of over-kill exhibited by the unfiltered extract as coagulation of cell cytoplasm and loss of cell

membrane observed was immediate occurring within 5 – 15 min of treatment (Figure 4.1); as opposed to the morphological change observed with filtered extract which occurred gradually (Figure 4.4 – 4.9).

The separation of the cytoplasmic membrane from outer membrane observed was initially thought to be due to plasmolysis of cells. Plasmolysis can occur, in this case, when a Gram-negative bacterium is challenged with sufficient concentration of a non-penetrating solute, such as a strong saline or sugar solution that causes water to be sucked out of the cell (Schwarz and Koch, 1995). It is sometimes characterised by the presence of plasmolysis spaces which may form if the cell's cytoplasmic membrane separates from the peptidoglycan layer and the outer membrane (Schwarz and Koch, 1995). As such, based on the observation in the transmission electron micrographs of *H. pylori* cells treated with potato extract (see Appendix I Figure 4.8.1 for higher magnification), separation of the cell wall from the outer membrane may be due to plasmolysis of the *H. pylori* cells. This may also occur due to the high concentration of potato extract initially used in the analysis. As a result, lower concentration of potato extract was used in the assay. At 125 mg/ml, similar change in morphology as observed with 250 mg/ml was detected (Figure 4.4 – 4.9). *H. pylori* cells treated with 62.5 mg/ml potato extract also showed separation of cell cytoplasmic content but this change in morphology was only observed after 60 – 90 min of incubation with potato extract (result not shown). This result is complementary to the time-kill studies shown in Figure 4.3, where 62.5 mg/ml potato extract did not have any significant effect on *H. pylori* when compared to the PBS control. TEM analysis of *E. coli* cells treated with potato extract was also carried out, and no change in morphology was observed as indicated in the viable count assay previously carried out (Figure 3.1). These observations with lower concentrations of potato

extract indicate that the change in morphology of *H. pylori* is not as a result of plasmolysis as no change in morphology was observed in *E. coli*. In addition, the fact that a different change in morphology was observed in cells treated with unfiltered extract disqualified the plasmolysis theory.

Thus, the change in morphology is thought to be as a result of disruption of the cell envelope-embedded peptidoglycan layer. The peptidoglycan layer is essential for the maintenance of cell osmotic stability and cell shape in many bacteria, including *H. pylori* (Sycuro et al., 2010; Vollmer et al., 2008). *H. pylori* encodes 3 of the penicillin binding proteins (PBPs) needed for peptidoglycan synthesis and peptide crosslinking which brings about its helical shape (Tomb et al., 1997; DeLoney and Schiller, 1999). Disruption of the peptidoglycan layer may be the result of the separation of the cell wall and cytoplasmic content of *H. pylori* from the outer membrane observed, as the cells lose their shape. Also, alterations in the peptidoglycan layer may affect the penetration of solutes (Co and Schiller, 2006), thereby presenting the plasmolytic characteristics observed.

4.6.2 Membrane damage to *H. pylori* cells

Transmission electron microscopy revealed structural membrane damage to *H. pylori* cells treated with potato extract for 5 – 90 min. It is reasonable to speculate that the loss of cell morphology and integrity as a result of potato extract treatment may lead to loss of the permeability barrier and could be one of reasons for *H. pylori* cell death. Bacterial viability is characterized as a function of membrane integrity, and this has been evaluated using fluorescent dyes, Syto9 and propidium iodide (Laflamme et al., 2004; Berney et al., 2007). Therefore, membrane damage due to treatment of *H. pylori* with potato extract was monitored by using fluorescent dyes from the LIVE/DEAD BacLight bacterial viability kit,

through fluorescence microscopy. As expected, the potato extract-treated cells were stained by propidium iodide (PI) (exhibited by an increased red fluorescence), thereby indicating an increased membrane permeabilization due to membrane damage of *H. pylori* cells. In this assay, cells treated with chloramphenicol was used as a negative control since chloramphenicol inhibits protein synthesis (Gale and Folkes, 1953) and is not expected to be implicated in membrane damage. However, an increase in nuclear PI staining was observed in the chloramphenicol-treated cells, thereby indicating cell membrane damage. This observation suggests that the membrane damage observed may be a secondary effect of cell death caused by chloramphenicol treatment since PI staining is only expected in cells with damaged membranes. This result also suggests that the Live/Dead BacLight kit may not be the most reliable method of assessing membrane permeability because there is a possibility that the nuclear propidium iodide dye stained all dead cells irrespective of cell membrane damage. Consequently, a study has shown that the Syto9 - PI combination is not the most reliable viability testing method (Lehtinen et al., 2004). Factors cited includes increased rate of diffusion of Syto9 through damaged membranes in comparison to intact membranes, increased fluorescence with increasing Syto9 dye concentration, and competition for DNA binding sites between Syto9 and PI. Thereby, leading to distortion in the proportion of viable and dead cells and quantification of “false” live and dead cells (Lehtinen et al., 2004). As such, these findings may disproof the result of this experiment.

4.6.3 Membrane active pumping assessment

The resting membrane potential is one of the important parameters responsible for controlling cell homeostasis (Bot and Prodan, 2009). It is controlled by specific ion pumps that regulate the permeability and concentration of important ions in the cell. Plasma membrane potential is a function of the intracellular energy levels, and controls the exchange of information between the intracellular and extracellular medium (De Nicola et al., 2008). Interestingly, in this study, potato extract induced plasma membrane hyperpolarisation in *H. pylori* cells. A depolarisation effect was expected as potato extract has been implicated in causing membrane damage to *H. pylori* cells as seen in the TEM analysis and fluorescence membrane permeability assay. It was thought that membrane damage will promote the permeability of the ion channels and diffusion of ions in and out of the cell, thereby dissipating the membrane potential. However, this was not the case. In this experiment, *H. pylori* cell sample treated with filtered 62.5 mg/ml potato extract was included so as to determine if the membrane hyperpolarisation observed is related to the antibacterial activity of the extract. However, membrane hyperpolarisation was also observed in cells treated with filtered 62.5 mg/ml potato extract, which does not have significant antibacterial effect on *H. pylori* in comparison to higher concentrations (as shown in Figure 4.2 and 4.3). Thus, we concluded that the change in plasma membrane potential observed suggests a change in cell behaviour, however this does not affect cell viability since the hyperpolarisation effect observed in cells treated with filtered 62.5 mg/ml potato extract do not lead to significant antibacterial killing of cells or changes in cell morphology (result not shown) as compared to cells treated with higher concentrations of potato extract. Studies have shown that plasma membrane hyperpolarisation may occur due to pH change from

acidic to neutral, or as a result of diffusion of K^+ outside the cell membrane through ion channels, thereby disrupting the cell homeostasis (Bot and Prodan, 2009). Thus, hyperpolarisation observed in this study may be due to cellular interference caused by potato extract, which does not interfere with the overall cell viability.

Consequently, when the metabolic ATP concentration of *H. pylori* was measured in the presence of potato extract, the result showed a slight but significant reduction of ATP in cells treated with 250 mg/ml potato extract. Whereas, no effect was observed in cells treated with lower concentrations of the extract. This result is inconclusive because ATP reduction was observed in cells treated with 250 mg/ml potato extract and not in cells treated with 125 mg/ml; both of which exhibit a similar level of damage to the morphology and membranes of *H. pylori*. With respect to the result of the membrane potential test, it is thought that potato extract affect membrane stability without effectively or directly interfering with active transport of the cell membrane.

In conclusion, potato extract disrupts the morphology and damages the membrane of *H. pylori* cells. It induces plasma membrane hyperpolarisation that disrupts the cell homeostasis, but does not significantly affect ATP concentration within the cell.

Chapter 5

Investigating the Target of Potato Extract Encoded by the *H. pylori* Genome

5.1 Introduction

In previous chapters, the antibacterial activity and mode of action of potato extract against *H. pylori* was evaluated. A third aim of this study is to identify genes/processes targeted by potato extract, encoded by the *H. pylori* genome. Therefore, in this chapter, *H. pylori* mutants resistant to potato extract were generated by chemical mutagenesis. Phenotypic variations between the wild-type and mutant strains were evaluated and identified. Subsequently, the genome sequences of wild-type and mutant DNA were analysed to identify genomic variations in the DNA that could have brought about resistance of the mutant strains to potato extract.

5.2 Generation and screening of *H. pylori* mutants resistant to potato extract

H. pylori strains resistant to potato extract were generated by chemical mutagenesis. Wild-type *H. pylori* strain 26695 was subjected to treatment with ethyl methanesulfonate (EMS). EMS is an alkylating chemical mutagen that induces point mutation in DNA at an approximate frequency of 1 base change per gene (Segal et al., 1992). Mutation is said to occur primarily by guanine alkylation forming O-6-ethylguanine which pairs with thymine rather than cytosine, thereby resulting in G-C being substituted by A-T (Lawley and Brookes, 1961; Loveless, 1969).

H. pylori cells were treated with EMS for 15 min. The mutagenised *H. pylori* cells obtained were assayed for urease activity as a means to determine if

the mutagenesis was successful. Of 600 colonies screened, 5 colonies were urease negative, thereby indicating the success of the chemical mutagenesis. The remaining mutagenised *H. pylori* stock was then screened for susceptibility to potato extract by culturing on agar plates supplemented with potato extract. Firstly, the MIC of potato extract was determined using the agar dilution method, and recorded as 6.25 mg/ml (refer to Table 3.3). The mutagenised *H. pylori* stock (2 x 10^6 CFU/ml) was then screened for resistance to potato extract on agar plates supplemented with 12.5 mg/ml (2x MIC). Mutagenised *H. pylori* stock cultured on agar plates without potato extract, wild-type strain cultured on agar plates supplemented with 12.5 mg/ml potato extract, and without extract were used as the negative control. After 8 days of incubation, a total of 67 colonies had appeared on potato extract-agar plates for screening the mutagenised *H. pylori* cells, and no colony was observed on the control plates. The colonies were then numbered and henceforth identified by their allocated number as individual mutant strains. These were classified as being less susceptible to potato extract than the wild-type strain. The mutant colonies were re-streaked on agar plates supplemented with potato extract at 2x MIC. This screening was carried out three times. Of the 67 colonies initially obtained from 2 x 10^6 CFU/ml of mutagenised *H. pylori* screened, 29 colonies were found to remain resistant to potato extract. These 29 mutant colonies were verified as *H. pylori* by Gram staining and a positive urease test (a few of the mutants showed delayed urease activity). The mutant strains were cultured individually on agar plates and stored as glycerol stocks for further analysis. A further screening, with agar supplemented with higher concentration of potato extract (25 mg/ml), was carried out so as to obtain the most resistant strains; however, none of the mutant strains were resistant at this high concentration.

5.3 Minimum inhibitory concentration (MIC) of potato extract and antibiotics against selected *H. pylori* mutants resistant to potato extract

5.3.1 MIC of potato extract against selected *H. pylori* mutants in comparison to that of the wild type strain

Following successful generation of *H. pylori* mutant strains resistant to potato extract, the MIC of potato extract was evaluated against these mutants and compared to that of the wild type strain. Few potato extract-resistant *H. pylori* mutant strains were selected for this purpose, based on their growth rate on agar supplemented with 12.5 mg/ml potato extract. Typically, mutant strains with the fastest growth rate of within 2-3 days were selected.

Using the viable count assay, potato extract at concentrations ranging from 0.12 mg/ml to 250 mg/ml was tested against 6 selected mutant strains. Cells treated with PBS were used as a negative control while *H. pylori* cells treated with potato extract was used as a positive control. Results show that the wild type strain is more susceptible to the extract than the mutant strains (Figure 5.1). The MIC at which potato extract completely kill the wild type strain is approx. 15.6 mg/ml; while MIC for the mutant strains is > 15.6 mg/ml, except mutant #20 with an MIC of 15.6 mg/ml (Figure 5.1). At 3.9 mg/ml, mutant strains #1, 18, 20 and 40 became completely resistant to potato extract, while mutants #4 and 6 remain susceptible. At 0.12 – 0.97 mg/ml, potato extract had no effect on all the mutant strains tested (Figure 5.1). In comparison, the wild type strain remains more susceptible with a percentage kill of approx. 31 % at 0.12 mg/ml (Figure 5.1).

