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Studies on the Interaction of Helicobacter pylori with Extracellular Matrix Proteins

A thesis Submitted for the Degree of Doctor of Philosophy (Ph.D.)

By

Denis Finn

B.Sc. Hons. (Zoology and Molecular Genetics), M.Sc. (Molecular Medicine)

At

University of Dublin, Trinity College
2007

Supervisor:
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The Department of Clinical Medicine & The Institute of Molecular Medicine,
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Ireland.
Declaration

I hereby declare that this thesis has not been previously submitted for a degree at this or any other university and that the work described in this thesis is entirely my own work. I hereby give my permission to the library of Trinity College Dublin to lend or copy this thesis upon request for the purpose of study.

Denis Finn
To my parents,
Kay and Jim Finn,
and to my family - past, present and future.
Summary

*Helicobacter pylori* is a common human pathogen that persistently colonises the stomach of half the world's population. The majority of people infected with *H. pylori* are asymptomatic, however, a proportion develop severe gastro-duodenal pathologies including gastritis, gastric/duodenal ulcers and gastric cancers. *H. pylori* has also been associated with other diseases such as iron-deficiency anaemia and autoimmune gastritis. *H. pylori* membrane proteins, such as adhesins BabA and AlpA, play an important role in the successful invasion and colonisation of host gastric mucosa. Damage to the gastric mucosa caused by the inflammatory response to *H. pylori* infection as well as microlesions caused by some foods exposes the extracellular matrix (ECM) to further sites of attachment for *H. pylori*. Indeed, numerous studies have shown that *H. pylori* can interact and associate with ECM ligands, including laminin, but few receptors to these ligands have been identified to date. An important host defence mechanism against infecting pathogens is to create a low iron environment. Iron is an essential micronutrient and its acquisition from the environment is critical for bacterial survival. However, pathogenic bacteria have evolved mechanisms to adapt to and acquire iron from the host, thereby, avoiding clearance.

The studies described in this thesis used a combination of molecular and proteomic tools to characterise the interactions between *H. pylori* and several ECM proteins in addition to the iron-binding protein lactoferrin. Two potential laminin-binding proteins were identified by affinity chromatography. These were HpaA (HP0797) and HorF (HP0671). Surface plasmon resonance was used also to characterise the adhesive interactions between *H. pylori* and the ECM ligands laminin, collagen IV and fibronectin under iron-reduced conditions. Significantly, *H. pylori* adhesion to the ECM ligands was increased under conditions of low iron availability. In addition, *H. pylori* displayed increased adhesion to the human iron-binding protein, lactoferrin. Furthermore, the increased adhesion to both ECM and lactoferrin ligands was independent of the transcriptional regulator, ferric uptake regulator protein (Fur). The effect of iron availability on the modulation of expression of *H. pylori* membrane proteins was carried out using two-dimensional gel electrophoresis and iTRAQ™.
labelling. Quantitative differences in the expression of membrane proteins, including adhesins HpaA, AlpA and virulence factors, such as NapA, CagA and urease were found when *H. pylori* was grown under iron-reduced conditions.

The combined data presented in this study suggests *H. pylori* adhesins play an important role in its pathogenicity. The ability to increase its adhesive properties under low iron conditions may be an important virulence mechanism not yet recognised. The inclusion of adhesins as vaccine candidates may prove to be successful in treating and preventing further infection by *H. pylori*.
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Associated Publications

Paper


Poster Presentations


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<th>Definition</th>
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<tr>
<td>1D</td>
<td>One dimensional</td>
</tr>
<tr>
<td>2DE</td>
<td>Two Dimensional gel Electrophoresis</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP- Binding Cassette</td>
</tr>
<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain Heart Infusion</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BSS</td>
<td>Blum Silver Stain (modified)</td>
</tr>
<tr>
<td>CB</td>
<td>Coomassie Blue</td>
</tr>
<tr>
<td>CCB</td>
<td>Colloidal Coomassie Blue</td>
</tr>
<tr>
<td>CCUG</td>
<td>Culture Collection University of Göteborg (Sweden)</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate</td>
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<tr>
<td>Chapt</td>
<td>Chapter</td>
</tr>
<tr>
<td>Cm</td>
<td>Chloroamphenicol</td>
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<tr>
<td>CNBr</td>
<td>Cyanogen Bromide activated sepharose™ 4B</td>
</tr>
<tr>
<td>Col</td>
<td>Collagen IV</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>DC Specific intracellular adhesion molecule-3 (ICAM) – Grabbing Non-integrin</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleotide mix</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiotreitol</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<td>-------------</td>
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<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>Ethyl-N-(3-diethylaminopropyl)carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EHS</td>
<td>Engelbreth-Holm-Swarm</td>
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<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<td>FCS</td>
<td>Fetal Calf Serum</td>
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<td>Fibronectin</td>
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<td>FUR</td>
<td>Ferric Uptake Regulator</td>
</tr>
<tr>
<td>GERD</td>
<td>Gastro-esophageal Reflux Disease</td>
</tr>
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<td>HBS</td>
<td>Hepes Based Saline</td>
</tr>
<tr>
<td>Hem</td>
<td>Hemin</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>IAA</td>
<td>Iodoacetamide</td>
</tr>
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<td>IDA</td>
<td>Iron Deficient Anaemia</td>
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<td>IEF</td>
<td>Isoelectric Focusing</td>
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<td>IFNγ</td>
<td>Interferon γ</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IM</td>
<td>Inner Membrane</td>
</tr>
<tr>
<td>IPG</td>
<td>Immobilised pH Gradient</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1 thiogalactopyranoside</td>
</tr>
<tr>
<td>IR</td>
<td>Iron Reduced</td>
</tr>
<tr>
<td>iTRAQ</td>
<td>isobaric Tag for Relative and Absolute Quantitation</td>
</tr>
<tr>
<td>Kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>Laf</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td>Lam</td>
<td>Laminin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>LBP</td>
<td>Laminin Binding Protein</td>
</tr>
<tr>
<td>Le</td>
<td>Lewis (antigen) e.g., Lex = Lewis X antigen</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosa-associated Lymphoid Tissue</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MHC I</td>
<td>MHC class I molecule</td>
</tr>
<tr>
<td>MHC II</td>
<td>MHC class II molecule</td>
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<tr>
<td>MS</td>
<td>Mass Spectrometry</td>
</tr>
<tr>
<td>MSCRAMM</td>
<td>Microbial Surface Components Recognising Adhesive Matrix Molecules</td>
</tr>
<tr>
<td>MW</td>
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</tr>
<tr>
<td>N</td>
<td>Normal</td>
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<tr>
<td>NCTC</td>
<td>National Collection of Type Culture (U.K.)</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NLBH</td>
<td>Neuraminyl-Lactose-Binding Haemagglutinin</td>
</tr>
<tr>
<td>O.D.</td>
<td>Optical Density</td>
</tr>
<tr>
<td>OM</td>
<td>Outer Membrane</td>
</tr>
<tr>
<td>OMP</td>
<td>Outer Membrane Protein</td>
</tr>
<tr>
<td>OMV</td>
<td>Outer Membrane Vesicles</td>
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<tr>
<td>OPD</td>
<td>O-phenlenediamine</td>
</tr>
<tr>
<td>PAI</td>
<td>Pathogenicity Island</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Based Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>Pfu</td>
<td>Pyrococcus furiosus</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPI</td>
<td>Proton Pump Inhibitor</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RU</td>
<td>Response Units</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
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<td>-------------</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS PolyAcrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean (statistics)</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface Plasmon Resonance</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris + Acetic acid + EDTA (buffer)</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Based Saline</td>
</tr>
<tr>
<td>TBS-hs</td>
<td>TBS with high salt content</td>
</tr>
<tr>
<td>Tc</td>
<td>T cytotoxic (T-cell)</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TE</td>
<td>Tris + EDTA (buffer)</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylenediamine</td>
</tr>
<tr>
<td>Tf</td>
<td>Transferrin</td>
</tr>
<tr>
<td>TFE</td>
<td>Trifluoroethanol</td>
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<tr>
<td>Th</td>
<td>T helper (T-cell)</td>
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<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor α</td>
</tr>
<tr>
<td>Tween-20®</td>
<td>Polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
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CHAPTER I
GENERAL INTRODUCTION
Chapter I

Helicobacter pylori

Helicobacter pylori was first described in 1983 by Warren and Marshall, and isolated in 1984 (Marshall and Warren, 1984, Warren and Marshall, 1983). Initially the new species was classified as Campylobacter pyloridis but was later re-classified as Helicobacter pylori (Goodwin et al., 1989). H. pylori is a Gram-negative, microaerophilic, spiral- or curved-shaped organism with flagella (Figure 1.1). Identification is by rapid urease+, catalase+ and oxidase+ reactions (Marshall, 1994). H. pylori normally infects human hosts but can naturally infect other mammals (Versalovic and Fox, 2001). Marshall himself demonstrated the pathogenic nature of H. pylori (Figure 1.2) by swallowing a culture of a H. pylori isolate; he developed gastritis over 14 days which was successfully treated following the eradication of the H. pylori infection (Marshall et al., 1985).