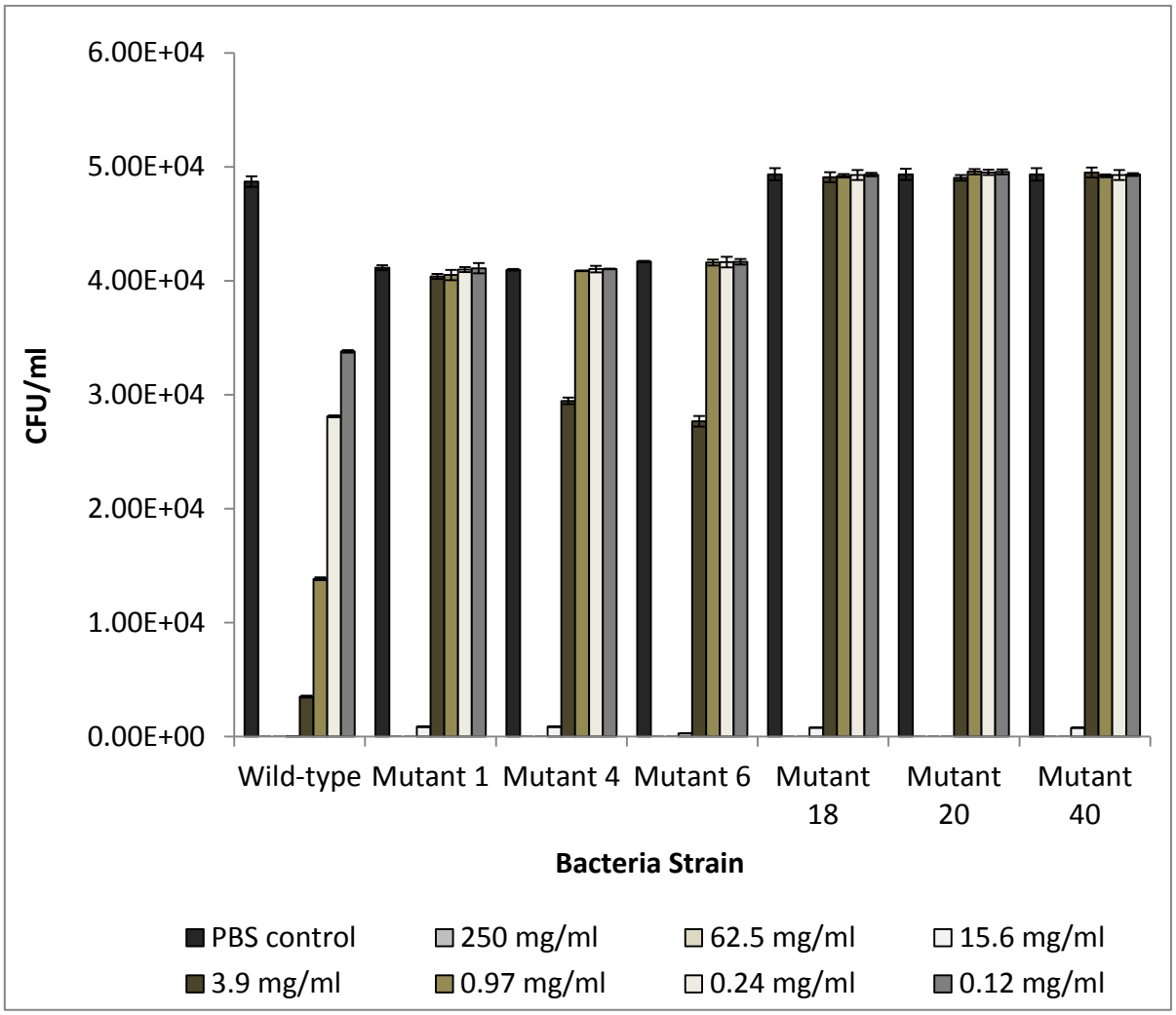


Figure 5.1 Comparison of anti-*H. pylori* activity of potato extract between *H. pylori* 26695 wild-type strain and potato extract-resistant *H. pylori* mutant strains

Viable cell count of *H. pylori* 26695 and resistant mutant strains after incubation with known concentrations of potato extract for 90 min. Experiment was carried out in duplicates and repeated three times; and the results are the mean ± standard error of the mean obtained. The mean difference is considered significant at $P \leq 0.05$.

5.3.2 MIC of antibiotics against *H. pylori* mutant strains in comparison to the wild-type strain

The MIC of antibiotics, ampicillin, tetracycline and metronidazole, currently used in the treatment of *H. pylori* infection was evaluated so as to identify mutants that have become more or less susceptible to the antibiotics as a

result of the chemical mutagenesis. The Oxoid MIC evaluator strip was used in this assay.

Results showed a variation in the susceptibility of the mutant strains to antibiotics (Table 5.1). As expected, some of the mutants had become more or less susceptible to the antibiotics tested. The pattern of susceptibility is such that a mutant may retain the same susceptibility to a given antibiotic as the wild type and be more or less susceptible to another antibiotic. Such as in the case of mutant 9 when compared to the wild type, retains susceptibility to ampicillin (AMP), becomes less susceptible to tetracycline (TET), and more susceptible to metronidazole (MET) (Table 5.1). Another mutation pattern is seen in mutant 12, which has become less susceptible to AMP and TET while being more susceptible to MET. Looking at the result of mutant #13, 20, 23, 25, 28, 30, 32, 37, 38, and 39, it was observed that all these mutants showed a marked increase in susceptibility to all the antibiotics tested (Table 5.1). Also, it was observed that all mutant strains except mutant #7 and 18 became more susceptible to metronidazole as compared to the wild type strain.

Table 5.1 MIC of antibiotics against potato extract-resistant *H. pylori* mutant strains

Antibiotic (µg) / Strain/ Mutant #	AMP	TET	MET
Wild-type	0.015	0.12	8
1	0.03	0.06	0.25
2	< 0.015	0.03	4
4	< 0.015	0.06	4
6	0.015	0.12	4
7	0.015	0.25	8
9	0.015	0.25	1
11	< 0.015	0.25	4
12	0.03	0.25	4
13	< 0.015	0.015	1
14	0.015	0.12	1
15	0.015	0.12	4
16	< 0.015	0.12	4
17	0.03	0.12	4
18	0.015	0.12	8
19	0.015	0.06	2
20	< 0.015	0.015	1
21	0.015	0.12	4
22	0.015	0.12	4
23	< 0.015	0.015	1
25	< 0.015	0.015	1
28	< 0.015	0.015	1
30	< 0.015	0.015	1
32	< 0.015	0.03	1
35	0.015	0.12	2
37	< 0.015	0.06	1
38	< 0.015	0.015	0.5
39	< 0.015	0.03	0.5
40	< 0.015	0.12	4
41	0.015	0.12	4

AMP- Ampicillin, TET- Tetracycline, MET- Metronidazole.

MIC of antibiotics was evaluated against *H. pylori* wild type strain and potato extract-resistant mutants. The values shown are the MIC of each antibiotic (in µg) against each strain.Experiment was carried out in duplicate and repeated three times.

5.4 Analysis of the membrane profile of potato extract-resistant *H. pylori* mutant strains in comparison to the wild type

Based on results of previous experiments in this study showing membrane damage to *H. pylori* cells, it is hypothesised that the cell membrane plays an active role in the antibacterial activity of potato extract. It is, therefore, reasonable to assume that mutational changes in the cell membrane may confer resistance to potato extract. Thus, the total membrane proteins, periplasmic proteins and lipopolysaccharide of *H. pylori* mutant cells and wild type strain were analysed.

5.4.1 Protein profile of the total membrane of *H. pylori* 26695 and that of resistant mutants are similar

The bacteria cell membrane is known to regulate cell permeability, especially the diffusion of biological compounds in and out of the cell (Salton, 1987). Membrane proteins such as porins have been shown to serve as a medium for the entry of biological compounds such as antibiotics (Burns and Smith, 1987). Thus, it is thought that resistance of *H. pylori* mutant strains to potato extract may be as a result of mutational changes to membrane proteins important for membrane function, for instance proteins altering the permeability of the extract to the cell membrane.

The total membrane proteins, comprising of the outer and inner membrane proteins was extracted by cellular fractionation as described in Section 2.3.1, and analysed by SDS-PAGE. Membrane proteins profile of potato extract-resistant mutants was compared to that of the wild type 26695 strain, the *galE* 26695 mutant strain and *H. pylori* 11637 strain. The SDS-PAGE analysis showed that there was no distinct difference between

the total membrane protein profile of the resistant mutant strains and that of the wild type strain, *galE* mutant and *H. pylori* 11637 strain (Figure 5.2).

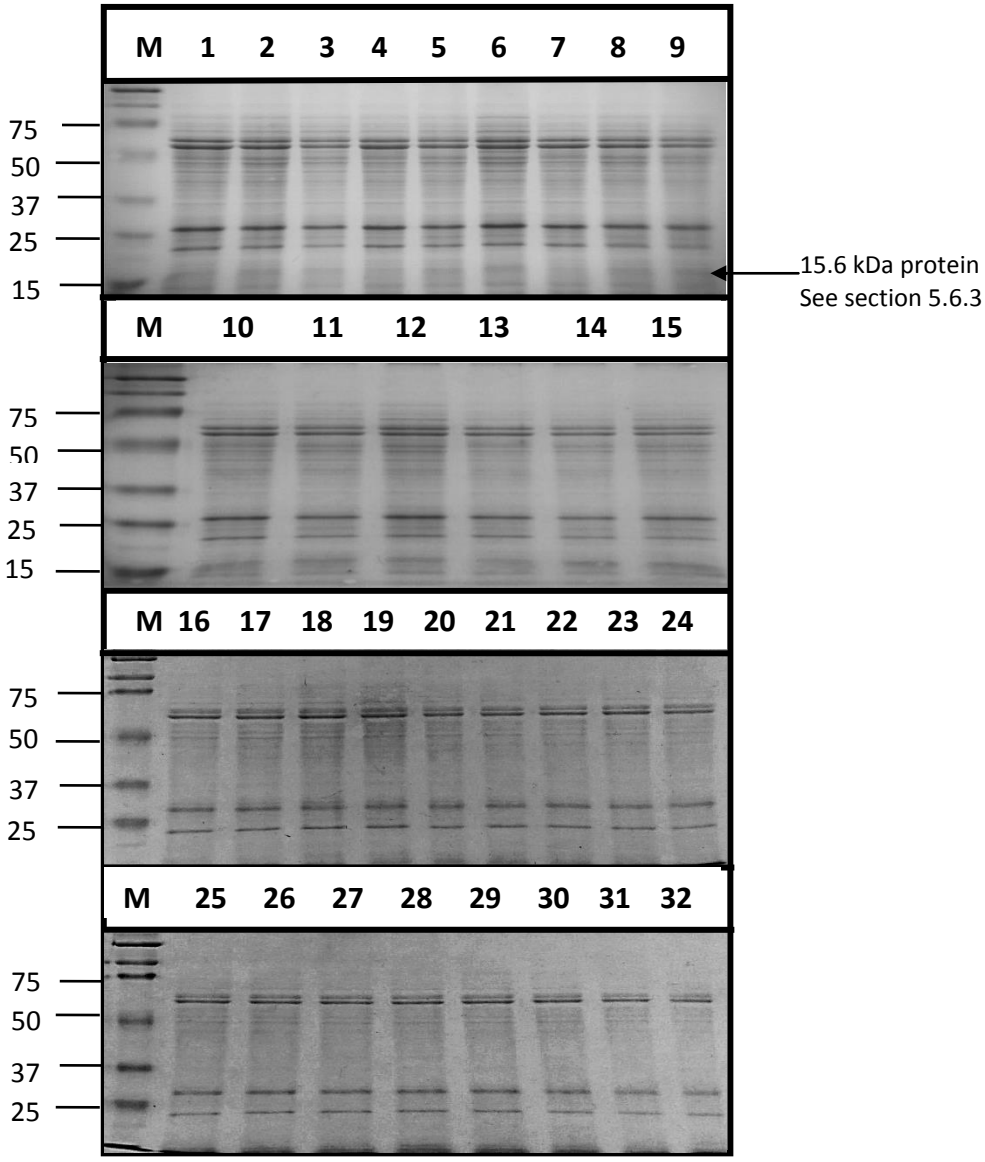


Figure 5.2 SDS-PAGE Analysis of *H. pylori* resistant mutant total membrane proteins

Typically, 10 µl of the total membrane protein samples standardised in SDS loading dye were loaded onto the gel along with molecular weight standard. A 12% denaturing gel was used. Molecular weights (in kDa) are indicated.
M, prestained marker (Precision Plus Protein Standard, BioRad); Lane 1-32, total membrane proteins from *H. pylori* 26695, *galE* mutant, *H. pylori* 11637, mutant #1, 2, 4, 6, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 28, 30, 32, 35, 37, 38, 39, 40, and 41, respectively.

5.4.2 Protein profile of the periplasmic extract of *H. pylori* 26695, *galE* mutant and potato extract-resistant *H. pylori* mutants

Electron microscopic analysis carried out on *H. pylori* cells treated with potato extract showed detachment of the plasma membrane from the outer membrane (Figure 4.4 – 4.9), thereby indicating a possible interaction of potato extract with components of the periplasmic space such as proteins and peptidoglycan. Periplasmic proteins have several functions important for cell survival such as transport of nutrients and small molecules into the cytoplasm, and detoxification of toxic materials (Pugsley, 1993). Thus, following analysis of the total membrane proteins of the mutant strains, the periplasmic proteins of the potato extract-resistant mutant strains, *galE* mutant strain, wild type strain and *H. pylori* 11637 strain were extracted by cellular fractionation and analysed by SDS-PAGE. The SDS-PAGE analysis of the periplasmic proteins showed that the protein profile of the resistant mutants was no different to that of the wild type strain, *galE* mutant and *H. pylori* 11637 strain (Figure 5.3).