Prevalence of H. pylori

Helicobacter pylori has colonised humans throughout our history. Recent phylogeographic studies suggest that the evolution of H. pylori populations mirrors the migration patterns (and evolution) of humans from a centre of origin located in Africa (Falush et al., 2003, Wirth et al., 2004, Linz et al., 2007). H. pylori is a common bacterial pathogen of humans that persistently colonises the stomach of approximately 50% of the world population (Parsonnet, 1998, Taylor and Blaser, 1991). However, the prevalence varies greatly among countries; a higher prevalence is associated with lower socioeconomic conditions. Prevalence ranges from over 80 percent of middle-aged adults in developing countries to 20-50 percent in industrialized countries. The infection is acquired by oral ingestion of the bacterium and is mainly transmitted within families in early childhood (Suerbaum and Michetti, 2002).
Figure 1.1: Electron micrograph of *Helicobacter pylori*. Bar is 0.5 μm. The characteristic spiral shape and flagella facilitate motility in the gastric mucosa. Image taken from Atherton (2006).
Figure 1.2: Light micrograph of *Helicobacter pylori* in association with the gastric mucosa. *H. pylori* demonstrates tropism for Trefoil factor family 1 (TFF1) and mucin MUC5AC expressed by gastric mucosa and is more normally found here. However bacteria can colonise the duodenum where gastric metaplasia has occurred. The bacterium can be seen swimming in the mucus but a proportion is closely associated with the epithelial surface. Adherence plays a critical role in disease development. Image taken from Atherton (2006).
Infection overview & outcome

Although *H. pylori* colonisation usually occurs in childhood, infection will persist lifelong in the absence of treatment. The majority of people infected with *H. pylori* are asymptomatic allowing *H. pylori* to persist in its niche environment. However, variation among *H. pylori* strains as well as individual hosts and/or environmental factors can upset the equilibrium. As a result, over time, 15-20% of infections develop into severe gastro-duodenal pathologies (Ernst and Gold, 2000, Blaser and Berg, 2001) (Figure 1.3), including stomach and duodenal ulcers, distal adenocarcinomas and B cell mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach (Petersen and Krogfelt, 2003). More recently, *H. pylori* has been associated with iron-deficiency anaemia (Barabino, 2002, DuBois and Kearney, 2005) and autoimmune gastritis (Negrini et al., 1997, Amedei et al., 2003) which can lead to pernicious anaemia. Interestingly, *H. pylori* has been linked to protection against severe gastro-esophageal reflux disease (GERD) and its complications, Barrett’s oesophagus and adenocarcinoma of the oesophagus (Ye et al., 2004, Vaezi et al., 2000).
Figure 1.3. Natural progression of *Helicobacter pylori* infection. Infection usually occurs during childhood but adults can also become infected. The initial symptoms of acute gastritis are non-specific and transient, rarely leading to early diagnosis. If left untreated the infection develops into chronic active gastritis that persists life-long. The infection can take multiple courses. The majority of people infected with *H. pylori* are asymptomatic. However, 15-20% of infections will develop ulcer disease, gastric adenocarcinoma, and MALT lymphoma. Figure taken from Suerbaum & Josenhans (2007).
**Host immune response to *H. pylori* infection**

The innate immune system provides a non-specific, first line defence, against a pathogen and includes mechanisms such as physical barriers (e.g., skin), mucosal secretions, chemical mediators (e.g., cytokines and chemokines) and phagocytic cells (e.g., macrophages and neutrophils). The innate immune system can defend against many common microbes but more infectious microbes often overcome and evade these defences. The adaptive immune system includes lymphocytes (T- and B-cells) that have evolved to provide a more targeted and dynamic defence to eliminate infections as well as providing increased protection against subsequent infections by the same pathogen (Davey, 1992, Janeway *et al.*, 2004).

T-cells have three main functions: to kill host cells that have become infected with pathogens, to maintain an inflammatory response at the site of persistent infection and to regulate immune cells from both the innate and adaptive immune system. T-cells require foreign antigens to be presented to them in the form of a complex between the antigen and a Major Histocompatibility Complex (MHC) molecule recognised by either T-cytotoxic, (Tc), T-cells or T-helper, (Th), cells. Virtually all cells in the body express MHC class I (MHC I) molecules. When a host cell is infected with a virus or other intracellular microorganisms, antigenic fragments, associated with MHC I molecules, are presented on the cell surface. Cytotoxic T-cells recognise the antigen/MHC I complex and set about destroying the host cell using toxic and pore-forming chemicals. In contrast, Th cells recognise foreign antigens associated with MHC class II (MHC II) molecules, presented to them by activated immune cells, such as dendritic cells (DC), and resulting in a prolonged inflammatory response where other immune cells, such as macrophages (phagocytosis) or B-cells (antibody production), are activated to help clear the infection. Macrophages and B-cells can also act as antigen presenting cells using MHC II molecules. T helper cells are further characterised by the type of immune response they initiate via the secretion of cytokines and chemokines. A Th1 response occurs following infection by an intracellular pathogen and results in the recruitment of immune cells, such as cytotoxic T-cells, that eventually lead to the destruction of an infected host cell. A Th2 response occurs in response to extracellular pathogens and results in the
production of specific antibodies against the pathogen leading to its eventual clearance (Abbas and Lichtman, 2006, Janeway et al., 2004).

Unusually for an extracellular pathogen, *H. pylori*, initiates primarily a Th1 response even though it occurs as an extracellular pathogen. This has been demonstrated in human and animal studies in response to *H. pylori* infection (D'Elios et al., 1997, Luzza et al., 2001, Mohammadi et al., 1996, Mattapallil et al., 2000, Rossi et al., 2000). The main Th1 components found are interferon-γ (IFNγ), tumour necrosis factor-α (TNFα), interleukin-12 (IL-12), and IL-17 while Th2 components include IL-4 and IL-10. This polarized Th1 response most likely leads to the damage caused to the epithelial lining of the gastrointestinal tract. Mice genetically modified to have a Th1 response developed severe gastritis when infected with *H. pylori* (Mohammadi et al., 1997, Smythies et al., 2000). In contrast, IFNγ deficient mice displayed less inflammation and less atrophy when infected with *Helicobacter* (Smythies et al., 2000, Obonyo et al., 2002).

Controlling and maintaining a mixed Th1/Th2 immune response by *H. pylori* may facilitate persistent colonisation and prevent development of severe atrophic gastritis and the subsequent loss of its ecological niche. Dendritic cells (DC) are potent antigen presenting cells and important mediators between the innate and acquired immune system (Kranzer et al., 2004). *H. pylori* co-culture with DC induces maturation and activation of both Th1 and Th2 cytokines (Guiney et al., 2003). However, lipopolysaccharide (LPS) Lewis (Le) antigens, present on the surface of *H. pylori*, can be switched on and off which in turn control the type of Th response. Bergman et al. demonstrated that *H. pylori* Le⁺ variants are able to bind to DC-specific intracellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin (DC-SIGN) and block Th1 cell development. Conversely Le⁻ variants did not bind DCs resulting in a strong Th1 response (Bergman et al., 2004). Similar interactions with DC-SIGNs are used by other human pathogens such as *Leishmania mexicana* (Alexander and Bryson, 2005) *Schistosoma mansoni* (Pearce et al., 2004) and *Mycobacterium tuberculosis* (Geijtenbeek et al., 2003) to modulate the immune response for their competitive advantage.
Co-infection with parasitic helminths has been shown to reduce gastric atrophy in mice with concurrent *Helicobacter* infection. The Th1 response was down-regulated despite chronic inflammation and high density *Helicobacter* colonisation (Fox *et al.*, 2000). Co-infection may account for the low rate of *Helicobacter*-associated gastric cancer rates in Africa where parasitic infections are endemic despite high *H. pylori* prevalence (Holcombe, 1992).

**Factors affecting *H. pylori* infection outcome**

Although infection with *H. pylori* usually results in inflammation of the gastric mucosa, the distribution and severity of the clinical outcome can vary widely among populations. The different outcomes of disease are influenced mainly by a combination of the host immune response and the virulence of the infecting *H. pylori* strain as summarised in Figure 1.4.

**Host cytokine/chemokine polymorphisms**

The location of *H. pylori* induced gastritis can affect the outcome of infection. Duodenal ulceration is associated with antral-predominant inflammation leading to increased acid secretion (Atherton *et al.*, 1996, Gillen *et al.*, 1998). Gastric ulceration and adenocarcinoma are associated with pan-gastritis or corpus-predominant gastritis resulting in reduced acid secretion (Blaser and Atherton, 2004). The reasons for the different patterns of inflammation are unclear. However, the cytokines IL-1β, TNFα, IFNγ and IL-2 all suppress acid production (Padol and Hunt, 2004) and polymorphisms in these genes may contribute to the differing course of *H. pylori* infection.

IL-1 is a pro-inflammatory cytokine involved in T-cell and macrophage activation and is a potent inhibitor of gastric acid secretion (Janeway *et al.*, 2004). Secretion of IL-1 is genetically determined and polymorphisms in the IL-1β gene and the IL-1 receptor-antagonist gene (IL-1RN) increase the risk of gastric atrophy, hypochlorhydria, intestinal metaplasia and gastric adenocarcinoma (El-Omar *et al.*, 2000, Furuta *et al.*, 2002, Rad *et al.*, 2003). The IL-1β and IL-1RN gene polymorphisms lead to a reduced acid environment coupled to an enhanced level of inflammation. In addition, polymorphisms in TNFα and IL-10 genes have also been associated with increased
risk for gastric cancer (El-Omar et al., 2003b, Rad et al., 2004). A recent study of Japanese patients infected with *H. pylori* suggested a link between an IL-8 polymorphism and increased risk of gastric cancer and gastric ulcer (Ohyauchi et al., 2005). IL-8 is a chemokine and is involved in the recruitment of neutrophils and T-cells to sites of infection. Ohyauchi et al. (2005) have also shown that the IL-8 promoter activity of a specific polymorphic region is enhanced in response to IL-1β and TNFα, leading to more active gastritis. Another Japanese study reported that a polymorphism in the IL-2 gene (secreted by T-cells to promote T-cell proliferation) is associated with an increased risk of gastric atrophy induced by *H. pylori* infection and might predispose to gastric cancer (Togawa et al., 2005). Furthermore, a cumulative effect may occur in patients possessing a combination of polymorphisms. Recent studies on patients possessing 2 or more polymorphisms, such as IL-1, TNFα and IL-10, show a significantly higher risk of developing gastric cancer than patients with none (Machado et al., 2003, El-Omar et al., 2003).
Host differences in cytokine genes

$H.\text{pylori}$ strain virulence

$+ \text{ or } -$ co-infection e.g., parasite

Severity of gastric inflammation

Asymptomatic persistent infection

Corpus-predominant or pan gastritis (inflamed gastric corpus)

\[ \text{Acid production} \]

Risk of gastric ulceration and gastric adenocarcinoma

Antral-predominant gastritis (healthy gastric corpus)

\[ \text{Risk of duodenal ulceration} \]

Figure 1.4. The key factors underlying $Helicobacter\text{ pylori}$-associated disease (boxed) and the external effects modulating them (orange). $H.\text{pylori}$ infection induces inflammation, but strain, host and environmental factors such as co-infection may alter its severity. High-level inflammation increases the risk of all $H.\text{pylori}$-associated diseases. The location of stomach inflammation can also determine the outcome. Polymorphisms in host cytokines affect the pattern of inflammation in the stomach, however, $H.\text{pylori}$ strain determinants and additional factors such as co-infection may also contribute. Figure adapted from Atherton (2006).
**H. pylori virulence factors**

The host immune systems possess an array of defence mechanisms, such as stomach acid, macrophages and T cells, to help protect and clear infections by microorganisms. However, many pathogenic microbes can overcome these defence systems as well enhance their survival, and pathogenic potential, in their chosen niche by possessing and employing a set of components and physical characteristics known as virulence factors. *H. pylori* possesses a variety of virulence factors including toxins, motility mechanisms and adhesins that are summarised in Table 1.1. The main virulence factors are described below. Adhesins are discussed later in the chapter.