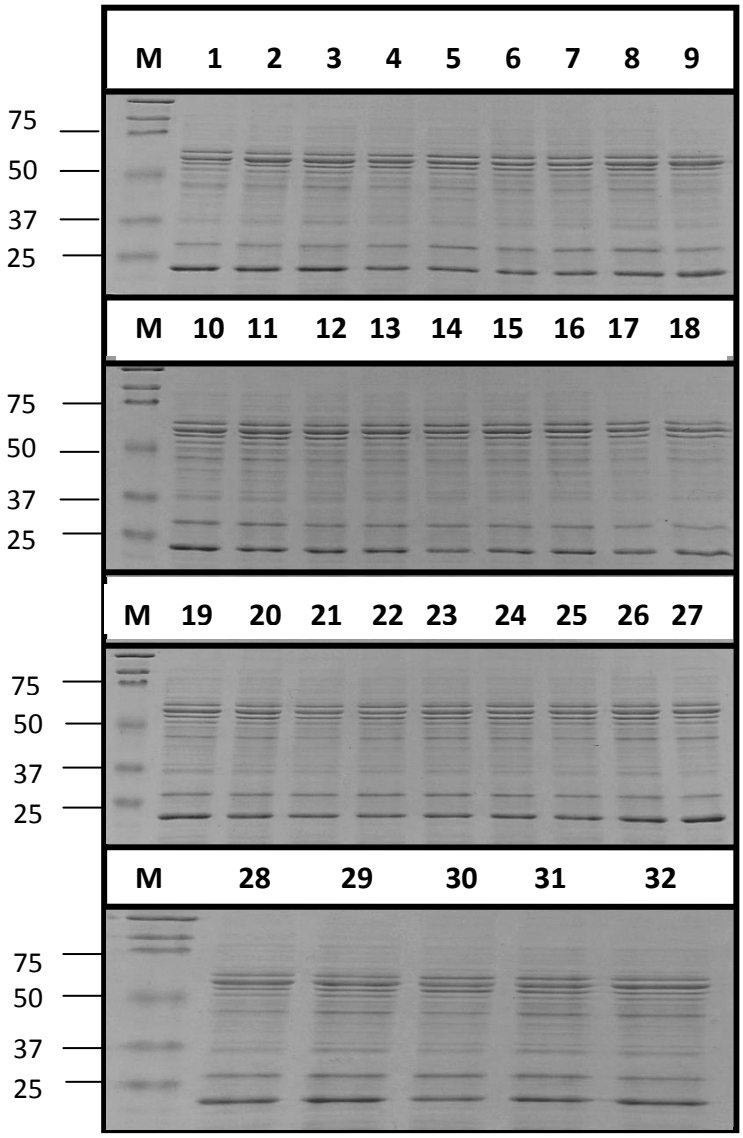


Figure 5.3 SDS-PAGE Analysis of *H. pylori* mutant periplasmic proteins

Typically, 10 µl of the periplasmic protein samples standardised in SDS loading dye were loaded onto the gel along with molecular weight standard. A 12% denaturing gel was used. Molecular weights (in kDa) are indicated.
M, prestained marker (Precision Plus Protein Standard, BioRad); Lane 1-32, periplasmic proteins from *H. pylori* 26695, *galE* mutant, *H. pylori* 11637, mutant #1, 2, 4, 6, 7, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 25, 28, 30, 32, 35, 37, 38, 39, 40, and 41, respectively.

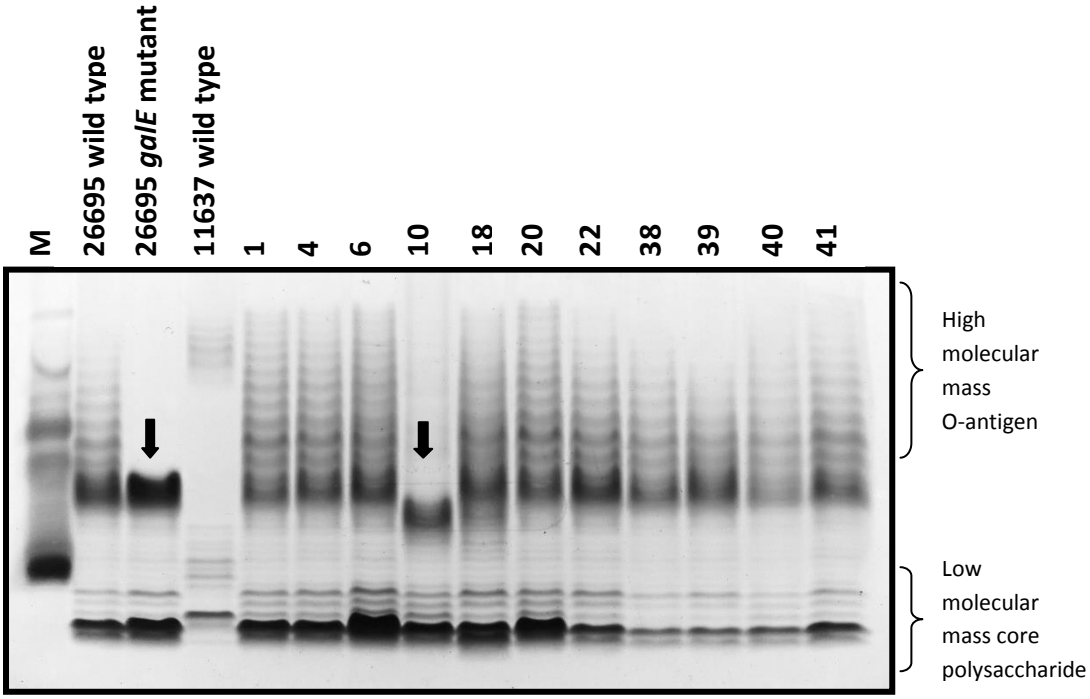
5.4.3 Profile of the lipopolysaccharide structure of *H. pylori* 26695, *galE* mutant and potato extract-resistant *H. pylori* mutants

From previous experiment carried out in this study, changes in the lipopolysaccharide (LPS) structure of *H. pylori* is thought to influence its sensitivity to potato extract (see Figure 3.5). Therefore, the LPS profile of the potato extract-resistant *H. pylori* mutant strains was analysed and compared to that of the wild type strain. The LPS was separated by T-SDS-PAGE and silver-stained as described in Section 2.4.

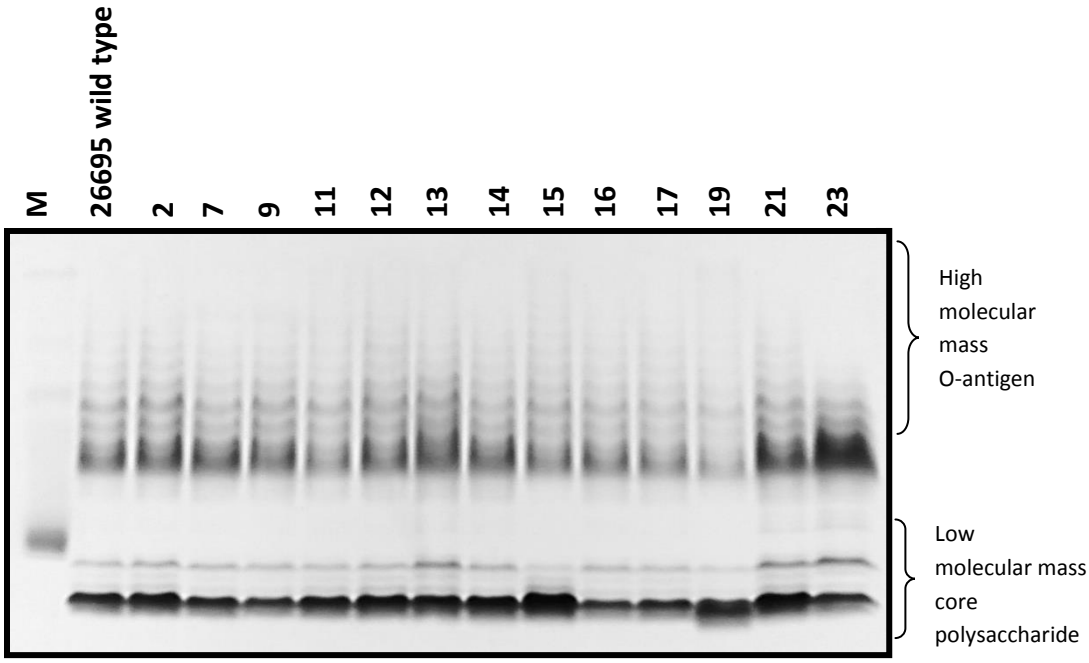
The potato extract-resistant mutant strains presented a similar O-antigen polysaccharide profile to that of the *H. pylori* 26695 wild type strain, while slight variations existed in the core polysaccharide profile (Figure 5.4). The variation is thought to be due to varying concentration of core polysaccharide in the LPS samples. The LPS of *H. pylori* 11637 strain was analysed to compare with that of *H. pylori* 26695 wild type and mutant strains. The result showed a significant variation in LPS profile, with the 11637 strain having a completely different profile to that of the 26695 strains (Figure 5.4A). One *H. pylori* 26695 mutant strain, mutant #10, generated from the chemical mutagenesis was also included in the analysis. Mutant #10 was initially resistant to potato extract, being able to grow on agar plates supplemented with potato extract. However, after the LPS analysis it was found that it had lost its O-antigen polysaccharide chain (Figure 5.4A). Considering the fact that loss of O-antigen chain in *H. pylori* 26695 *galE* mutant is thought to confer increased susceptibility to potato extract, mutant #10 was again screened for resistance to potato extract. The result showed that mutant #10 is unable to grow on agar supplemented with potato extract; thereby showing that it is indeed

sensitive to potato extract, and not resistant as previously observed (data not shown).

A)



B)



C)

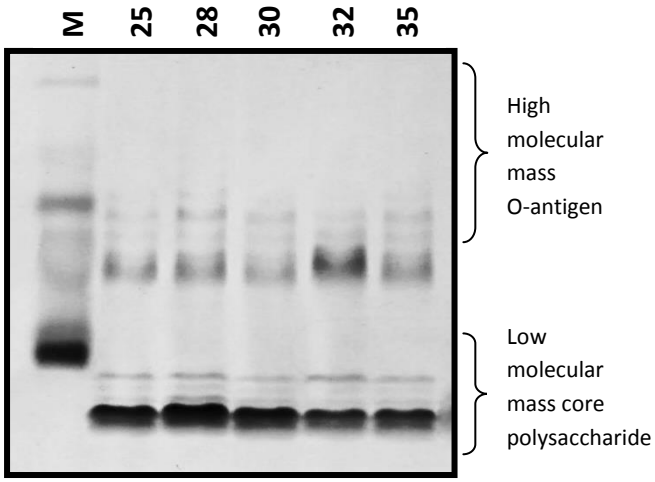


Figure 5.4 T-SDS-PAGE Analysis of LPS from *H. pylori* 26695, *galE* mutant, *H. pylori* 11637 strain and potato extract-resistant *H. pylori* mutant strains indicated

A, LPS profile of *H. pylori* 26695, *galE* mutant, *H. pylori* 11637 and potato extract resistant mutants indicated; **B**, LPS profile of *H. pylori* 26695 and potato extract resistant mutants indicated; **C**, LPS profile of potato extract resistant mutants indicated; **M**, prestained marker (Precision Plus Protein Standard, BioRad); **Mutant #10**, non-resistant *H. pylori* mutant. LPS extracted from each *H. pylori* strain was separated by T-SDS-PAGE and visualised by silver staining. The high molecular mass region consists of the O-antigen chains while low molecular region consists of the lipid-A-core. The black arrow indicates strains that have lost the O-antigen.

5.5 Sequence analysis of *H. pylori* mutants in comparison to wild-type strain

The analysis of the total membrane, periplasmic protein profile and lipopolysaccharide profile of the mutant strains in comparison to the wild type strain provided no insight into the mechanism of resistance of mutant *H. pylori* strains. Therefore, the complete genome of selected mutant strains and wild type strain was sequenced so as to identify mutations in the genome, common to all the mutant strains but absent in the wild type strain. This will provide insights into the possible mechanism by which the mutants developed resistance to potato extract. Mutant strains sequenced were selected based on similar phenotypic characteristic; the phenotype being strains that presented similar colonies on agar plates and grew at the same rate within 2-3 days. As such, 6 mutant strains were selected and their genome sequenced. The genome sequencing was carried out at Leiden Genome Technology Center, Netherlands. Following sequencing, genome sequence analysis was carried out to identify single nucleotide polymorphism (SNPs) present in all mutant strains but absent in the wild type strain. The genome sequences were analysed against *Helicobacter pylori* 26695 reference genome, NCBI Reference Sequence: NC_000915.1. The Bioinformatics Core Facility at the University of Manchester carried out the genome sequence analysis. All further analysis was carried using the Intergrative Genomics Viewer (Broad Institute) and Geneious software.

Results of the genome sequence analysis carried out by the Bioinformatics Core Facility are presented in Appendix II Table 5.2, showing SNPs present in the mutants and absent in the wild type strain; and Appendix II Table 5.3, showing SNPs present in both mutant and wild type strains. The SNPs of interest are those occurring in all 6 mutant genomes but absent in the wild

type lab strain. Five SNPs were identified in the following locations: 1344891, 639930, 1643821, 1643822 and 1643824 (Appendix II Table 5.2). Details of the mutations, that is, base/amino acid change and gene in which the SNPs occur are presented in Appendix II Table 5.2.