**Urease**

Urease is used by *H. pylori* to survive and persist in the acidic environment of the stomach. There are seven genes, found in a single cluster (ureABIEFGH), associated with the production and function of urease (Clayton et al., 1990, Labigne et al., 1991, Hu et al., 1992, Cussac et al., 1992). *ureA* and *ureB* encode the two structural subunits UreA (29 kDa) and UreB (66kDa) (Hu and Mobley, 1990, Dunn et al., 1990) which form a dodecameric assembly (Ha et al., 2001). The *ureE-F* genes encode proteins involved in the delivery and incorporation of nickel ions to the urease enzyme to produce its active form (Montecucco and Rappuoli, 2001, Cussac et al., 1992). An acid-gated channel (encoded by *ureI*) regulates the uptake of urea into *H. pylori*’s cytoplasm (Weeks et al., 2000). Urea undergoes hydrolysis to yield ammonia, which helps to neutralise hydrochloric acid – resulting in a neutral layer surrounding the bacterial surface. The importance of urease is demonstrated by the failure of urease negative *H. pylori* to colonise the gastric mucosa (Suerbaum and Josenhans, 1999, Weeks et al., 2000).

Urease activity is used as a diagnostic tool for *H. pylori*. Patient gastric biopsies are placed onto gel containing urea and a pH indicator; hydrolysis of urea raises the pH (McNulty and Wise, 1985, Marshall et al., 1987). A non-invasive method, called the Urea Breath Test, involves patients taking an oral dose of either $\text{[}^{13}\text{C}\text{]}$ or $\text{[}^{14}\text{C}\text{]}$ labelled urea. If an active *H. pylori* infection is present, the urea will be hydrolysed and $^{13}\text{CO}_2$ or $^{14}\text{CO}_2$ will eventually be exhaled and measured (Graham et al., 1987, Bell et al., 1987).
Cytotoxin-associated gene (Cag) Pathogenicity Island (PAI)

A pathogenicity island (PAI) is a distinct DNA fragment containing virulence genes that has been incorporated in the genome of a pathogen by horizontal transfer. The cag PAI of H. pylori is between 35 and 40 kb long, flanked by 39 bp DNA repeats and contains approximately 30 genes (Censini et al., 1996, Akopyants et al., 1998). H. pylori strains can possess the entire island, partial island or no island (Sozzi et al., 2001). The cag PAI contains the cagA gene that encodes the antigenic CagA protein (128 kDa). Infection by strains possessing cagA results in increased inflammation in the stomach (Perez-Perez et al., 1999) and increased risk for peptic ulcer disease (Nomura et al., 2002) and gastric cancer (Blaser et al., 1995). CagA is injected into epithelial cells by a Type IV secretion system (Odenbreit et al., 2000) - also encoded by the cag PAI – where it undergoes tyrosine-phosphorylation and interferes with normal cell signalling pathways affecting the shape, migration and adhesion of epithelial cells (Segal et al., 1999, Higashi et al., 2002). Phase variation occurs within the cagA gene, allowing the expression of CagA proteins with differing numbers of phosphorylation sites (Aras et al., 2003); strains with multiple phosphorylation sites are more virulent and associated with gastric cancer (Atherton, 2006). CagA has also been shown to disrupt cell junction complexes by recruitment of tight junction components to the site of bacterial attachment on polarised epithelial cells (Amieva et al., 2003). Another contribution of the cag PAI to the pathogenicity of H. pylori is demonstrated by its role in inducing pro-inflammatory chemokines (Crabtree et al., 1994, 1995). Strains possessing the cag PAI induce higher levels of IL-8 production in epithelial cells than strains without the cag PAI. The effect is independent of CagA protein expression but dependent on expression of the Type IV secretion system (Fischer et al., 2001b), perhaps suggesting that other unknown components are injected into the host.

Vacuolating toxin A (VacA)

Although the vacA gene is found in all strains of H. pylori, its virulence is determined by the combination of two gene region types, the signalling (s1 and s2) and the mid (m1 and m2) regions (Cover et al., 1994). Strains possessing types s2/m1 or s2/m2 vacA are rarely associated with disease and their products are non-toxic (Atherton, 2006). Strains possessing both type s1 vacA combinations are toxic to cells, but the combination of s1/m1 adheres to a broader range of cells and has been associated with
gastric adenocarcinoma (Blaser and Atherton, 2004). VacA is composed of two subunits, 58 kDa and 37 kDa, which assemble into a rosette structure—a multimer consisting of the 58 kDa and 37 kDa subunits (Cover and Blaser, 1992, Cover et al., 1997, Lanzavecchia et al., 1998). It is secreted by *H. pylori* and attaches to the epithelial cell surface before activation by acid exposure. VacA inserts into the plasma membrane of cells, where it forms anion-specific channels allowing anions and other molecules, such as urea, to flow out of the cell. The toxin pores are eventually endocytosed, developing into late-endosomal compartments and later forming large acidic vacuoles. VacA has also been shown to interfere with cell tight junctions, increasing the flow of nutrients across the mucosal barrier (Atherton, 2006, Montecucco and Rappuoli, 2001, Blaser and Atherton, 2004).

**Motility and Flagella**

Motility of *H. pylori* is required for colonisation. *H. pylori* possesses a bundle of four to eight unipolarly arranged sheathed flagella, which it uses to provide motility in the gastric lumen and mucosa (Suerbaum and Josenhans, 1999). The sheath covering the flagellar filaments is continuous with the outer membrane and has a similar composition of lipopolysaccharides, phospholipids and proteins (Geis et al., 1993). The flagella filaments are composed of two flagellin subunits, FlaA and FlaB. The published *H. pylori* genomes (26695 and J99) contain over 40 genes related to motility and the structural assembly of flagella (Josenhans and Suerbaum, 2001). Eaton et al. (1996) demonstrated that *H. pylori* deficient in either flaA or flaB displayed weak colonisation using a piglet model, while *H. pylori* deficient in both genes displayed no colonisation. This suggests partial motility can support colonisation, whereas, full motility is required for high levels of infection. Furthermore, Ottemann and Lowenthal (2002) demonstrated that even if the flagella are present, movement of the filaments is required for colonisation in the murine model. *H. pylori* deficient in the motB gene, which encodes the MotB flagellar motor protein, were unable to colonise the stomach of mice. Other studies have shown the importance to colonisation of a fully functional motility system (Foynes et al., 1999, Kim et al., 1999).
<table>
<thead>
<tr>
<th>Factor</th>
<th>Function</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>CagA</td>
<td>Interferes with cell signalling pathways.</td>
<td>(Naumann, 2002)</td>
</tr>
<tr>
<td>VacA</td>
<td>Lead to formation of vacuoles inside infected epithelial cell.</td>
<td>(Blaser and Atherton, 2004)</td>
</tr>
<tr>
<td><strong>Enzymes:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urease</td>
<td>Protects against acid destruction.</td>
<td>(Suerbaum and Joscnhans, 1999, Weeks et al., 2000)</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Protect against reactive oxygen species produced as part of the host immune response.</td>
<td>(Seyler et al., 2001, Basu et al., 2004)</td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Adhesins:</strong></td>
<td>Bind to Lewis group antigens expressed on the gastric epithelium</td>
<td>(Odenbreit, 2005)</td>
</tr>
<tr>
<td>BabA, BabB</td>
<td></td>
<td></td>
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<tr>
<td>SabA,</td>
<td>Bind to gastric epithelium</td>
<td>(de jonge et al., 2004)</td>
</tr>
<tr>
<td>AlpA, AlpB</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Others:</strong></td>
<td>Promotes adhesion to endothelial cells and plays a role in protection against oxidative stress. Can also bind to iron.</td>
<td>(Evans et al., 1995, Cooksley et al., 2003)</td>
</tr>
<tr>
<td>LPS</td>
<td>Protection against digestive enzymes and plays a role in antigen mimicry and adhesion.</td>
<td>(Bergman et al., 2004)</td>
</tr>
<tr>
<td>Heat shock proteins</td>
<td>Induce Th1 immune response and stress induced adhesion.</td>
<td>(Lin et al., 2006, Huesca et al., 1998)</td>
</tr>
<tr>
<td>Flagella</td>
<td>Facilitate movement in the host gut.</td>
<td>(Eaton et al., 1996, Ottmann and Lowenthal, 2002)</td>
</tr>
</tbody>
</table>
**H. pylori strain variation**

*H. pylori* strains display high genetic variability. Most isolates from unrelated patients possess their own unique genetic fingerprint (Akopyanz *et al.*, 1992b, Akopyanz *et al.*, 1992a). Individuals can be colonised with multiple strains which have been shown to change their genetic complement during infection (Kuipers *et al.*, 2000, Kersulyte *et al.*, 1999). Much of the genetic variation in *H. pylori* is achieved by a high mutation rate, possibly resulting from the lack of DNA repair genes in addition to a high frequency of recombination events at sites of repetitive DNA sequences causing “on-off” switching of genes resulting in phase variation of different gene products (Suerbaum and Josenhans, 2007, Blaser and Atherton, 2004, Bergman *et al.*, 2004). The recombination events arising at these sites of repetitive DNA sequences occur by a mechanism known as ‘slipped strand mis-paring’, summarised in Figure 1.5. Finally, *H. pylori* is naturally competent and can acquire DNA/gene fragments from other *H. pylori* strains. (Suerbaum and Josenhans, 2007, Blaser and Atherton, 2004). Although not fully understood, the horizontal transfer and DNA uptake system is thought to be associated with a type IV secretion system encoded by the *comB* operon and is independent of the type IV secretion system encoded by the *cag-Pl* (Hofreuter *et al.*, 2001). The uptake and incorporation of DNA in this way leads to the generation of a high degree of genetic diversity.
Figure 1.5. Slipped strand mis-pairing occurs during DNA replication resulting in loss or addition of base pair repeat units. Short tandem repeat units are represented by arrows. During replication, mis-pairing between complementary DNA strands can occur, leading to 'bulging' at the point of mismatch. As a result, repeat units are either inserted (if the bulging occurs in the nascent strand) or deleted (if the bulging occurs in the template (parental) strand) in the newly synthesized DNA strand. Multiple bulging can occur on a single strand resulting in multiple insertion/deletion of repeat units. Figure taken from van Belkum et al. (1998).
**Treatment and vaccines**