SNP occurring at position 1344891 is located within gene HP1272 which encodes NADH-ubiquinone oxidoreductase subunit M. This SNP induces an amino acid change from serine to isoleucine (S27I). BLAST analysis of the genomic DNA of this gene in the NCBI database indicated presence in 76 *H. pylori* strains, *H. acinonychis* (sheeba strain), and *H. cetorum* (MIT 99-5656 strain). The genomic DNA sequence of HP1272 has 91 % similarity to the *nuoM* gene, encoding NADH dehydrogenase I in *H. acinonychis*; and 83 % similarity to the gene HCD00465, encoding NADH-ubiquinone oxidoreductase subunit 4 (chain M) in *H. cetorum*. It is interesting to note that *H. acinonychis* is the most susceptible to potato extract out of all the other bacteria strains of *Helicobacter* and *Campylobacter* species previously screened for susceptibility to potato extract (Figure 3.6).

SNP occurring at position 639930 is located within gene HP0603 which is identified as a predicted coding region/hypothetical protein. It induces an amino acid change from tryptophan to glycine (W100G). The BLAST analysis of the genomic DNA of this gene in the NCBI database indicated presence in 77 *H. pylori* strains and in *H. acinonychis* (sheeba strain). The genomic DNA sequence of HP0603 is 88 % identical to the geneHac1400, encoding a conserved hypothetical protein in *H. acinonychis.* Further analysis of the DNA and protein sequence of HP0603 using EMBL, NCBI and ExPASy online tools such as UniProt, InterPro, and Conserved domain database indicated that this protein is uncharacterised with no detected putative conserved domains, protein family, molecular function or cellular component. Also, no

signal peptide or transmembrane helix, which provides insights into the localisation of a protein within the cell, was detected for this protein HP0603. Therefore, we are unable to predict the possible location or function of this protein. In-depth details of the physical properties and cellular localisation analysis carried out on HP0603 are presented in Appendix III.

SNPs occurring between positions 1643821-4 are located in an intergenic region between genes HP1561 and HP1562 which both encodes iron (III) ABC transporter substrate binding protein (ceuE). The SNPs occur between 40 - 43 bp downstream HP1561 and 157 - 160 bp upstream HP1562, and are unlikely to affect gene activity since no protein target is involved.

Furthermore, SNPs occurring in 4 – 5 of the mutant strains were also analysed in NCBI. These are SNPs at position 895934, 815419 and the cluster of SNPs between positions 775013-18. SNP at position 895934 occurs in HP0844 encoding thiamine biosynthesis protein. BLAST analysis of shows that the genomic DNA sequence of HP0844 has alignment with sequences in 74 *H. pylori* strains and in *H. cetorum* (85 % similarity), but absent in *H. acinonychis*. SNP at position 815419 occur in a pseudogene HP0760. BLAST analysis reveals alignment in 76 *H. pylori* strains, *H. acinonychis* (91 % similarity), and in two *H. cetorum* strains (MIT 99-5656 and MIT 00-7128 strain, with 84 and 79 % similarity respectively). The cluster of SNPs around 775013-8 hit a pseudogene HP0722 which is annotated as a putative outer membrane protein *omp16*, with unknown function; and BLAST analysis reveals significant alignment of this gene sequence in *H. pylori* strains only.

5.6 Discussion

5.6.1 Antibacterial activity of potato extract and antibiotics against *H. pylori* resistant mutants

MIC studies of potato extract against selected *H. pylori* resistant mutants, generated by chemical mutagenesis with ethyl methanesulfonate (EMS), showed that the mutants gradually became resistant to the extract starting from 3.9 mg/ml to 0.12 mg/ml (Figure 5.1). The wild type strain remained susceptible to the extract at the same concentration, exhibiting approx. 31 % killing at 0.12 mg/ml (Figure 5.1). Based on the result showing susceptibility of the wild type strain to potato extract at 0.12 mg/ml, it can be predicted that under normal conditions, potato extract will totally lose activity against *H. pylori* at approx. 0.03 mg/ml. In light of this hypothesis, the present assay showed a decrease in susceptibility of *H. pylori* resistant mutants strains by over 32-fold in comparison to the wild type strain. This represents a huge difference in susceptibility of the wild type strain and the resistant mutants to potato extract, and thus confirms the development of resistance in *H. pylori* strains subjected to chemical mutagenesis.

Furthermore, the susceptibility of the resistant mutant strains to antibiotics, ampicillin, metronidazole and tetracycline, was evaluated in comparison to the wild type strain. A varied pattern of increase and decrease in susceptibility was observed among the mutants, with some becoming more susceptible to all antibiotics and others being more or less susceptible to one or two of the antibiotics (Table 5.1). *H. pylori* is considered susceptible to ampicillin at ≥ 0.06 – 0.25 µg, tetracycline at ≥ 0.25 – 2 µg, and metronidazole at ≥ 0.5 – 8 µg (Table 1.1); thus, the lab strain 26695 used in this study is susceptible to all antibiotics tested (Table 5.1). It was observed that the MIC of mutant strains to the antibiotics

remained within the range of susceptibility. In addition to this, all the mutant strains except mutants 7 and 18 became more susceptible to metronidazole.

With the antibiotics all having different mode of action against *H. pylori*, it is difficult to establish/determine the "culprits" responsible for the susceptibility pattern observed. However, by studying/comparing the genome sequence analysis of mutant #1, 4, 6, 18, 20 and 40 (Appendix II Table 5.2 and 5.3) and their relative susceptibility pattern to the antibiotics, it is possible to arrive at a logical conclusion. The genes associated with the mode of action for each antibiotic and those responsible for conferring resistance (as reviewed in section 1.6.2) were carefully investigated in the genome sequence analysis data in order to detect whether mutation in these genes were present. It is thought that presence of SNPs in these genes will give an insight into the observed variation in susceptibility of the mutant strains to antibiotics. However, no such mutation was observed except in mutant #40 which has a point mutation within its penicillin binding protein 1A (PBP1A) gene, and is more susceptible to ampicillin compared to the wild type strain. PBPs are integral to the function of penicillin-like antibiotics such as ampicillin (DeLoney and Schiller, 1999) and point mutations in the PBP1A gene have been implicated in conferring antibiotic resistance (Co and Schiller, 2006). Thus, the SNP observed in PBP1A gene of mutant 40 may be responsible for its increased susceptibility to ampicillin; although no PBP1A mutation was observed in other mutants that became more susceptible to ampicillin. Furthermore, it was observed that each mutant strain had several mutations in their individual genome that was absent in the other mutant strains. This is thought to have an impact on the differences in susceptibility of each mutant strain to the antibiotics tested. Therefore, a reasonable conclusion

is that multiple mutations in each mutant genome played a role in the susceptibility of the mutant strains to the antibiotics tested.

5.6.2 Evaluation of *H. pylori* resistant mutant membrane proteins and LPS profile

Antimicrobial agents, especially from plant extracts have been shown to disrupt bacteria cell membrane as a means to increase cell permeability leading to the disruption of many cellular processes within the cell (Sanchez et al., 2010; Nazzaro et al., 2013). The cell membrane provides a permeability barrier that controls cell homeostasis; therefore, any injury to it will destabilise cellular function and ultimately lead to cell death. In addition to this, results of experiments in this study have shown that the cell membrane is a crucial organelle in the antibacterial activity of potato extract against *H. pylori*.

As most of the membrane function is mediated by proteins, the lipopolysaccharide, the membrane and periplasmic proteins from *H. pylori* resistant mutants was compared to that of the wild type in order to identify any contrast. SDS-PAGE analysis showed no distinct differences in the profiles assessed, that is the wild type and resistant mutants had similar protein and LPS profile. With respect to the LPS profile, this observation suggests that the LPS components may not be directly involved in conferring resistance to potato extract on *H. pylori*. The similarity observed in the membrane and periplasmic protein profiles is thought to be due to the fact that mutations conferring resistant in the *H. pylori* mutants did not lead to a complete deletion or inexpression of the protein(s) encoded in the mutated gene(s). Since, no stop codon was observed in the mutated gene sequences. As such, a two-dimensional gel electrophoresis may be employed in future so as to determine the relative abundance of each

mutant membrane protein in comparison to that of the wild type. Lastly, it is a possibility that the genes conferring resistance do not encode proteins present in the cell membrane; although results in this study suggests that potato extract acts by interfering with the cell wall/membrane of *H. pylori*.

5.6.3 Analysis of whole genome sequences of *H. pylori* mutant strains resistant to potato extract

The whole genome of six *H. pylori* resistant mutant strains, selected on the basis of their rapid growth on agar supplemented with potato extract was sequenced. Analysis of the sequenced genome involved identifying SNPs that are present in all 6 mutants but absent in the wild type strain. The SNPs identified occur in gene HP1272, HP0603 and in the intergenic region between gene HP1561 and HP1562. Since the SNPs in the intergenic region do not encode proteins, HP1272 and HP0603 were identified as highly probable genes involved in inducing resistance.

HP1272 encodes NADH-ubiquinone oxidoreductase subunit M, which is a component of respiratory enzyme, NADH-ubiquinone oxidoreductase (Complex I) (Leif et al., 1995). Complex I is the first of many respiratory chain complexes that generates the proton motive force used for energy utilizing processes such as ATP synthesis (Friedrich and Scheide, 2000). It catalyses the transfer of electrons from NADH to ubiquinone while serving as a proton pump translocating protons across the cytoplasmic membrane (Smith et al., 2000; Leif et al., 1995). Basically, the oxidation of NADH to NAD^+ contributes to the creation of the proton gradient that generates the proton motive force which in turn drives processes such as ATP synthesis.

$$\text{NADH} + \text{Ubiquinone} + 5H^+_N \longrightarrow NAD^+ + \text{Ubiquinol} + 4H^+_P$$

Complex I, found in all subdivisions of proteobacteria is made up of 14 genes clustered in a conserved order and known as the *nuo* genes (A-N) (Smith et al., 2000). Subunit M encoded in HP1272 is one of the *nuo* genes. This explains the presence of similar genomic DNA sequence for *H. pylori* HP1272 in *H. acinonychis* and *H. cetorum*. The NADH-complex I is poorly studied in *H. pylori* hence it is difficult to identify the exact function of subunit M in which SNPs common to all *H. pylori* resistant mutant was found; as well as predicting its phenotype. However, in *E. coli* in which Complex I is well characterised, subunit M is identified as an integral membrane protein (Leif et al., 1995) predicted to be involved in H^+ translocation (Friedrich, 1998) and required for the correct assembly of NADH complex I (Torres-Bacete et al., 2011). Torres-Bacete et al (2011) showed that the deletion of subunit M resulted in the partial assembly of complex I and total loss of its activity. Smith et al. (2000) has suggested that the presence of similar respiratory chain structures in *H. pylori* and *C. jejuni* may mean that the respiratory complexes are important for their growth and survival under microaerophilic conditions. Therefore, inhibition of all or part of the respiratory complex function may have bactericidal or bacteriostatic effects on *H. pylori*; and this may be the mechanism by which potato extract affects *H. pylori*.

It was observed that all the resistant mutants sequenced except one had increased susceptibility to metronidazole (Table 5.1), a nitro-imidazole that needs to accept electrons from an electron transport protein for its activity (Edwards, 1980). The rate of diffusion of this antibiotic into the cell is enhanced by the rate of intracellular reduction (Ings et al., 1974). Thus, since complex I is an electron transporter, this observation suggests that it may play a role in the antibacterial activity of metronidazole and a mutation in the complex specifically subunit M shown to be required to

maintain its function, may impact on reduction of metronidazole and its consequent antibacterial activity.

Proton translocation which is a predicted function of HP1272 subunit M, provides the proton motive force that drives ATP synthesis. However, in our experiments, potato extract did not significantly affect ATP concentration (Figure 4.15). This may be due to the presence of other respiratory complexes driving ATP synthesis in *H. pylori*(Marais et al., 1999). Basically, how or if this gene HP1272 is involved in inducing resistant to potato extract remains to be determined.

The second gene identified to be possibly involved in the resistance of *H. pylori* to potato extract is HP0603. All the mutants sequenced had a SNP in this gene that was not present in the wild type. The SNP caused a change in amino acid from tryptophan to glycine (W100G), which is a major change in the protein considering the fact that glycine being the smallest of the 20 amino acids has a single hydrogen substituent in its side chain while tryptophan has an aromatic ring system. Thus, this amino acid change is considered capable of disrupting the protein function.

HP0603 is a predicted coding region encoding a hypothetical protein that is uncharacterised, having no known conserved domains, molecular function or homologue in the protein database. As such, it is difficult to determine how this protein may impact on the resistance of *H. pylori* to potato extract.

A recent study has carried out a structural annotation of the proteome of *H. pylori* 26695. Using computational methods, Singh et al (2014) studied the three-dimensional (3D) structural organisation of each protein so as to determine the protein fold, structure and active site, and thus predict

protein function. They predicted that HP0603 is outer membrane protein H1 (Singh et al., 2014).