The current treatment regime for *H. pylori* infection is a triple therapy consisting of two antimicrobial agents along with a proton-pump inhibitor (PPI) for 7-14 days (Cavallaro *et al.*, 2006, Kim *et al.*, 2007). The main antibiotics used are metronidazole, amoxicillin or clarithromycin (Figure 1.6). Eradication rates are normally > 90%, however, this can be as low as 80% (Cavallaro *et al.*, 2006). Antibiotic resistance is a major cause of treatment failure in addition to non-compliance and other patient complications such as smoking (Suerbaum and Michetti, 2002, Banerjee and Michetti, 2001, Kabir, 2007). The high cost of antibiotics and high re-infection rate in developing countries suggests an alternative approach to treating *H. pylori* infection is required (Del Giudice *et al.*, 2001). Vaccination with several antigens, including UreB, VacA, CagA, NapA and whole cell preparations, have been shown to confer protection against *Helicobacter* infection in animal models (Kabir, 2007, Del Giudice *et al.*, 2001). Although the feasibility of vaccination has been proven in animal models, the mechanism is poorly understood and a suitable vaccine for human use is still a number of years away. Membrane proteins, particularly those exposed on the surface of *H. pylori*, such as adhesins, may prove to be effective vaccine candidates. The importance of adhesins, such as BabA, SabA and AlpA, for successful *H. pylori* colonisation has been demonstrated by others, suggesting adhesins may prove to be ideal vaccine targets (Odenbreit, 2005, Mahdavi *et al.*, 2002, de Jonge *et al.*, 2004).
First-line therapy

PPI (RBC) b.d. + clarithromycin 500 mg b.d. (C) + amoxicillin 1000 mg b.d. (A) or
metronidazole R 500 mg b.d. (M) for a minimum of 7 days
*CA is preferred to CM as it may favour best results with second-line PPI quadruple therapy

In case of failure

Second-line therapy

PPI b.d. + bismuth subsalicylate/subcitrate 120 mg q.d.s. + metronidazole 500 mg t.d.s. +
tetracycline 500 mg q.d.s. for a minimum of 7 days
If bismuth is not available, PPI-based triple therapies should be used

Subsequent failures should be handled on a case-by-case basis. Patients failing second-line
therapy in primary care should be referred

Figure 1.6. Summary of the recommended treatment strategy for the eradication of
*Helicobacter pylori*. PPI = proton pump inhibitor; RBC = ranitidine bismuth citrate. Figure
Composition and structure of the cell envelope of *H. pylori*

The envelope of all Gram-negative bacteria is composed of an inner (IM) and outer (OM) membrane separated by a thin layer (periplasm) of peptidoglycans (Figure 1.7). The IM is in direct contact with the cytoplasm/periplasm and contains integral IM proteins and lipoproteins. Many of the IM proteins are involved in oxidative phosphorylation, lipid biosynthesis and protein translocation. The periplasm is an aqueous layer containing high concentrations of proteolytic enzymes and transport proteins. The peptidoglycans in the periplasm act as a "cytoskeleton" contributing to cell shape and flexibility. The OM consists of lipopolysaccharides (LPS), lipoproteins and integral OM proteins. Many integral OM proteins function as selective porin channels controlling the entry of nutrients and other essential molecules (e.g., urea) and the exit of toxic waste products. Other proteins function as enzymes or adhesins. The OM also serves as an anchor for surface organelles such as flagella used for bacterial motility (Ruiz *et al*., 2006).

![Diagram of Gram-negative cell envelope](image)

**Figure 1.7. Structure of Gram-negative cell envelope.** The cytoplasm is surrounded by a cell envelope, which is composed of the inner membrane (IM), the periplasm and the outer membrane (OM). The role and composition of the membrane is described in the text. Figure taken from Talaro & Talaro (2002).
**Outer membrane components of H. pylori**

**Lipopolysaccharide (LPS)**

Lipopolysaccharides are a family of toxic phosphorylated glycolipids composed of a lipid moiety (lipid A), a core oligosaccharide, and a polymeric O-specific polysaccharide chain (Figure 1.8). The structure of lipid A is conserved whereas the core oligosaccharide and O-specific chain are more variable (Moran, 2001).

![Figure 1.8. General structure of lipopolysaccharide. LPS is composed of lipid A (endotoxin), core oligosaccharide and O-antigen. Figure taken from Moran (2001).](image)

*H. pylori* LPS functions as a barrier to digestive enzymes and detergents as well as playing a role in antigen mimicry. It has low endotoxic and antigenic activity compared to LPS of other bacteria (Bergman *et al.*, 2006). The induction of cytokines such as IL-1, IL-6 and TNFα is significantly lower than that by *Escherichia coli* LPS (Birkholz *et al.*, 1993). Lipid A moieties have been shown to be poorly recognised by toll-like receptor 4 (Chmiela and Michetti, 2006). The reduced immune response to *H. pylori* LPS may contribute to the persistent and prolonged colonisation of the human stomach.

The O-specific chains of *H. pylori* LPS possess Lewis blood-group antigens (Le) similar to the Le expressed on the mucosal surface of the human stomach (Bergman *et
The extensive phenotypic variation displayed by *H. pylori* LPS can result in strains displaying one or multiple Le (Aspinall and Monteiro, 1996). The genes controlling the expression of these antigens can be switched on or off to create populations with highly diverse LPS glycosylation patterns (Suerbaum and Josenhans, 2007). The varied Le phenotype displayed by *H. pylori* may help to maintain a balanced Th1/Th2 immune response. Strains expressing Le associate with DC-SIGNs, resulting in a reduced Th1 immune response, while non-Lewis antigen strains fail to bind DC-SIGNs, resulting in a continued Th1 response (Bergman et al., 2004).

LPS has also been linked with *H. pylori* adhesion to gastric epithelium (Osaki et al., 1998, Edwards et al., 2000) and the extra-cellular matrix (ECM) protein laminin (Valkonen et al., 1994). Edwards et al. found that *H. pylori* without Lewis x (Lex) failed to adhere to gastric epithelium whereas strains with Lex did (Edwards et al., 2000, Moran, 1996). Two laminin-binding sites were identified by Valkonen et al. on LPS; depending on the strain it was either on a phosphorylated (haemagglutinating strains) site or a non-phosphorylated (non-haemagglutinating strains) site in the core oligosaccharide of LPS (Valkonen et al., 1994).
**Adhesins**

A small subset of *H. pylori* organisms adhere to the gastric mucosa, and this is important in pathogenesis as adhesion triggers bacterial and host events outlined in Figure 1.9. Adhesion by *H. pylori* to the gastric mucosa has been demonstrated in animal models and cell lines (Petersen and Krogfelt, 2003). Patients with gastric pathology appear to have more attached bacteria (Hessey *et al.*, 1990).

The ability of *H. pylori* to bind to gastric epithelial cells is a critical step in the colonisation and persistence in the human stomach, counteracting the forces of liquid flow and peristaltic movement of the gut (Odenbreit, 2005). Adherence allows strains containing a functional *cag* PAI to inject CagA into the host cells where it becomes tyrosine-phosphorylated, eventually affecting normal cell shape, migration and adhesion (Odenbreit *et al.*, 2000, Tsutsumi *et al.*, 2003). The close contact may also be utilised by other bacterial factors which induce IL-8 production, possibly also via the Type IV secretion system (Tsutsumi *et al.*, 2003). In addition, the genome of *H. pylori* contains a large super-family of 32 paralogous genes encoding Hop or Hop-related (Hor) outer membrane proteins (Tomb *et al.*, 1997, Alm *et al.*, 2000). Several of these outer membrane proteins are involved in adherence to the gastric epithelium.