As studied in *Pseudomonas aeruginosa*, outer membrane protein H1 (OprH) is a divalent cation-regulated protein that provides increased stability to the outer membrane by interacting with the LPS molecules (Edrington et al., 2011). It has been implicated in inducing multiple drug resistance in *P. aeruginosa* (Nicas and Hancock, 1980). It was observed that *P. aeruginosa* strains cultured in Mg^{2+} deficient medium acquired non-mutational resistance to antibiotic polymyxin B, aminoglycosides and EDTA (Gilleland et al., 1974; Brown and Melling, 1969; Shand et al., 1988); and also featured an increased expression of OprH (Nicas and Hancock, 1980). The LPS is sustained by interactions between divalent cations such as Mg^{2+}, and negatively charged ions in the lipid A and core regions (Nikaido and Vaara, 1985). This interactions can be destroyed by the removal of the divalent cations by EDTA or displacement by polymyxin B (Bell and Hancock, 1989). Thus, it is suggested that OprH replaces divalent cations (Mg^{2+}) on the LPS binding site of strains grown in Mg^{2+} deficient medium thereby protecting this site from EDTA and antibiotic treatment (Nicas and Hancock, 1980; Bell and Hancock, 1989).

Little is known of the exact analysis carried out by Singh et al (2014) for which HP0603 is annotated as outer membrane protein H1; thus, we are unable to validate this annotation. However, going by the above review of OprH being associated with the LPS and involved in inducing drug resistance in *P. aeruginosa*, these characteristics can be related to the activity of potato extract. In this study, it has been shown that LPS plays a role in the activity of potato extract, where we showed that *H. pylori* 26695 *galE* mutant strain which produces a truncated LPS structure is more

susceptible to the extract (Figure 3.5). Therefore, it is hypothesised that the protein(s) involved in inducing resistance to potato extract may interact with the LPS.

Amino acid sequence of OprH shows that it is a 21 kDa slightly basic polypeptide made up of 178 residues (18 basic residues, 2 histidines and 17 acidic residues), having little similarity to other bacteria surface proteins of known sequences (Bell and Hancock, 1989). In comparison, HP0603 is a 15.6 kDa basic polypeptide of 141 residues, having 16 basic residues, 1 histidine and 7 acidic residues. Upon further analysis of the SDS-PAGE of total membrane proteins of the resistant mutants (see Figure 5.2), protein bands representing molecular weight of HP0603 (15.6 kDa) was detected and this band was present in all of the mutant strains including the wild type. Signal peptide and transmembrane analysis, to predict protein localisation, confirmed that OprH is a membrane protein (result not shown) while localisation of HP0603 remains unknown. By comparing OprH and HP0603, we are unable to confirm the possibility that HP0603 is outer membrane protein H1 as predicted by Singh et al (2014). Based on the magnitude of the amino acid change induced by the SNP in HP0603 (W100G- from tryptophan to glycine), we predict that the function of this protein would be altered, either becoming non-functional or inducing a different function/phenotype. However, more investigative analysis and experiments need to be carried out to determine if HP0603 is involved in inducing resistance of *H. pylori* to potato extract.

It is important to note that in addition to SNPs common to some or all of the mutants, each mutant strain have genomic mutations unique to it; that is, SNPs absent in the other mutants and the wild type strain (Appendix II Table 5.2). Also, each mutant strain has genomic mutations that are

present in the wild type strain and in other mutant strains (Appendix II Table 5.3). It is thought that a combination of these different mutations may also impact on the susceptibility of the strains to potato extract and to the antibiotics tested. However, new investigative experiments need to be carried out to determine the exact gene(s) responsible for conferring resistance to potato extract on *H. pylori*.

Overall, in this chapter, *H. pylori* strain 26695 was subjected to chemical mutagenesis and mutant strains resistant to potato extract were generated. However, the exact mechanism of resistance is yet to be elucidated.

Chapter 6

General Discussion

Eradication of *H. pylori* and treatment of its associated diseases has proven difficult due to the continuous emergence of antibiotic resistance among *H. pylori* isolates. As a result, numerous research investigations into alternative therapies for successful and complete eradication of *H. pylori* are currently underway. Alternative therapies from plant sources are the most widely researched due to the promising results of plant bioactive compounds against infectious microbes. Although many research studies and publications have proved the bioactivity of plants extracts and their compounds, most have failed to identify the mechanism of action of these compounds against microbes. This is due to the fact that plant antimicrobial research is largely focused on antimicrobial activity, with little attention given to how the plant extracts exhibit these activities. With the identification of antibacterial activity in potato extract against *H. pylori*, this study aims to investigate its mode of action within the cell and its gene target(s) within the genome.

Data presented demonstrate that potato extract is bactericidal against *H. pylori*, while showing no activity against *E. coli*. Antibacterial activity was also extended to *H. acinonychis* and *H. felis*, both of which are closely related to *H. pylori* by phylogenetic analysis (see Figure 1.1), while showing little to no activity towards other *Helicobacter*, *Campylobacter* and *Wolinella* species tested. Therefore, indicating that potato extract has specificity for *H. pylori*.

Bactericidal activity of potato extract towards clinical antibiotic resistant *H. pylori* isolates suggest that it can serve as an alternative treatment regimen

for eradicating strains that have become difficult to kill as a result of resistance to multiple antibiotics. However, it may not be effective in combination therapies with antibiotics; as in vitro assays testing its synergy with antibiotics showed minimal activity in the reference lab strain and no synergistic activity in the clinical isolates.

Disruption of *H. pylori* cell morphology observed by transmission electron microscopy suggests that potato extract's first point of engagement, where it elicits its toxic action, is the cell envelope. This change in morphology, characterised by the detachment of the outer membrane from the plasma membrane, suggests peptidoglycan disruption since the peptidoglycan crosslinking is mostly responsible for maintaining cell shape in *H. pylori* (Sycuro et al., 2010). Fluorescence microscopy shows membrane damage in cells treated with potato extract; thus, further confirming the hypothesis that potato extract's initial point of toxic contact is the cell envelope. Higher susceptibility of *H. pylori galE* mutant (which produces a truncated LPS) to potato extract also supports this hypothesis. Membrane damage to *H. pylori* cells may have led to cell death due to loss of cellular homeostasis. It was quite intriguing that potato extract induced plasma membrane hyperpolarisation in *H. pylori* cells despite indicating membrane damage. However, this membrane hyperpolarisation is not considered to be a killing effect of potato extract because it was also observed in cells treated with low concentrations of potato extract that neither induces membrane damage nor cell death. Bio-imaging observations of *H. pylori* cells treated with potato extract led to the hypothesis that the anti-*H. pylori* activity of potato extract follow sequential events presented in Figure 6.1.

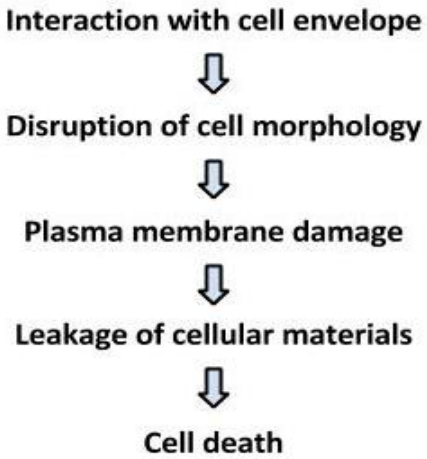


Figure 6.1 Hypothesized sequence of events carried out by potato extract against *H. pylori*

Following treatment of *H. pylori* cells with potato extract, potato extract first interacts with the outer membrane; it then disrupts the cell morphology and induces membrane damage, leading to cell death. In addition, alteration of intracellular processes not detectable in bio-imaging may also contribute to cell death.

Analysis of mutant *H. pylori* strains that are resistant to potato extract for antibiotic susceptibility demonstrate that the mutants became more or less susceptible to antibiotics; the mechanism of which is yet to be identified. The mutants remained within the susceptibility value range for each antibiotic tested. It is hypothesised that the presence of multiple mutations within individual mutant genome contributed to this phenotype. Analysis of mutant cell membrane profiles in comparison to that of the wild type did not provide any insights into identifying the cellular target of potato extract. Sequence analysis of mutant genome to identify SNPs common to all mutants as a means to identify the mutation conferring resistance to potato extract resulted in the identification of 2 possible gene(s) of interest. These are; HP1272- NADH ubiquinone oxidoreductase subunit M, and HP0603- predicted hypothetical protein. HP1272, being an integral component of an oxidoreductase, is thought to be involved in the activity

of metronidazole against the mutant *H. pylori* strains, as all but one mutant became more susceptible to the antibiotic. In terms of potato extract bioactivity, it is not known if this gene is involved, as our hypothesis (based on observations in this study) is that potato extract interacts with *H. pylori* cell membrane.

On the other hand, HP0603 is a hypothetical protein; and all the protein prediction methods employed to characterise and predict its cellular location/function did not provide any conclusive result. Thus, it is unknown whether this protein is involved in inducing resistance to potato extract. It is thought that the presence of several mutations common to the mutants and those unique to each mutant contribute to their resistance to potato extract and also to their individual phenotype.

Future work will need to be carried out in order to determine whether the identified genes are involved in inducing resistance to potato extract, and to also identify the target of potato extract within the *H. pylori* genome. This will involve genetic manipulation of *H. pylori* 26695 wild type strain to induce the exact mutation present in the resistant mutant strains so as to generate HP0603 and HP1272 isogenic mutants. The DNA of the newly mutated strain will then be used to transform another *H. pylori* wild type strain, which will then be tested for susceptibility to potato extract. Transfer of potato extract resistance to the transformed strains will confirm the involvement and provide insights into the role of these genes in the antibacterial activity of potato extract.

Ultimately, the active components in potato extract need to be identified so as to ease the identification of the mechanism of action of potato extract. Previous work has been carried out by a neutraceutical company, Provexis Plc that is focused on commercialisation of functional foods and

dietary supplements, in order to identify the active compounds in potato extract. However, their research did not yield a conclusive/positive result. As such, future work needs to be carried out in this area so as to identify and characterise the active compounds in the extract.

As reviewed by Nazzaro et al (2013), mechanism of action of potato extract is said to be dependent on chemical composition of the plant bioactive compounds. However, in the case of potato extract, the bioactive compounds are yet to be identified and this posed a limitation to this study. Irrespective of this setback, this research focused on understanding the mechanism of action of potato extract against *H. pylori*. Potato extract has specific bactericidal activity against *H. pylori* as well as clinical antibiotic resistant isolates. Anti-*H. pylori* activity is hypothesised to be mediated by cell membrane damage, and a series of potato extract bioactivities leading to cell death is proposed (Figure 6.1). Specific gene(s) involved in mediating bactericidal activity are yet to be identified; however, it is proposed that anti-*H. pylori* activity may be as a result of several reactions involving several genes and/or the entire bacterial cell.

In summary, this study provides a clearer understanding of the antibacterial activity of potato extract as well as its mode of action against *H. pylori*. It emphasises the practical value of potato extract in therapeutic applications for the treatment of *H. pylori* infection.

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Appendix I TEM analysis of *H. pylori* (higher magnification)

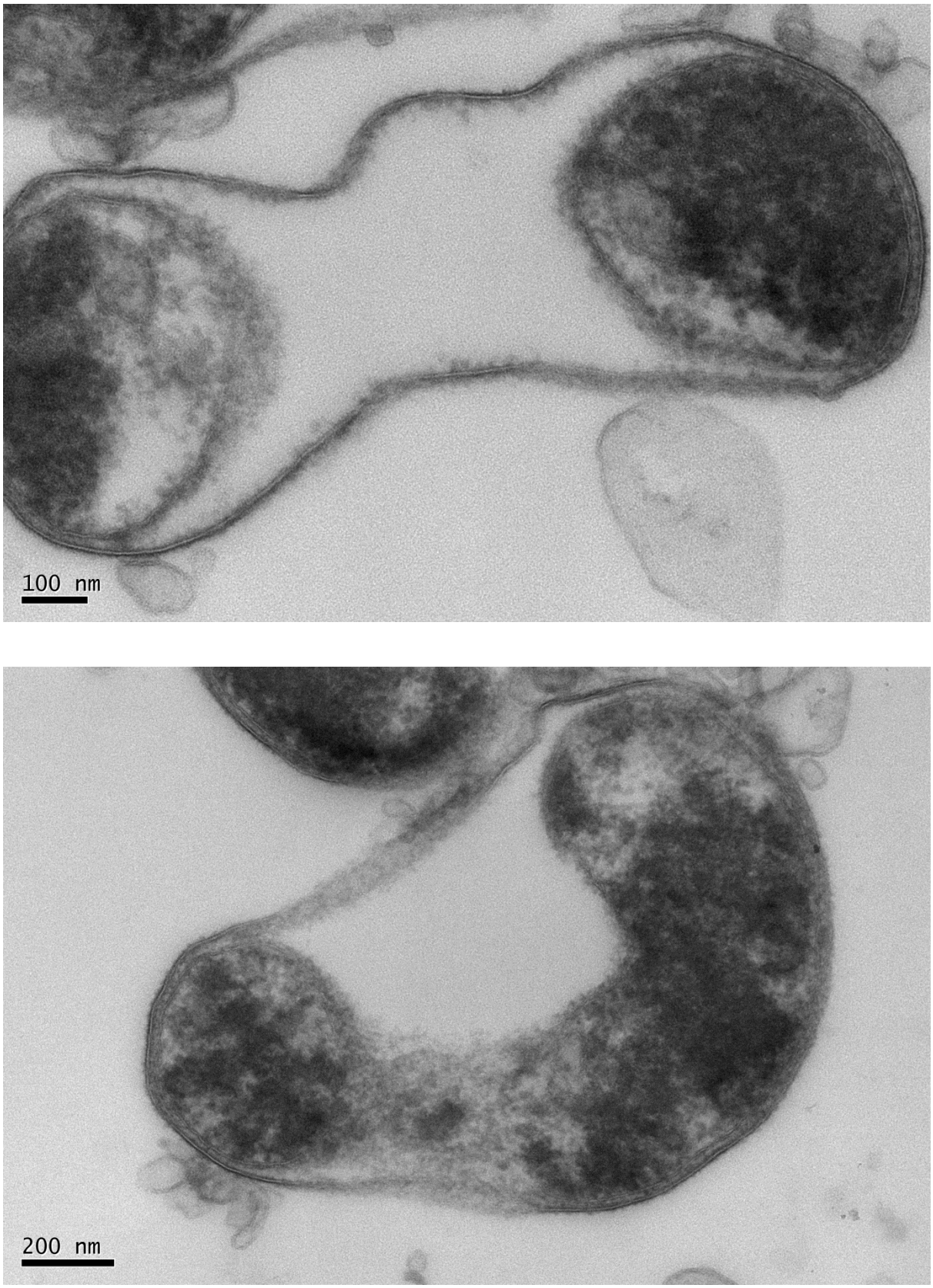

Figure 4.8.1 Transmission electron micrographs, showing the effect of potato extract on *H. pylori* cell following treatment for 60 min (≥ 13000x magnification)

Appendix II Genome sequence analysis of *H. pylori* 26695 wild type strain and potato extract-resistant mutants

Table 5.2 Mutational changes in potato extract-resistant *H. pylori* mutant strains, absent in the wild type strain

SNP	No. of Mutants SNP occur	Type of mutation (amino acid/ base change)	Gene Description	Mutant #
1344891T	6	HP_1272 S27I (aGc/aTc)	NADH-ubiquinone oxidoreductase, NQO13 subunit (NQO13)	1, 18, 20, 4, 40, 6
639930G	6	HP_0603 W100G (Tgg/Ggg)	predicted coding region HP0603	1, 18, 20, 4, 40, 6
1643821C	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1643822C	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1643824T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
895934A	5	HP_0844 G17V (gGt/gTt)	thiamine biosynthesis protein (thi)	1, 18, 20, 4, 40
815419T	4	Intergenic	N/A	20, 4, 40, 6
775015T	4	Intergenic	N/A	18, 20, 40, 6
775018G	4	Intergenic	N/A	18, 20, 40, 6
775017T	3	Intergenic	N/A	18, 20, 40
775013A	3	Intergenic	N/A	18, 20, 40
775016T	3	Intergenic	N/A	18, 20, 40
1317485A	3	Intergenic	N/A	18, 40, 6
1272488G	2	HP_1198 Y1521S (tAt/tCt)	DNA-directed RNA polymerase, beta subunit (rpoB)	1, 4

927376T	2	Intergenic	N/A	40, 6
323504T	1	HP_0305 T115M (aCg/aTg)	predicted coding region HP0305	1
426532T	1	HP_0413 G103S (Ggc/Agc)	transposase-like protein, PS3IS	1
1381829T	1	HP_1321 T150I (aCc/aTc)	conserved hypothetical ATP-binding protein	1
276808T	1	HP_0266 A276V (gCt/gTt)	dihydroorotase (pyrC)	1
25158A	1	HP_0025 P101L (cCg/cTg)	outer membrane protein (omp2)	1
572445T	1	HP_0540 G94D (gGc/gAc)	cag pathogenicity island protein (cag19)	1
1399809T	1	HP_1340 L93F (Ctt/Ttt)	biopolymer transport protein (exbD)	1
1062845A	1	HP_1000 synonymous	PARA protein	1
1417865A	1	HP_1355 R9C (Cgc/Tgc)	nicotinate-nucleotide pyrophosphorylase (nadC)	1
1055070A	1	HP_0994 synonymous	predicted coding region HP0994	1
462181A	1	HP_0443 synonymous	predicted coding region HP0443	1
1506001T	1	HP_1433 S3N (aGc/aAc)	predicted coding region HP1433	1
316644T	1	HP_0298 P354S (Cct/Tct)	dipeptide ABC transporter, periplasmic dipeptide-binding protein (dppA)	1
813456T	1	HP_0759 synonymous	conserved hypothetical integral membrane protein	1
340632T	1	HP_0324 S33N (aGc/aAc)	outer membrane protein (omp10)	1
1602794T	1	HP_1523 R389H (cGc/cAc)	DNA recombinase (recG)	1
1461690A	1	HP_1400 S87N (aGc/aAc)	iron(III) dicitrate transport protein (fecA)	1
45076T	1	HP_0047 V322M (Gtg/Atg)	hydrogenase expression/formation protein (hypE)	1
1114260T	1	HP_1051 synonymous	predicted coding region HP1051	1

1233011T	1	HP_1167 R137C (Cgc/Tgc)	predicted coding region HP1167	1
985232T	1	HP_0922 T1934I (aCt/aTt)	toxin-like outer membrane protein	1
563595T	1	HP_0530 R134K (aGa/aAa)	cag pathogenicity island protein (cag10)	1
499056T	1	HP_0477 P13L (cCt/cTt)	outer membrane protein (omp12)	1
797310A	1	HP_0742 synonymous	phosphoribosylpyrophosphate synthetase (prsA)	1
948644T	1	HP_0896 A355T (Gcg/Acg)	outer membrane protein (omp19)	1
1281856T	1	HP_1205 R8K (aGa/aAa)	translation elongation factor EF-Tu (tufB)	1
728466T	1	HP_0679 synonymous	lipopolysaccharide biosynthesis protein (wbpB)	1
136605A	1	HP_0123 synonymous	threonyl-tRNA synthetase (thrS)	1
1466241T	1	HP_1402 R499Q (cGa/cAa)	type I restriction enzyme R protein (hsdR)	1
292894A	1	HP_0284 synonymous	conserved hypothetical integral membrane protein	1
1273716T	1	HP_1198 D1112N (Gat/Aat)	DNA-directed RNA polymerase, beta subunit (rpoB)	1
1345888T	1	HP_1272 synonymous	NADH-ubiquinone oxidoreductase, NQO13 subunit (NQO13)	1
894580T	1	HP_0843 S200N (aGc/aAc)	thiamin phosphate pyrophosphorylase/hyroxyethylthiazole kinase (thiB)	4
1018800A	1	HP_0960 A191V (gCa/gTa)	glycyl-tRNA synthetase, alpha subunit (glyQ)	4
219360A	1	HP_0212 S297L (tCg/tTg)	succinyl-diaminopimelate desuccinylase (dapE)	4
1198836A	1	HP_1136 S127F (tCt/tTt)	ATP synthase F0, subunit b (atpF)	4
1084279A	1	HP_1021 V50I (Gtt/Att)	response regulator	4
1526129A	1	HP_1455 S45L (tCa/tTa)	predicted coding region HP1455	4
1299916T	1	HP_1222 synonymous	D-lactate dehydrogenase (dld)	4

1076826A	1	HP_1012 A428T (Gcc/Acc)	protease (pqqE)	4
752833A	1	HP_0701 D108N (Gat/Aat)	DNA gyrase, sub A (gyrA)	4
651249A	1	HP_0610 G88E (gGg/gAg)	toxin-like outer membrane protein	4
937543A	1	HP_0886 V251M (Gtg/Atg)	cysteinyl-tRNA synthetase (cysS)	4
591400A	1	Intergenic	N/A	4
1365015A	1	HP_1289 A96T (Gcg/Acg)	predicted coding region HP1289	4
1264647A	1	HP_1193 synonymous	aldo-keto reductase, putative	4
329328A	1	HP_0313 synonymous	nitrite extrusion protein (narK)	4
1211775A	1	HP_1148 P175L (cCt/cTt)	tRNA (guanine-N1)-methyltransferase (trmD)	4
1202560T	1	HP_1141 synonymous	methionyl-tRNA formyltransferase (fmt)	4
350226A	1	HP_0338 synonymous	predicted coding region HP0338	4
631716A	1	HP_0597 synonymous	penicillin-binding protein 1A (PBP-1A)	4
77102A	1	HP_0072 synonymous	urease beta subunit (urea amidohydrolase) (ureB)	4
23889T	1	HP_0025 R524H (cGc/cAc)	outer membrane protein (omp2)	4
1134476A	1	HP_1075 P259L (cCt/cTt)	conserved hypothetical secreted protein	4
700553A	1	HP_0654 synonymous	conserved hypothetical protein	4
1233080T	1	HP_1167 R160C (Cgc/Tgc)	predicted coding region HP1167	4
1187614A	1	HP_1121 synonymous	cytosine specific DNA methyltransferase (BSP6IM)	4
993883A	1	HP_0931 G124E (gGg/gAg)	predicted coding region HP0931	4
993906C	1	HP_0931 T132P (Acc/Ccc)	predicted coding region HP0931	4

727822A	1	HP_0677 A35V (gCa/gTa)	conserved hypothetical integral membrane protein	6
957101T	1	HP_0906 A206V (gCg/gTg)	predicted coding region HP0906	6
127930A	1	Intergenic	N/A	6
745581A	1	Intergenic	N/A	6
590758A	1	HP_0556 H102Y (Cat/Tat)	predicted coding region HP0556	6
860793A	1	HP_0806 C146Y (tGc/tAc)	predicted coding region HP0806	6
1403015C	1	Intergenic	N/A	6
1183462A	1	HP_1118 synonymous	gamma-glutamyltranspeptidase (ggt)	6
841581T	1	HP_0786 L668F (Ctt/Ttt)	preprotein translocase subunit (secA)	6
1597379T	1	HP_1521 synonymous	type III restriction enzyme R protein (res)	6
1429861A	1	HP_1368 synonymous	type IIS restriction enzyme M2 protein (mod)	6
1101761A	1	HP_1041 E279K (Gaa/Aaa)	flagellar biosynthesis protein (flhA)	6
927352T	1	Intergenic	N/A	6
1279568A	1	HP_1203 L152F (Ctc/Ttc)	transcription termination factor NusG (nusG)	6
251305A	1	HP_0240 L89F (Ctc/Ttc)	octaprenyl-diphosphate synthase (ispB)	6
1232922A	1	HP_1167 G107D (gGt/gAt)	predicted coding region HP1167	6
1225855A	1	HP_1159 D4N (Gac/Aac)	cell filamentation protein (fic)	6
1605098A	1	HP_1526 R227C (Cgc/Tgc)	exodeoxyribonuclease (lexA)	6
813357A	1	Intergenic	N/A	6
311905A	1	HP_0294 G295S (Ggt/Agt)	aliphatic amidase (aimE)	6

533017A	1	HP_0506 G343S (Ggc/Agc)	conserved hypothetical secreted protein	6
305553A	1	HP_0289 synonymous	toxin-like outer membrane protein	6
232565T	1	HP_0224 synonymous	peptide methionine sulfoxide reductase (msrA)	6
1024434T	1	HP_0965 A50T (Gct/Act)	predicted coding region HP0965	6
859101T	1	HP_0804 synonymous	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone 4-phosphate synthase (ribA, ribB)	6
806868A	1	HP_0752 G10E (gGg/gAg)	flagellar hook-associated protein 2 (fliD)	6
1492210A	1	HP_1422 A825V (gCc/gTc)	isoleucyl-tRNA synthetase (ileS)	6
553234A	1	HP_0525 synonymous	virB11 homolog	6
1382950T	1	Intergenic	N/A	18
373670A	1	Intergenic	N/A	18
1155903T	1	Intergenic	N/A	18
1251373T	1	Intergenic	N/A	18
259510A	1	Intergenic	N/A	18
147243A	1	Intergenic	N/A	18
1400005T	1	Intergenic	N/A	18
112014A	1	Intergenic	N/A	18
772846T	1	Intergenic	N/A	18
556920T	1	Intergenic	N/A	18
305237T	1	HP_0289 P2405L (cCc/cTc)	toxin-like outer membrane protein	18

506626T	1	Intergenic	N/A	18
1417864T	1	Intergenic	N/A	18
152262A	1	Intergenic	N/A	18
1284390A	1	Intergenic	N/A	18
519511A	1	Intergenic	N/A	18
188626T	1	Intergenic	N/A	18
422914T	1	Intergenic	N/A	18
911433T	1	Intergenic	N/A	18
1310225T	1	Intergenic	N/A	18
260198A	1	Intergenic	N/A	18
402080T	1	Intergenic	N/A	18
436592T	1	Intergenic	N/A	18
1112511T	1	Intergenic	N/A	18
387681T	1	Intergenic	N/A	18
450412T	1	Intergenic	N/A	18
467854A	1	Intergenic	N/A	18
969713A	1	Intergenic	N/A	18
153966T	1	HP_0142 G250R (Ggg/Agg)	A/G-specific adenine glycosylase (mutY)	20
1543428T	1	Intergenic	N/A	20
250184A	1	HP_0239 H155Y (Cat/Tat)	glutamyl-tRNA reductase (hemA)	20