Two of the better known adhesins are Le b (Leb) blood group antigen binding adhesin (BabA) (Ilver *et al.*, 1998) and sialic acid binding adhesin (SabA) (Mahdavi *et al.*, 2002). BabA binds to Leb while SabA binds to sialyl Lex (sLex) antigens present on the surface of gastric epithelial cells. Strains possessing the *babA2* gene, which encodes active BabA2, are associated with increased epithelial proliferation and inflammation (Yu, 2002). In addition, *H. pylori* strains possessing *cagA*, *vacA* (type s1) and *babA2* genes are associated with an increased risk of gastric adenocarcinoma and peptic ulcer (Gerhard *et al.*, 1999). The adherence properties of *H. pylori* can adapt quickly to host immune responses by changing the binding characteristic of either BabA or SabA due to phase variation of their respective genes. For example, expression of sLex increases in inflamed mucosa, and inflammation may therefore provide selection for strains that express SabA (Mahdavi *et al.*, 2002), (Figure 1.10).
H. pylori possesses several adhesins, such as SabA, BabA and AlpA that help it to bind and colonise gastric epithelial cells. Strains harbouring the cag PAI are then able to inject CagA into epithelial cells where it undergoes phosphorylation, leading to interference of normal cell signalling and, eventually, host cell integrity. Secreted VacA also attaches to, and eventually becomes internalised into host epithelial cells, resulting in the loss of electrolytes and other micronutrients from infected cells. Both CagA and VacA interfere with the normal function of tight junctions, causing leakage of nutrients into the gastric lumen. The presence of toxins and of secreted or shed H. pylori products, such as porins, urease and LPS, induce IL-8 secretion from infected cells and host immune cells, leading to an increase in macrophage, neutrophil and T-cell recruitment to the site of infection. In parallel, H. pylori and its products also induce an increase in pro-inflammatory cytokines, including TNF-α, IL-1β, and IFN-γ. The colonisation of epithelial cells, coupled to the increase in immune cells and pro-inflammatory chemokines/cytokines, leads to disruption of the gastric mucosal layer, induction of changes in gastric acid secretion and homeostasis, and apoptosis of infected cells. In addition, changes in the expression of surface proteins such as the Le antigens on LPS (antigen switching) can also alter the type of immune response.
induced by the infected host. Dendritic cells, which mediate the innate and adaptive immune response, react to different Le antigens to induce cycles of either Th1 (inducing cell mediated response) or Th2 (inducing antibody production) immune defence systems. Furthermore, *H. pylori* may infiltrate the epithelial sheet, possibly via tight junctions, and attach to basal lamina components, such as laminin and fibronectin, thus compromising the ECM-epithelial cell interactions. The failure to clear the *H. pylori* infection leads to a prolonged and persistent inflammatory response, which, in turn, leads to continuous impairment of the host's gastric tissue structure and function. Figure adapted from Monack et al. (2004) and Suerbaum & Michetti (2002).
Figure 1.10. Adaptation of *H. pylori* adhesins to differing Lewis antigen expression on inflamed stomach tissue. (A) *H. pylori* (in green) binds to Lewis b (Leb) blood group antigen (blue chain), expressed on the surface of gastric epithelium cells, and mediated in part by *H. pylori* BabA adhesins (green Y). (B) As the epithelial tissue becomes inflamed (gastritis) the Le pattern of expression changes on the gastric surface with the up-regulation of sialylated Le x (sLex) antigen (red triangle). *H. pylori* is able to bind intimately with the epithelial surface using SabA (red Y) to bind to Lex antigens. (C) Host immune cells (orange ‘bleb’) are recruited to sites of local inflammation and those *H. pylori* sub-clones that have lost sLex-binding capacity due to ON/OFF frame shift mutation can escape close contact with (sialylated) immune cells. The ability of *H. pylori* to vary the expression of Le antigens receptors may contribute to persistent and chronic infection. Figure taken from Mahdavi *et al.* (2002).
Further evidence of the ability of *H. pylori* to adapt its functional adhesive properties to suit the host environment was highlighted by the work of Aspholm-Hurtig *et al.* (2004). They found that *H. pylori* strains infecting an indigenous South American population, where blood group O antigen is dominant, preferentially bind blood group antigen via BabA, when exposed to all the ABO blood group antigens. In contrast, strains taken from Asian and European populations displayed no differences in binding to any of the ABO blood group antigens.

Several other adhesins have been identified but are less well characterised. HpaA (HP0797) is a neuraminyl-lactose-binding haemagglutinin (NLBH) (Evans *et al.*, 1993) which has been shown to function via a sialic-acid binding motif (Miller-Podraza *et al.*, 1997a, Miller-Podraza *et al.*, 1997b). Adherence associated lipoprotein (Alp) A and AlpB also function as adhesins, as isogenic *alpA* and *alpB* mutants displayed reduced adherence to human gastric tissue and gastric epithelial cells (Odenbreit *et al.*, 2002). Namavar *et al.* (1998) have suggested that *H. pylori* neutrophil activating protein (NapA) plays a role in adhesion to mucin molecules. LPS has also been linked with *H. pylori* adhesion to the gastric epithelium (Edwards *et al.*, 2000, Osaki *et al.*, 1998) and the ECM protein, laminin (Valkonen *et al.*, 1994).

As adhesins have been shown to be essential colonisation factors (Odenbreit, 2005, Mahdavi *et al.*, 2002, de Jonge *et al.*, 2004) the development of vaccines to block or inhibit their function may prove successful in preventing the establishment of a persistent *H. pylori* infection.

**Outer Membrane Vesicles (OMV)**

Outer membrane vesicles occur due to ‘blebbing’ and eventual shedding of small circular vesicles from the outer membrane of Gram-negative bacteria during growth and are produced by a wide variety of bacteria including *Escherichia coli*, *Pseudomonas aeruginosa* and *Neisseria* species (Kuehn and Kesty, 2005). Shedding of OMV may be a mechanism for the delivery of virulence factors and antigenic products to the gastric mucosa of the host. *H. pylori* has also been shown to shed OMV (Keenan *et al.*, 1997, Keenan *et al.*, 2000a). These OMV contain LPS, porins, and bacterial antigens, including VacA and Lpp20 lipoprotein (Keenan *et al.*, 2000a, Keenan *et al.*, 2000b). OMV have been observed in *H. pylori*-colonised human
epithelium of gastric biopsies (Fiocca et al., 1999, Keenan et al., 2000a). Purified OMV protected mice against a challenge with *H. pylori*, possibly mediated by Lpp20 (Keenan et al., 2000b, Hocking et al., 1999). Ismail et al. (2003) demonstrated that *H. pylori* OMV increased proliferation of gastric epithelial cells and at higher doses led to cell growth arrest, increased toxicity and IL-8 production. They suggest that constitutively shed OMV play a role in promoting low-grade gastritis associated with *H. pylori* infection.

**Basic structure and function of the human stomach**

The surface of the stomach is composed of a sheet of epithelial cells arranged into a series of folds, forming gastric glands (Figure 1.12). The gastric gland contains several cell types that secrete mucus, acid or hormones (Figure 1.13). Both the surface mucosa cell and mucous neck cell secrete mucus, forming a protective barrier that prevents digestion of the stomach wall. The parietal cell secretes hydrochloric acid that denatures proteins, converts pepsinogen into pepsin and kills ingested microbes. The parietal cell also secretes intrinsic factor, which is required for vitamin B₁₂ absorption in the small intestine. The chief cell secretes pepsinogen that is converted to pepsin by hydrochloric acid, and is used to break down proteins into peptides. Chief cells also secrete gastric lipase that is used to break down fats. Secretion by parietal and chief cells is stimulated by the hormone gastrin and is secreted by the G cell. Gastrin also stimulates contractions of the gastric wall allowing food to mix with the gastric juices during digestion and eventual passage to the small intestine by peristalsis. The gastric glands in the pylorus region of the stomach also contain D cells adjacent to the G cells. D cells secrete the hormone somatostatin, which inhibits the secretion of gastrin (Tortora and Derrickson, 2006, Martini, 2004).

The base of the epithelial sheet is bound to a thin basal lamina (basement membrane) produced by the basal surface of the epithelia and the underlying connective tissue of the lamina propria (Figure 1.14, A). The epithelial cells are tightly bound together by adhesins forming a barrier between the gut lumen and underlying tissue (Figure 1.14, B) (Martini, 2004). The basal lamina is composed of ECM proteins which include laminin, collagen and fibronectin. Laminin, a major component of the basal lamina,
is a large glycoprotein composed of α, β, and γ chains which combine to form a cruciform shaped molecule (Figure 1.15). Different regions of laminin bind to cell surface receptors and other ECM components. Laminin promotes several cellular activities including adhesion, growth, polarisation and differentiation (Martin and Timpl, 1987).

A large number of receptors have been found to bind laminin. These range from ECM protein, collagen IV (Rao et al., 1985); cells from gastric (Eckstein and Shur, 1989), gingival (Sengupta et al., 1991), neural (Douville et al., 1988) and muscle (Lesot et al., 1983) tissue, in addition to several microorganisms, such as Treponema pallidum (Cameron, 2003), Streptococcus anginosus (Allen and Hook, 2002), Streptococcus pyogenes (Terao et al., 2002) Echinococcus granulosus (Zhang et al., 1997), Leishmania donovani (Ghosh et al., 1999) and Staphylococcus aureus (Carneiro et al., 2004).

Although a number of laminin-binding proteins have been identified the specific amino acid motif responsible for binding to laminin is less well characterised. The amino acids responsible for binding to laminin have been identified for epithelial surface proteins, elastin binding protein (Hinek et al., 1993) and laminin receptor (Castronovo et al., 1991) in addition to bacterial proteins, Yersinia pestis plasminogen activator (Benedek et al., 2005) and laminin-binding protein from Treponema pallidum (Cameron et al., 2005). The epithelial laminin-binding protein binds via the palindromic amino acid sequence LMWWML (Castronovo et al., 1991) while the elastin-binding protein binds via the domain VVGSPSAQDFASPL (Hinek et al., 1993). The plasminogen activator protein from Y. pestis binds to laminin via the amino acid domains WSLLTPA and YPYIPTL (Benedek et al., 2005) while the laminin-binding protein from T. pallidum binds via three short amino acid sequences, PVQT, LWIQ and TAIS (Cameron et al., 2005).
Figure 1.12. **Outline of the stomach structure.** The main regions of the stomach are indicated; corpus (main body), antrum and pylorus. A section of the stomach is enlarged to show the tissue structure of the surface and gut wall. The lamina propria contains connective tissue, vascular supplies and immune cells such as macrophages and T-cells. Figure taken from Tortora & Derrickson (2006).
Figure 1.13. **Structure of the gastric gland and cell types.** The gastric glands contain different cell types that specialise in secreting mucus, acid and hormones. Parietal and chief cells are found mainly in gastric glands located in the corpus region of the stomach whereas mucous secreting cells are mainly found in gastric glands located in the antrum and pylorus region of the stomach. Figure taken from Tortora & Derrickson (2006).
Figure 1.14. Gastric epithelial cells form a tightly bound sheet attached to the basal lamina. (A) The epithelial cells and connective tissue produce a thin basal lamina composed of extracellular matrix proteins such as laminin, collagen and fibronectin. (B) The epithelial sheet is held together and to the underlying basal lamina by a number of adhesive mechanisms. Epithelial cells lining the lumen of the stomach are bound together by adhesins forming specialised cell junctions. Tight junctions seal cells together, preventing the movement of small molecules from either side of the epithelial sheet. Cadherins and integrins mechanically attach cells (and their cytoskeletons) to their neighbour or to the extracellular matrix. Gap junctions facilitate cell-cell communication. Both CagA and VacA have been shown to interfere with cell tight junctions resulting in an increase in nutrient transfer across the mucosal barrier. Figure A taken from Martini (2004) and Figure B taken from Alberts et al. (1994).
Figure 1.15. **Structure of laminin, a large heterotrimeric multiadhesive matrix protein found in all basal lamina.** Schematic model of the laminin molecule showing the typical cruciform shape formed by the $\alpha$ and $\beta$ chains. Laminin can bind to cell surface receptors and other extracellular matrix proteins, such as collagen. Binding to laminin has been suggested to play a role in the pathogenicity of many bacterial infections including *H. pylori*. Figure taken from Lodish & Darnell (2004).
The gastric mucosa and *H. pylori*

*H. pylori* only colonises gastric epithelial cells, and it is thought that this is due to its tropism for the mucin glycoprotein, MUC5AC, and Trefoil factor family 1 (TFF1) protein, expressed on the surface of the gastric mucosa (Van den Brink *et al.*, 2000, Clyne *et al.*, 2004).