421652A	1	HP_0408 S7N (aGt/aAt)	predicted coding region HP0408	20
383973T	1	Intergenic	N/A	20
1443502T	1	HP_1379 P369S (Cct/Tct)	ATP-dependent protease (lon)	20
1243222A	1	Intergenic	N/A	20
657572T	1	HP_0612 synonymous	predicted coding region HP0612	20
1505851A	1	HP_1433 A53V (gCt/gTt)	predicted coding region HP1433	20
582651A	1	HP_0547 A911T (Gct/Act)	cag pathogenicity island protein (cag26)	20
685833T	1	HP_0639 synonymous	conserved hypothetical protein	20
1286599A	1	HP_1209 synonymous	ulcer-associated gene restriction endonuclease (iceA)	20
122987T	1	HP_0115 synonymous	flagellin B (flaB)	20
1188599T	1	Intergenic	N/A	20
340586A	1	HP_0324 synonymous	outer membrane protein (omp10)	20
314391A	1	Intergenic	N/A	20
535877A	1	HP_0509 A223T (Gcg/Acg)	glycolate oxidase subunit (glcD)	20
1234929A	1	HP_1168 synonymous	carbon starvation protein (cstA)	20
529398A	1	HP_0501 synonymous	DNA gyrase, sub B (gyrB)	20
645567T	1	HP_0607 P703L (cCg/cTg)	acriflavine resistance protein (acrB)	20
927369T	1	Intergenic	N/A	20
205497A	1	HP_0199 G73R (Ggg/Agg)	predicted coding region HP0199	20
112247A	1	HP_0104 P143S (Ccg/Tcg)	2',3'-cyclic-nucleotide 2'-phosphodiesterase (cpdB)	20

301545A	1	HP_0289 synonymous	toxin-like outer membrane protein	20
1399137A	1	HP_1339 synonymous	biopolymer transport protein (exbB)	20
1436392T	1	HP_1372 synonymous	rod shape-determining protein (mreC)	20
1373041T	1	HP_1301 A11T (Gct/Act)	ribosomal protein L15 (rpl15)	20
253104A	1	HP_0244 synonymous	signal-transducing protein, histidine kinase (atoS)	20
329575A	1	HP_0313 S173F (tCc/tTc)	nitrite extrusion protein (narK)	20
310555A	1	HP_0293 A469T (Gcg/Acg)	para-aminobenzoate synthetase (pabB)	20
1199540A	1	HP_1137 synonymous	ATP synthase F0, subunit b' (atpF')	20
420995A	1	HP_0407 G640D (gGc/gAc)	biotin sulfoxide reductase (bisC)	20
632624A	1	HP_0597 R66C (Cgt/Tgt)	penicillin-binding protein 1A (PBP-1A)	40
1666400T	1	HP_1588 E131K (Gaa/Aaa)	conserved hypothetical protein	40
742345A	1	HP_0691 D201N (Gat/Aat)	3-oxoadipate coA-transferase subunit A (yxjD)	40
1607181A	1	HP_1527 A72V (gCa/gTa)	predicted coding region HP1527	40
620035A	1	Intergenic	N/A	40
1040044T	1	HP_0977 A299V (gCg/gTg)	conserved hypothetical secreted protein	40
253197T	1	HP_0244 synonymous	signal-transducing protein, histidine kinase (atoS)	40
774722A	1	Intergenic	N/A	40
331623T	1	Intergenic	N/A	40
742354T	1	HP_0691 H204Y (Cac/Tac)	3-oxoadipate coA-transferase subunit A (yxjD)	40
613155A	1	HP_0582 synonymous	predicted coding region HP0582	40

200104T	1	HP_0193 G225E (gGg/gAg)	fumarate reductase, cytochrome b subunit (frdC)	40
1465664A	1	HP_1402 synonymous	type I restriction enzyme R protein (hsdR)	40
1347930A	1	HP_1274 synonymous	paralysed flagella protein (pflA)	40
494578A	1	HP_0472 A121V (gCc/gTc)	outer membrane protein (omp11)	40
1589928A	1	Intergenic	N/A	40
462329T	1	HP_0444 G504R (Gga/Aga)	predicted coding region HP0444	40
1028681A	1	HP_0969 T495M (aCg/aTg)	cation efflux system protein (czcA)	40
151120T	1	HP_0140 A260V (gCt/gTt)	L-lactate permease (lctP)	40
558603A	1	HP_0527 L463F (Ctt/Ttt)	cag pathogenicity island protein (cag7)	40
194841T	1	HP_0188 synonymous	predicted coding region HP0188	40
1232632G	1	HP_1167 F10L (ttT/ttG)	predicted coding region HP1167	40
1405559A	1	HP_1345 synonymous	phosphoglycerate kinase	40
1237341A	1	HP_1170 G39R (Ggg/Agg)	glutamine ABC transporter, permease protein (glnP)	40
65220A	1	HP_0060 G402E (gGg/gAg)	predicted coding region HP0060	40
397931T	1	HP_0388 synonymous	conserved hypothetical protein	40
758298A	1	HP_0705 P598S (Cct/Tct)	excinuclease ABC subunit A (uvrA)	40
525438A	1	HP_0499 synonymous	phospholipase A1 precursor (DR-phospholipase A)	40
484286T	1	HP_0464 E553K (Gaa/Aaa)	type I restriction enzyme R protein (hsdR)	40
647297A	1	HP_0609 R14H (cGc/cAc)	predicted coding region HP0609	40
701056A	1	HP_0655 A108T (Gct/Act)	protective surface antigen D15	40

1291216T	1	HP_1214 synonymous	conserved hypothetical protein	40
671456T	1	HP_0624 R12K (aGa/aAa)	solute-binding signature and mitochondrial signature protein (aspB)	40

Intergenic- mutation occurring between genes or in a pseudogene; Synonymous- SNP that does not result in an amino acid change; (?)- unknown amino acid; N- unknown nucleotide base.

Table 5.3 Mutational changes in potato extract-resistant *H. pylori* mutant strains, present in the wild type strain

SNP	No. of Mutants SNP occur	Type of mutation (amino acid/ base change)	Gene Description	Mutant #
1644954G	6	HP_1562 L10F (ttA/ttC)	iron(III) ABC transporter, periplasmic iron-binding protein (ceuE)	1, 18, 20, 4, 40, 6
990814T	6	HP_0927 E183D (gaG/gaT)	heat shock protein (htpX)	1, 18, 20, 4, 40, 6
20966T	6	HP_0021 R59H (cGc/cAc)	predicted coding region HP0021	1, 18, 20, 4, 40, 6
1367889T	6	HP_1293 E290K (Gaa/Aaa)	DNA-directed RNA polymerase, alpha subunit (rpoA)	1, 18, 20, 4, 40, 6
500090G	6	HP_0477 M358V (Atg/Gtg)	outer membrane protein (omp12)	1, 18, 20, 4, 40, 6
632150T	6	HP_0597 D224N (Gat/Aat)	penicillin-binding protein 1A (PBP-1A)	1, 18, 20, 4, 40, 6
1644951A	6	HP_1562 synonymous	iron(III) ABC transporter, periplasmic iron-binding protein (ceuE)	1, 18, 20, 4, 40, 6
1446976C	6	HP_1384 R62G (Cgg/Ggg)	predicted coding region HP1384	1, 18, 20, 4, 40, 6
573116C	6	HP_0541 synonymous	cag pathogenicity island protein (cag20)	1, 18, 20, 4, 40, 6
1643807G	6	Intergenic	N/A	1, 18, 20, 4, 40, 6

331893T	6	HP_0317 synonymous	outer membrane protein (omp9)	1, 18, 20, 4, 40, 6
1273983C	6	HP_1198 T1023A (Act/Gct)	DNA-directed RNA polymerase, beta subunit (rpoB)	1, 18, 20, 4, 40, 6
1644985G	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
629979G	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
132283T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
557269T	6	HP_0527 ?907E (gaN/gaA)	cag pathogenicity island protein (cag7)	1, 18, 20, 4, 40, 6
915894A	6	HP_0863 synonymous	predicted coding region HP0863	1, 18, 20, 4, 40, 6
745425A	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1027030A	6	HP_0968 ?19Y (taN/taT)	predicted coding region HP0968	1, 18, 20, 4, 40, 6
132290T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
780255C	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1077873T	6	HP_1014 synonymous	7-alpha-hydroxysteroid dehydrogenase (hdhA)	1, 18, 20, 4, 40, 6
394519A	6	HP_0384 ?191F (Ntt/Ttt)	predicted coding region HP0384	1, 18, 20, 4, 40, 6
1427512G	6	HP_1365 synonymous	response regulator	1, 18, 20, 4, 40, 6
458644T	6	HP_0440 ?229E (gaN/gaA)	DNA topoisomerase I (topA)	1, 18, 20, 4, 40, 6
500089G	6	HP_0477 synonymous	outer membrane protein (omp12)	1, 18, 20, 4, 40, 6
745426C	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
130106C	6	HP_0119 synonymous	predicted coding region HP0119	1, 18, 20, 4, 40, 6
331896G	6	HP_0317 R732S (agA/agC)	outer membrane protein (omp9)	1, 18, 20, 4, 40, 6
500091C	6	HP_0477 M358T (aTg/aCg)	outer membrane protein (omp12)	1, 18, 20, 4, 40, 6

589778G	6	HP_0555 ?17A (Nct/Gct)	predicted coding region HP0555	1, 18, 20, 4, 40, 6
745422A	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1264197A	6	HP_1193 synonymous	aldo-keto reductase, putative	1, 18, 20, 4, 40, 6
143733A	6	HP_0133 A368V (gCg/gTg)	serine transporter (sdaC)	1, 18, 20, 4, 40, 6
331872A	6	HP_0317 synonymous	outer membrane protein (omp9)	1, 18, 20, 4, 40, 6
130111G	6	HP_0119 K220Q (Aaa/Caa)	predicted coding region HP0119	1, 18, 20, 4, 40, 6
604769C	6	HP_0572 D28G (gAc/gGc)	adenine phosphoribosyltransferase (apt)	1, 18, 20, 4, 40, 6
1417580T	6	HP_1355 A104T (Gct/Act)	nicotinate-nucleotide pyrophosphorylase (nadC)	1, 18, 20, 4, 40, 6
1027027A	6	HP_0968 synonymous	predicted coding region HP0968	1, 18, 20, 4, 40, 6
750323A	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1077872A	6	HP_1014 V27E (gTa/gAa)	7-alpha-hydroxysteroid dehydrogenase (hdhA)	1, 18, 20, 4, 40, 6
516418A	6	HP_0489 synonymous	predicted coding region HP0489	1, 18, 20, 4, 40, 6
780250A	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1322343C	6	HP_1247 synonymous	predicted coding region HP1247	1, 18, 20, 4, 40, 6
1077871T	6	HP_1014 V27L (Gta/Tta)	7-alpha-hydroxysteroid dehydrogenase (hdhA)	1, 18, 20, 4, 40, 6
1259151C	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
93148C	6	HP_0088 Q607R (cAg/cGg)	RNA polymerase sigma-70 factor (rpoD)	1, 18, 20, 4, 40, 6
500098T	6	HP_0477 synonymous	outer membrane protein (omp12)	1, 18, 20, 4, 40, 6
1055148T	6	HP_0994 synonymous	predicted coding region HP0994	1, 18, 20, 4, 40, 6
1446975G	6	HP_1384 R62P (cGg/cCg)	predicted coding region HP1384	1, 18, 20, 4, 40, 6

745424T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
394520A	6	HP_0384 ?190A (gcN/gcT)	predicted coding region HP0384	1, 18, 20, 4, 40, 6
500024G	6	HP_0477 M336V (Atg/Gtg)	outer membrane protein (omp12)	1, 18, 20, 4, 40, 6
132261T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
897444A	6	HP_0846 S667I (aGc/aTc)	type I restriction enzyme R protein (hsdR)	1, 18, 20, 4, 40, 6
500107T	6	HP_0477 synonymous	outer membrane protein (omp12)	1, 18, 20, 4, 40, 6
597135T	6	HP_0564 ?12V (gtN/gtT)	predicted coding region HP0564	1, 18, 20, 4, 40, 6
32006A	6	HP_0031 A16T (Gct/Act)	predicted coding region HP0031	1, 18, 20, 4, 40, 6
1264198G	6	HP_1193 L142V (Ctt/Gtt)	aldo-keto reductase, putative	1, 18, 20, 4, 40, 6
331898G	6	HP_0317 synonymous	outer membrane protein (omp9)	1, 18, 20, 4, 40, 6
745427T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
224638T	6	HP_0215 synonymous	CDP-diglyceride synthetase (cdsA)	1, 18, 20, 4, 40, 6
780253T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
223640A	6	HP_0214 G474E (gGg/gAg)	sodium-dependent transporter (huNaDC-1)	1, 18, 20, 4, 40, 6
1055241T	6	HP_0994 M68I (atG/atT)	predicted coding region HP0994	1, 18, 20, 4, 40, 6
1643806G	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
1077870G	6	HP_1014 F26L (ttT/ttG)	7-alpha-hydroxysteroid dehydrogenase (hdhA)	1, 18, 20, 4, 40, 6
797663T	6	HP_0742 A297V (gCg/gTg)	phosphoribosylpyrophosphate synthetase (prsA)	1, 18, 20, 4, 40, 6
1643134T	6	HP_1561 synonymous	iron(III) ABC transporter, periplasmic iron-binding protein (ceuE)	1, 18, 20, 4, 40, 6
130157A	6	HP_0119 synonymous	predicted coding region HP0119	1, 18, 20, 4, 40, 6

1264199C	6	HP_1193 L142P (cTt/cCt)	aldo-keto reductase, putative	1, 18, 20, 4, 40, 6
698037G	6	HP_0651 V19A (gTc/gCc)	fucosyltransferase	1, 18, 20, 4, 40, 6
780254G	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
777607G	6	HP_0724 synonymous	anaerobic C4-dicarboxylate transport protein (dcuA)	1, 18, 20, 4, 40, 6
780246T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
526507T	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
132267A	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
130052T	6	HP_0119 synonymous	predicted coding region HP0119	1, 18, 20, 4, 40, 6
1600774A	6	Intergenic	N/A	1, 18, 20, 4, 40, 6
223736C	5	HP_0214 V506A (gTc/gCc)	sodium-dependent transporter (huNaDC-1)	1, 18, 20, 4, 40
1437746G	5	HP_1373 V100A (gTg/gCg)	rod shape-determining protein (mreB)	1, 18, 20, 4, 40
1437912T	5	HP_1373 G45R (Gga/Aga)	rod shape-determining protein (mreB)	1, 18, 20, 4, 40
1370263C	5	HP_1297 N13K (aaC/aaG)	ribosomal protein L36 (rpl36)	1, 18, 20, 4, 40
1563753A	5	HP_1490 ?222V (gNg/gTg)	conserved hypothetical protein	1, 18, 20, 4, 40
1240765A	5	HP_1174 G311V (gGc/gTc)	glucose/galactose transporter (gluP)	1, 18, 20, 4, 40
370872A	5	HP_0360 R238K (aGg/aAg)	UDP-glucose 4-epimerase	1, 18, 20, 4, 40
301322T	5	HP_0289 S1100I (aGc/aTc)	toxin-like outer membrane protein	1, 18, 20, 4, 40
1644934A	5	HP_1562 A17V (gCg/gTg)	iron(III) ABC transporter, periplasmic iron-binding protein (ceuE)	1, 18, 20, 40, 6
1642725G	5	HP_1560 ?381S (aNc/aGc)	cell division protein (ftsW)	1, 18, 20, 4, 40
997932G	5	Intergenic	N/A	1, 18, 20, 4, 6

38693T	4	HP_0039 synonymous	predicted coding region HP0039	18, 4, 40, 6
1001151G	4	HP_0941 ?375V (gtN/gtC)	alanine racemase, biosynthetic (alr)	1, 18, 4, 6
1001215G	3	HP_0941 ?354A (gNt/gCt)	alanine racemase, biosynthetic (alr)	1, 4, 6
1001087T	3	Intergenic	N/A	1, 4, 6
999250A	3	HP_0938 R99C (Cgt/Tgt)	predicted coding region HP0938	1, 4, 6
947690A	2	HP_0896 synonymous	outer membrane protein (omp19)	1, 4
947691C	2	HP_0896 synonymous	outer membrane protein (omp19)	1, 4
1207862T	2	rRNA HP_r04	N/A	1, 4
1511903C	2	rRNA HP_r07	N/A	1, 4
127996T	1	HP_0118 E375K (Gaa/Aaa)	predicted coding region HP0118	20
127992A	1	HP_0118 A376V (gCa/gTa)	predicted coding region HP0118	20

Intergenic- mutation occurring between genes or in a pseudogene; Synonymous- SNP that does not result in an amino acid change; (?)- unknown amino acid; N- unknown nucleotide base.

Appendix III Analysis of HP0603, predicted coding region

In order to characterise the hypothetical protein HP0603, it was analysed for the following parameters: physical properties, cellular localisation, signal peptides and transmembrane regions. However, these analyses did not provide a definite characterisation of the protein, that is whether it is a membrane or cytoplasmic protein. Detailed results of analyses are provided below.

HP0603: 639633-640202, NCBI ORF Finder: 639777-640202

DNA sequence

TTGGCCAAAAAGGATTGGAATTTTTTCAAACCTTTAGAGCCTACTAAAAAATATTTTGGCTCTTT
TAAAATCGGCTATCTTTACCAGCATGCAGAAACGACTAAAAGATCCCCCATCCGCCCTAAAAAC
CGCCCTCCTATTTTAATGGATAAAACTTACCATGACGCTTCTTTAGGCTTTCAGGTAGGGTTCGT
TTTAAAAAAGAAAGCTTTATTAGGGGGGTATTTGGATGCAGGAATGGGCGATTCGTATTTCATG
AGCGCTGGGTTTATGGCTGGGGTTAGGCTTTTTAAGGGGTGGGTTATCCCTAAAATCGCCTTAG
GCTATCAGCTTCAAATTTTAGGGGCTAAGATTGATAAGTATCAATTCAATATCCAATCAGCGGT
GGGGAGTGTGGGCTTGTTTTTCAATGCGGCTAAAAATTTTGGCTTGAGCATAGAAGCAAGGGG
TGGTATCCCTTTTTATTTTATCCAGAGCAGGTTTTCTAAGGCTTTCGGCACGCCACGATTAAATAT
CTATTCTGTTGGTATTACATTCACTTTTTATGATTTTACGAGATTTTTAGGGTAA

Amino acid sequence

LAKKDWNFFKPLEPTKKYFGSFKIGYLYQHAETTKRSPIRPKNRPPILMDKTYHDASLGFQVGFVLKK
KALLGGYLDAGMGDSYFMSAGFMAGVRLFKGWVIPKIALGYQLQILGAKIDKYQFNIQSAVGSVGL
FFNAAKNFGLSIEARGGIPFYFIQSRFSKAFGTPRLNIYSVGITFTFYDFTRFLG

The green, red and purple highlight denotes the start codon, stop codon and SNP position, respectively.

PHYSICAL PROPERTIES ANALYSIS FOR HP0603

Physical properties was analysed using ProtParam, an online ExPASy tool provided by the SIB Swiss Institute of Bioinformatics (Gasteiger et al., 2005). This tool provides physical and chemical properties of a given protein sequence. Details of the analysis are shown below;

User-provided sequence:

```
      10        20        30        40        50        60
MDKTYHDASL GFQVGFVLKK KALLGGYLDA GMGDSYFMSA GFMAGVRLFK GWVIPKIALG

      70        80        90       100       110       120
YQLQILGAKI DKYQFNIQSA VGSVGLFFNA AKNFGLSIEA RGGIPFYFIQ SRFSKAFGTP

     130       140
RLNIYSVGIT FTFYDFTRFL G
```

Number of amino acids: 141

Molecular weight: 15595.1

Theoretical pI: 9.80

Amino acid composition:

Ala (A)	12	8.5%
Arg (R)	5	3.5%
Asn (N)	4	2.8%
Asp (D)	6	4.3%
Cys (C)	0	0.0%
Gln (Q)	6	4.3%
Glu (E)	1	0.7%
Gly (G)	19	13.5%
His (H)	1	0.7%
Ile (I)	10	7.1%
Leu (L)	13	9.2%
Lys (K)	10	7.1%
Met (M)	4	2.8%
Phe (F)	17	12.1%
Pro (P)	3	2.1%
Ser (S)	9	6.4%
Thr (T)	5	3.5%
Trp (W)	1	0.7%
Tyr (Y)	8	5.7%
Val (V)	7	5.0%
Pyl (O)	0	0.0%
Sec (U)	0	0.0%
(B)	0	0.0%
(Z)	0	0.0%
(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 7
Total number of positively charged residues (Arg + Lys): 15

Atomic composition:

Carbon	C	736
Hydrogen	H	1103
Nitrogen	N	179
Oxygen	O	188
Sulfur	S	4

Formula: $C_{736}H_{1103}N_{179}O_{188}S_4$
Total number of atoms: 2210

Estimated half-life:

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, in vitro).
>20 hours (yeast, in vivo).
>10 hours (Escherichia coli, in vivo).

Instability index:

The instability index (II) is computed to be 33.31
This classifies the protein as stable.

Aliphatic index: 86.52

Grand average of hydropathicity (GRAVY): 0.298

CELLULAR LOCALISATION AND SIGNAL PEPTIDE ANALYSIS FOR HP0603

The presence of signal peptides and protein localisation was analysed using TargetP, hosted by the Center for Biological Sequence Analysis (CBS) (Emanuelsson et al., 2007), and PSORTb, a subcellular localisation prediction tool (Gardy et al., 2003; Gardy et al., 2005; Yu et al., 2010). Results are shown below;

Targetp v1.1 prediction results

Number of query sequences: 1
Cleavage site predictions included.
Using NON-PLANT networks.

Name	Len	mTP	SP	other	Loc	RC	TPlen
Sequence	**141**	**0.054**	**0.107**	**0.911**	**_**	**1**	**-**
cutoff		**0.000**	**0.000**	**0.000**			

Explanation of parameters assessed;

Name	Sequence name truncated to 20 characters	
Len	Sequence length	
cTP, mTP, SP, other	Final NN scores on which the final prediction is based (Loc, see below). Note that the scores are not really probabilities, and they do not necessarily add to one. However, the location with the highest score is the most likely according to TargetP, and the relationship between the scores (the reliability class, see below) may be an indication of how certain the prediction is.	
Loc	Prediction of localization, based on the scores above; the possible values are:	
	C	Chloroplast, i.e. the sequence contains **cTP**, a chloroplast transit peptide;
	M	Mitochondrion, i.e. the sequence contains **mTP**, a mitochondrial targeting peptide;
	S	Secretory pathway, i.e. the sequence contains **SP**, a signal peptide;
	_	Any other location;
	*****	"don't know"; indicates that cutoff restrictions were set (see instructions) and the winning network output score was below the requested cutoff for that category.
RC	Reliability class, from 1 to 5, where 1 indicates the strongest prediction. RC is a measure of the size of the difference ('diff') between the highest (winning) and the second highest output scores. There are 5 reliability classes, defined as follows: 1 : diff > 0.800 2 : 0.800 > diff > 0.600 3 : 0.600 > diff > 0.400 4 : 0.400 > diff > 0.200 5 : 0.200 > diff Thus, the lower the value of RC the safer the prediction.	
TPlen	Predicted presequence length; it appears only when TargetP was asked to perform cleavage site predictions	

PSORTb Results

SeqID: gi|15645228|ref|NP_207398.1| hypothetical protein HP0603 [Helicobacter pylori 26695]

Analysis Report:
CMSVM- Unknown [No details]
CytoSVM- Unknown [No details]
ECSVM- Unknown [No details]
ModHMM- Unknown [No internal helices found]
Motif- Unknown [No motifs found]
OMPMotif- Unknown [No motifs found]
OMSVM- Unknown [No details]
PPSVM- Unknown [No details]
Profile- Unknown [No matches to profiles found]
SCL-BLAST- Unknown [No matches against database]
SCL-BLASTe- Unknown [No matches against database]
Signal- Unknown [No signal peptide detected]

Localization Scores:
Cytoplasmic 2.00
Cytoplasmic Membrane 2.00
Periplasmic 2.00
Outer Membrane 2.00
Extracellular 2.00

Final Prediction:
Unknown

TRANSMEMBRANE REGION ANALYSIS FOR HP0603

Presence of transmembrane segments was detected using TMHMM, a server used to predict the presence of transmembrane helices in proteins (Krogh et al., 2001) so as to determine if HP0603 is a membrane protein. Results are presented below;

TMHMM

```
# WEBSEQUENCE Length: 141
# WEBSEQUENCE Number of predicted TMHs:  0
# WEBSEQUENCE Exp number of AAs in TMHs: 17.19262
# WEBSEQUENCE Exp number, first 60 AAs:  9.40386
# WEBSEQUENCE Total prob of N-in:	0.32659
WEBSEQUENCE	TMHMM2.0	outside	1	141
```

Explanation of Parameters assessed:

- Length: the length of the protein sequence.
- Number of predicted TMHs: The number of predicted transmembrane helices.
- Exp number of AAs in TMHs: The expected number of amino acids intransmembrane helices. If this number is larger than 18 it is very likely to be a transmembrane protein (OR have a signal peptide).
- Exp number, first 60 AAs: The expected number of amino acids in transmembrane helices in the first 60 amino acids of the protein. If this numbers more than a few, you should be warned that a predicted transmembrane helix in the N-term could be a signal peptide.
- Total prob of N-in: The total probability that the N-term is on the cytoplasmic side of the membrane.