*H. pylori* adheres to the gastric mucosa, triggering a cascade of events that eventually leads to epithelial tissue damage (*Figure 1.9*). The stimulation of various cytokines/chemokines, such as IL-12, IL-1, TNFα and IL-8, and other host defence mechanisms, such as macrophages and T-cells, results in cell apoptosis and impairment of cell migration processes. Furthermore, *H. pylori* infection can interfere with epithelial cell differentiation, leading to irregular cell proliferation typically associated with gastric cancer pathologies (Zarrilli *et al.*, 1999). Although binding to the gastric epithelium by adhesins, such as BabA, AlpA, and AlpB, are important for *H. pylori* colonisation (Prinz *et al.*, 2001, de Jonge *et al.*, 2004), the role played by *H. pylori* adherence to host ECM in disease progression is unclear. ECM proteins such as laminin and fibronectin are involved in signal transduction pathways that regulate cellular processes including actin rearrangement, cell cycle regulation, or cell survival (Schwartz and Shattil, 2000). *H. pylori* may undermine the mucosal integrity by penetrating the tight junctions of epithelial cells. Many microscopy studies of patient biopsies have demonstrated the presence of *H. pylori* located inside gastric epithelial cells, associated with intercellular tight junctions and on top of the basal lamina. However, other studies have failed to locate *H. pylori* intracellularly (Petersen and Krogfelt, 2003).

The ability to bind to ECM proteins may provide additional colonisation sites and enable bacteria to persist in the host. For example, pathogenic bacteria, such as *Streptococcus pyogenes* and *Neisseria gonorrhoeae*, use adherence to fibronectin as a platform for entry into host cells (Joh *et al.*, 1999). Many pathogenic bacteria have been shown to adhere to the ECM, including *Staphylococcus aureus* (Patti *et al.*, 1992, Liang *et al.*, 1995), *Streptococcus pyogenes* (Podbielski *et al.*, 1999, Hanski and Caparon, 1992) and *H. pylori* (Trust *et al.*, 1991, Valkonen *et al.*, 1993, Ascencio *et al.*, 1993). Attachment to laminin has been suggested to play a role in the
pathogenesis of numerous bacterial species (Fenno et al., 1996, Hytonen et al., 2001, Tanskanen et al., 2001) including H. pylori (Valkonen et al., 1997). Laminin-binding proteins have been identified on Treponema pallidum (Cameron, 2003), Staphylococcus aureus (Carneiro et al., 2004) and Streptococcus anginosus (Allen and Hook, 2002).

Potential H. pylori receptors for ECM components have been described before. The 58 kDa subunit from VacA has been shown to bind heparan (Utt et al., 2001) while a 25 kDa protein with laminin-binding properties mediated by LPS was described by Valkonen et al. (1994, 1997). During the course of this study Walz et al. (2005) identified SabA (70 kDa) as a laminin-binding protein. Studies, conducted in our own laboratory, suggested a possible role for laminin-binding in the pathogenesis of H. pylori infection. Briefly, H. pylori was harvested into tissue culture medium and left to stand for 20 minutes. The bacterium was collected by centrifugation and the supernatant (H. pylori extract), containing soluble factors shed or secreted by the bacterium, was incubated with T84 epithelial cells growing on a laminin-containing substrate (T84 monolayer is a model for the polarised and differentiated epithelial sheet found in the gastric mucosa and contains functional tight junctions). Coincubation of T84 cells with H. pylori extract resulted in perturbations to intracellular sodium levels (reduced), loss of barrier function (decreased transepithelial electrical resistance) and a reduction in adhesion of the monolayer to the laminin substrate. However, these physiological changes were not observed when the H. pylori extract was pre-incubated with laminin before being incubated with the T84 cells (Terres et al., 2003). Together, these data indicate an important role for the H. pylori-laminin interaction at least in vitro.

**Adaptation of H. pylori to changing gastrointestinal conditions**

H. pylori has to contend with adverse and unstable environmental conditions such as mild to strong acidity, fluctuating nutrient availability and an intense immune response from the host (de Reuse and Bereswill, 2007). The ability of H. pylori to change and adapt to its shifting environment facilitates persistence and pathogenicity. For example, catalase, superoxide dismutase, and alkylhydroperoxide reductase are
required to protect \textit{H. pylori} from reactive oxygen species released during the host immune response (Hazell \textit{et al.}, 2001). Greater motility is demonstrated under acidic conditions (Merrell \textit{et al.}, 2003a) while gene array studies show differential expression of a range of \textit{H. pylori} genes in response to variations in extracellular pH; from cell metabolism, virulence factors (such as urease, \textit{vacA} and \textit{cagA}), membrane proteins and cell motility (Gancz \textit{et al.}, 2006, Merrell \textit{et al.}, 2003a, Allan \textit{et al.}, 2001). Gene array studies have also demonstrated differential expression of genes under conditions of varying iron availability (Ernst \textit{et al.}, 2005, Merrell \textit{et al.}, 2003b, Szczebara \textit{et al.}, 1999), an important micronutrient to \textit{H. pylori}.

\textbf{Iron homeostasis}

Iron is critical for the survival of all living organisms, playing a vital role in cellular metabolism, respiration, enzyme catalysis and protein structure stabilisation (Wooldridge and Williams, 1993, Schaible and Kaufmann, 2004). Biologically available iron is not freely accessible and exists in either the predominantly oxidised Ferric form (Fe\textsuperscript{3+}) or the reduced ferrous form (Fe\textsuperscript{2+}). Ferric iron is insoluble (1.4 x 10\textsuperscript{-9} M) at pH 7, and requires an acidic pH to be reduced to a more soluble and available ferrous form (Chipperfield and Ratledge, 2000). The oxidation of Fe\textsuperscript{2+} to Fe\textsuperscript{3+} (known as the Fenton reaction) creates reactive oxygen species harmful to both host and pathogen, thus the control of iron homeostasis is of paramount importance. In humans, the majority of iron is intracellular, either coupled to metalloproteins such as haemoglobin or stored in iron storage proteins such as ferritin (Payne, 1993, Theil, 1987). Extracellular iron is stored/transported by transferrin (Tf) proteins found in serum (serum Tf) or extracellularly bound to lactoferrin (Barclay, 1985, Graham \textit{et al.}, 1992, Griffiths and Williams, 1999, Crichton and Charloateaux-Wauters, 1987). Bacteria also possess a range of iron acquisition, transport and storage proteins to achieve iron homeostasis (Schaible and Kaufmann, 2004).

Among bacteria, iron homeostasis has been thoroughly investigated in \textit{E. coli} (Andrews \textit{et al.}, 2003). \textit{E. coli} secrete high-affinity iron-binding molecules, known as siderophores, which capture iron from host proteins such as ferritin and Tf by virtue of their superior binding strength (Baig \textit{et al.}, 1986, Crosa, 1989). The iron-siderophore complex binds to outer membrane receptors, such as FecA, FepA and FhuA, where it is actively transported across the outer membrane by an ATP-
dependent TonB-ExbB-ExbD protein complex (Ratledge and Dover, 2000, Wandersman and Delepelaire, 2004). The iron-siderophore complex is then transported across the inner membrane by different ABC transporter systems, such as FecBCDE, FepBCDEFG and FhuBCD, and arrives in the cytoplasm where the iron is made available for use (Andrews et al., 2003, Wandersman and Delepelaire, 2004). E. coli can also take up iron directly via the Feo (Ferrous iron transport) system. Soluble iron, present in the extracellular environment, diffuses into the periplasm via an undefined mechanism and is actively transported into the cytoplasm by the proteins FeoA, FeoB and FeoC (Cartron et al., 2006). The iron can be stored using ferritins, such as FtnA, which can be used during periods of low iron availability (Andrews et al., 2003). Similar iron acquisition, transport and storage systems can be found in other pathogenic bacteria such as Haemophilus influenzae, Neisseria gonorrhoeae and Pseudomonas aeruginosa (Andrews et al., 2003, Ratledge and Dover, 2000).

The expression of many genes involved in bacterial iron homeostasis is under the control of the ferric uptake regulator (Fur). Fur homologues are found in numerous Gram-positive and Gram-negative bacteria (Escolar et al., 1999) including H. pylori (Bereswill et al., 1998a). H. pylori Fur has also been implicated in the regulation of acid resistance (Bijlsma et al., 2002, Bury-Mone et al., 2004, van Vliet et al., 2004), nitrogen metabolism (van Vliet et al., 2001, van Vliet et al., 2003) and oxidative stress resistance (Barnard et al., 2004, Cooksley et al., 2003, Harris et al., 2002).

Iron acquisition via siderophores is an important function in iron homeostasis, but the production of siderophores by H. pylori is unclear (Husson et al., 1993, Illingworth et al., 1993). However, the genomes of H. pylori strains 26695 and J99 contain homologues of a number of the iron transporter genes of E. coli. These include genes fecA1, fecA2, fecA3, the tonB-exbB-exbD complex and feoB. In addition, haem binding and lactoferrin binding proteins have been described for H. pylori (Worst et al., 1995, Dhaenens et al., 1997).

**Role of iron in bacterial pathogenesis**

The host's capacity to regulate the availability of free iron is an important bacterial defence mechanism. Iron is essential for the growth of pathogens and iron-binding
proteins produced by the host, such as lactoferrin in the stomach, serve to withhold free iron from infecting microbes. However, bacteria are equipped with mechanisms to extract iron from the host using siderophores or other iron-binding proteins. Indeed, host-mediated iron-restriction results in the expression of iron acquisition proteins by the infecting bacterium. In addition, the production of virulence factors from some pathogenic bacteria, such as *Escherichia*, *Shigella* and *Vibrio* species, is altered under iron-reduced conditions (Litwin and Calderwood, 1993b). Furthermore, increased levels of adhesion to epithelial cells have been observed by pathogens including *Pseudomonas aeruginosa* (Scharfman *et al.*, 1996) and *Corynebacterium diphtheriae* (Moreira Lde *et al.*, 2003) when grown under iron-reduced conditions.

Similarly, iron-restricted growth conditions have been shown to modulate protein expression by *H. pylori*. Lactoferrin and haem binding proteins have been described on *H. pylori* which displayed increased expression under iron-reduced conditions (Dhaenens *et al.*, 1997, Worst *et al.*, 1995). *H. pylori* OMV shedding also increased under iron-reduced conditions (Keenan and Allardyce, 2000). Furthermore, two proteolytic enzymes associated with the OMV membrane were produced only under iron limiting conditions.

Indeed, a combination of host cytokine polymorphisms, which reduce acid secretion, and infection with a *H. pylori* strain with an enhanced ability to obtain iron from its host as well as limiting the production of gastric acid, may explain the development, by infected patients, of refractory iron-deficient anaemia (IDA) (Annibale *et al.*, 2003, Capurso *et al.*, 2001, Dickey, 2002). A number of studies have shown that IDA in patients infected with *H. pylori* was refractory to iron therapy and was reversed only after eradication of the *H. pylori* infection (Choe *et al.*, 1999, Choe *et al.*, 2000, Choe *et al.*, 2001).
Thesis rationale and objectives

Binding of *H. pylori* to the ECM-rich basal lamina has been described for the interaction of *H. pylori* with gastric biopsies from infected patients. *H. pylori* can also bind ECM proteins directly, including laminin and collagen (Petersen and Krogfelt, 2003, Valkonen *et al.*, 1993, Trust *et al.*, 1991). In addition, an unidentified 25 kDa protein from *H. pylori* has been described that binds laminin through a mechanism mediated by *H. pylori* LPS (Valkonen *et al.*, 1993, 1994, 1997). Furthermore, studies conducted in our own laboratory identified a potentially important mechanism whereby *H. pylori*-laminin interactions could have implications for disease progression in vivo, as described earlier. Crucially, this *H. pylori*-mediated disruption to the barrier function and integrity of the polarised and differentiated epithelial monolayer could be blocked by pre-incubating the *H. pylori* extract with laminin (Terres *et al.*, 2003). Taken together, these studies clearly suggest a potential role for *H. pylori*-ECM interactions in disease progression – however, the mechanism is unclear. Thus, one objective of this study was to extend the observations made by Terrés *et al.* (2003) and others to further evaluate the interaction of *H. pylori* with ECM proteins. To this end, the interaction between *H. pylori* and a variety of ECM proteins was studied using various approaches in an attempt to elucidate the potential molecular mechanisms of interaction.

A second major objective of this thesis was to evaluate the effect of iron availability on the interaction of *H. pylori* with ECM proteins. The rationale for this is provided by the findings of others that iron availability can modulate the adhesive properties of pathogenic bacteria (Scharfman *et al.*, 1996, Moreira Lde *et al.*, 2003). In addition, it is likely that *H. pylori* inhabit an iron-restricted environment in vivo, as a primary innate defence mechanism of infected hosts is to withhold free iron to reduce its availability to the infecting pathogen. However, no information is available on the adhesive properties of *H. pylori* grown under iron-reduced conditions.
CHAPTER II

METHODS AND MATERIALS
Chapter II

Chemicals

Zwittergent 3-14® was from Calbiochem (UK). Cyanogen Bromide Activated Sepharose™ 4B was from Amersham Biosciences (UK). Silver Nitrate, Potassium Carbonate, Potassium Permanganate, Electran 2.6 premix acrylamide: N,N'-methylenebisacrylamide (29.2:0.8), all acids and alcohols were from BDH (U.K.) except trifluoroethanol (TFE) and trichloroacetic acid (TCA) which were from Sigma (U.K.). Brucella Broth was from Becton Dickinson (France). Agar and carbenicillin were from Duchafa Biochemie (The Netherlands). Agarose and IPTG were from Melford (U.K.). BHI, Tryptone and Yeast Extract Powder were from LabM (U.K.). DNA ladders were from Promega (U.K.). Coomassie Blue G-250, Bradford Reagent and Detergent Compatible Protein Assay kit reagents were from Bio-Rad (U.S.A). Fetal Calf Serum was from Gibco (Invitrogen, U.K.). 1,2-phenylenediamine dihydrochloride (OPD, 2 HCl) tablets were from DakoCytomation (Denmark). All other chemicals were from Sigma (U.K. and U.S.A.).

Antibodies

Rabbit Anti-Laminin was from Sigma (UK) and Sheep Anti-Laminin was kindly donated by Dr. Kaija Valkonen (University of Oulu, Finland). Mouse Anti-penta Histag was from Qiagen (U.K.) and Horseradish peroxidase was from DakoCytomation (Denmark).

Proteins

Laminin (L2020) and Collagen Type IV (C0543) from Engelbreth-Holm-Swarm (EHS) murine sarcoma, Fibronectin from human foreskin fibroblasts (F2518) and Lactoferrin (L0520) from human milk were from Sigma (UK). Laminin (3400-010-01) from EHS murine sarcoma was from Trevigen (AMS Biotechnology, UK). Recombinant laminin binding-protein (LBP) from Treponema pallidum (Tp0751) was kindly provided by Dr. Caroline Cameron (University of Washington, U.S.A) and used as a positive control for affinity chromatography, Western blot and ELISA experiments.
**Enzymes**

Enzymes *Hind*III and *EcoRI* were from Promega (U.K.). Trypsin – Proteomic Grade- was from Sigma (U.K.). Recombinant Pfu DNA polymerase was kindly provided by Dr. Kenneth Whelan (Trinity College Dublin, Ireland).

**Helicobacter pylori culture**

**H. pylori strains and growth conditions**

*Helicobacter pylori* (*H. pylori*) strain NCTC 11638 was obtained from the National Collection of Type Cultures (Colindale, U.K.) and used for all experiments. NCTC 11638 *sab*A-deficient spontaneous mutant was a gift from Professor Thomas Borën (Umea University, Sweden). NCTC 11638 *fur*-deficient mutant was a gift from Dr. Henry Windle (Trinity College Dublin, Ireland). Strains were maintained on plates of columbia blood agar (Oxid, U.K.) with 5% (v/v) lysed horse blood. Plates were incubated at 37°C under microaerophilic conditions generated using the MART Anoxomat system (The Netherlands) or alternatively using Gas Generating Kits (BR-0038) (Oxoid, U.K.). Liquid cultures were grown in Brain Heart Infusion (BHI) medium supplemented with 6.5% (v/v) FCS. These cultures were grown in 25 cm\(^3\) or 250 cm\(^3\) cell culture flasks (Nunclon, Denmark) under microaerophilic conditions at 37°C and shaking at 120 rpm in a SANYO orbital incubator (MIR-221U, SanyoGallenkamp, U.K.). *H. pylori* was harvested from plates at 48-72 h growth while liquid grown bacteria were used at 24-48 h of growth. To simulate iron-reduced conditions *H. pylori* was grown in liquid medium containing 100 µM Desferrioxamine methanesulfonate (Desferal), an iron chelator. Optical density readings were taken at 600 nm using a Unicam 5625 spectrometer (Unicam Ltd., U.K.)

**H. pylori culture purity and viability testing**

The *H. pylori* cultures were regularly tested to confirm their purity throughout the study. Confirmation was by a positive Rapid Urease Test and a negative Gram stain. Viability testing was carried out using colony counting on blood agar plates.
Rapid Urease Test

Broth grown bacteria were collected by centrifugation at 3,000 x g x 4 min while plate-grown bacteria were collected by scraping directly from the plate. A 1 µl loop-full of bacteria was added to 1ml Rapid Urease Test Solution [5 mM Tris-Cl, pH 6.8 + 100 mM Urea + trace phenol red] and gently mixed. The solution was left at room temperature (RT). Urease activity is confirmed by a colour change from yellow to bright pink within 1 h. A colour change was normally observed after 10 min.

Gram stain

Bacteria were harvested as described above. A 1 µl loop-full of bacteria was spread on an 19 mm microscope slide (Borozillicate glass, VWR Intl, U.K.) containing a drop of growth media. The back of the slide was heated momentarily under gas flame to permanently fix the bacteria to the slide. Slides were stored in a covered slide-box at RT if Gram staining could not be carried out immediately. The slide was incubated in Crystal Violet for 1 min, rinsed with tap water, Lugol’s Iodine for 2 min, rinsed with tap water, Acetone for 2-3 s, rinsed with tap water and finally in Dilute Carbol Fuchsin for 30 s, rinsed with tap water. The slide was viewed under 100 x by oil immersion. *H. pylori* produce a Gram-negative stain (Figure 2.1).

Figure 2.1. *H. pylori* magnified 100 x, heat fixed and Gram stained.
Viability – Colony count on blood agar plates

Overnight liquid cultures of *H. pylori* were collected by centrifugation at 500 x g x 15 min and re-suspended in 1 ml 50 mM Tris Buffered Saline (TBS) pH 7.4 [50 mM Tris-Cl + 150 mM NaCl] with or without Tween-20® (0.05% v/v) to an O.D. 600 nm of 0.5. The suspension was diluted 1/1000 with TBS and left to stand at RT. Three 50 µl samples were spread on separate columbia blood agar plates. Thereafter triplicate samples were taken every 30 min until 2.5 h elapsed. Plates were incubated at 37°C under microaerophilic conditions as each set of plates was inoculated.

Bacterial stock storage and revival

A Cryopreservation Solution of BHI was prepared containing, 10% (v/v) FCS and 20% (v/v) Glycerol. Bacteria were collected from a full plate or liquid culture (3,000 x g x 4 min) and re-suspended into a sterile cryovial (Sarstedt, Ireland) containing 0.5-1 ml ice-cold cryopreservation solution. The vial was immediately placed at −80°C for long term storage.

New culture stocks were started by inoculating a blood plate with a heaped 1µl loop-full of frozen cryopreserved bacterial suspension and incubated at 37°C for 4-5 days in microaerophilic conditions.

*H. pylori* membrane detergent fractionation

Plate grown bacteria

Plate grown bacteria were harvested into ice-cold 10 mM TBS pH 7.5 [10 mM Tris-Cl + 150 mM NaCl], 1 ml/plate. Suspended bacteria were placed on ice, adjusted to an O.D. 600 nm of 3, and then centrifuged at 7,500 x g x 10 min at 4°C. The pellet was washed 3 times in ice-cold 10 mM TBS, by centrifugation at 12,000 x g x 15 min at 4°C. The pellet of bacteria was suspended in 5 ml ice-cold 10 mM TBS and sonicated on ice, 8 x 20 s at 10 microns using a Soniprep 150 (SanyoGallenkamp, U.K.) sonicator. The sample was centrifuged at 1,000 x g x 15 min at 4°C to remove cell debris. 1 mM PMSF was added to the supernatant and the whole envelope fraction was collected by centrifugation at 100,000 x g x 1 h at 4°C. The supernatant containing mostly cytoplasmic proteins was aliquoted and stored at −20°C. The pellet
was suspended in 1ml ice-cold 10 mM TBS containing 1% (v/v) Zwittergent® 3-14 and left at RT for 30 min with occasional mixing. The detergent insoluble fraction was collected by ultra-centrifugation at 100,000 x g x 30 min at 4°C. Both insoluble (composed of mostly inner membrane) and soluble (composed of mostly outer membrane) fractions were stored at -20°C.

Plate grown bacteria were used to isolate potential laminin-binding proteins.

**Liquid grown bacteria**

Pellets from liquid grown cultures of *H. pylori*, stored at -20°C, were thawed, combined and washed twice in 50 mM TBS pH 7.4. The final pellet was re-suspended in 3 ml 50 mM TBS to an O.D. 600 nm between 4 and 7. 1 mM PMSF was added before sonicating on ice, 8 x 20 s at 10 microns using a Soniprep 150 (SanyoGallenkamp, U.K.) sonicator. The sample was centrifuged at 1,000 x g x 15 min at 4°C to remove cell debris. The whole envelope fraction was collected by centrifugation at 100,000 x g x 1 h at 4°C. The supernatant containing mostly cytoplasmic proteins was aliquoted and stored at -20°C. The pellet was suspended in 1ml ice-cold 50 mM TBS containing 1% (v/v) Zwittergent® 3-14 and left at RT for 30 min with occasional mixing. The detergent insoluble fraction was collected by ultra-centrifugation at 100,000 x g x 30 min at 4°C. Both insoluble (composed of mostly inner membrane) and soluble (composed of mostly outer membrane) fractions were stored at -20°C.

**H. pylori membrane trifluoroethanol fractionation**

Pellets from liquid grown cultures of *H. pylori*, stored at -20°C, were thawed, combined and washed twice in 50 mM TBS pH 7.4. The final pellet was re-suspended in 1 ml 50 mM TBS to an O.D. 600 nm between 4 and 7. 1 mM PMSF was added before sonicating on ice, 8 x 20 s at 10 microns using a Soniprep 150 (SanyoGallenkamp, U.K.) sonicator. The sample was spun at 4,500 x g x 10 min at 4°C to remove cell debris. The supernatant was transferred to another 1 ml tube and spun at 20,000 x g x 30 min at 4°C (Deshusses et al., 2003). The supernatant was
removed and the pellet further separated by TFE phase partition (Deshusses et al., 2003, Molloy et al., 1999). The pellet was re-suspended in 150 µl of 50 mM ammonium bicarbonate. 1 ml of a 2:1 solution of TFE:CHCl₃ was added with strong shaking and maintained at 4°C for 1 h with periodic vortexing. The mixture was separated into 3 phases by centrifugation at 10,000 x g x 4 min at 4°C. Both the top phase (TFE rich) and the bottom phase (CHCl₃ rich) were taken off with gel loading tips (narrow bore) while the middle phase (insoluble) usually stuck to the side of the tube. All phases were concentrated by vacuum centrifugation – medium heat (DNA110, Savant, U.S.A.)

**H. pylori – protease digestion of surface exposed domains of membrane proteins (‘shaving’)**

All reagents were sterile and sample preparations were carried out in a re-circulating hood to minimise foreign protein contamination. Trypsin was reconstituted in PBS-Mg pH 7.4 [PBS (Gibco – 10010 sterile liquid) + 5 mM MgCl₂]. The reaction volume was 200 µl.

Liquid cultures of *H. pylori* grown in normal conditions or 47-48 h cultures grown in iron-reduced conditions were collected in PBS-Mg pH 7.4 by centrifugation at 3,000 x g x 4 min and washed twice at 500 x g x 4 min. The bacteria were re-suspended in PBS-Mg containing 100 µg/ml Trypsin (Sigma, U.K) with 5 mM DTT, and incubated in a water bath at 37°C for 1 h. The protease reaction was quenched by adding 5 mM PMSF. The bacteria were collected by centrifugation at 20,000 x g x 4 min. The supernatant was transferred to another sterile 1 ml tube and spun at 20,000 x g x 4 min. This was preformed 3 times. The supernatant was concentrated by vacuum centrifugation – medium heat (DNA110, Savant, U.S.A.).
Protein concentration estimation, separation & detection

Concentration estimation
Protein concentration was determined using Bio-Rad Protein Assay Kit (U.K.), based on the Lowry assay, according to the manufacturer instructions. A standard curve was prepared using serially diluted BSA (20mg/ml), solubilised in appropriate buffer. The absorbance of protein solutions was read at 690 nm on a Tecan Spectra Fluor Plus spectrophotometer (Tecan Ltd., U.K.).

Sample and molecular weight standard preparation
Proteins were re-suspended in Sample Buffer pH 6.8 [62.5 mM Tris-Cl + 10% (v/v) Glycerol + 2% (w/v) SDS, trace Bromophenol Blue] as required. The protein samples were boiled at 100°C for 5 min and centrifuged at 10,000 x g briefly (30 s) to remove any insoluble material. Sigma Broad Range molecular weight standards (5 μl = 10 μg) were run alongside samples. When required proteins were precipitated with either acetone or trichloroacetic acid (TCA) using standard procedures.

SDS – Polyacrylamide Gel Electrophoresis (SDS-PAGE)
All SDS-PAGE gels were carried out using the ATTO gel electrophoresis system (ATTO Corporation, Japan). Proteins were separated under reducing conditions using a discontinuous buffer system as described by Laemmli (1970) and adapted by Sambrook & Gething (1989). Resolving and stacking acrylamide gels were prepared according to the required percentage acrylamide in the order indicated in Tables 2.1 and 2.2. APS (fresh) and TEMED were added last with gentle mixing. Electrophoresis was carried out in a Running Buffer pH 8.3 [25 mM Tris-Cl base + 192 mM glycine + 0.1% (w/v) SDS] at 35 mA/gel or 175 volts until the dye front had reached the base of the gel.
**Table 2.1: Composition of resolving gel for SDS-PAGE**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>7.5%</th>
<th>10%</th>
<th>12.5%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acryl/Bis</td>
<td>5.0 ml</td>
<td>6.7 ml</td>
<td>8.3 ml</td>
<td>13.2 ml</td>
</tr>
<tr>
<td>1.5M Tris-Cl, pH 8.8</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>9.7 ml</td>
<td>8.0 ml</td>
<td>6.6 ml</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 μl</td>
<td>10 μl</td>
<td>10 μl</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

**Table 2.2: Composition of stacking gel for SDS-PAGE**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acryl/Bis</td>
<td>0.88 ml</td>
</tr>
<tr>
<td>0.5 M Tris-Cl, pH 6.8</td>
<td>1.66 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>4.06 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>66 μl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 μl</td>
</tr>
</tbody>
</table>
Western blotting

Proteins separated by SDS-PAGE were immobilised onto either PVDF (0.45 µm, Pall Life Sciences, U.K.) or Nitrocellulose (0.45 µm, Amersham Biosciences, U.K.) membrane using the ATTO semi-dry transfer system (ATTO Corporation, Japan) as described by Towbin (1979). Prior to use the PVDF membrane was saturated with methanol for 10-15 sec while the nitrocellulose membrane was saturated with distilled water for 1 min. The membrane (PVDF or nitrocellulose) was then equilibrated in Transfer Buffer pH 8.5 [25 mM Tris-Cl base + 192 mM glycine + 20% (v/v) methanol + 0.1% (w/v) SDS] for 10-15 min. Whatmann filter paper (3 mm, Whatmann, U.K.) of similar dimension to membrane was also saturated in Transfer Buffer before the assembly of the blot stack (cathode; filter paper; membrane; filter paper and finally anode). Electrophoresis transfer was performed at 100 mA/gel for 1 h (1.7 mA/cm²). Following the transfer the lane containing the MW marker was cut from the membrane, stained with Coomassie Blue R-250 (1 min) and destained in 50% (v/v) methanol. The remaining membrane was processed by immuno detection.

Immunodetection & development

Membrane blocking and antibody incubations were carried out with gentle shaking. Blots were stored at 4°C or −20°C.

Laminin-Binding Protein detection (Laminin Blot)

The Laminin Blot used nitrocellulose membrane. Non-specific sites on the nitrocellulose membrane were blocked with freshly prepared BSA - Block Buffer I, pH 7.4 - [20 mM Tris Cl + 500 mM NaCl + 0.1 % (v/v) Tween-20® (TBS-hs) + 2.5% (w/v) BSA] for 1 h at RT. The blot was then washed 3 times (7.5 min) with Wash Buffer I, pH 7.4 [20 mM TBS-hs] and incubated with laminin (5 µg/ml) in Wash Buffer I for 1 h at RT. After 3 further washes the membrane was incubated with anti-laminin (antibody produced in rabbit) diluted in Block Buffer I (1/800) for 1 h at RT or overnight at 4°C. The blot was then washed 3 times and incubated with anti-rabbit immunoglobulins (antibody produced in swine) conjugated to HRP in Block Buffer I for 1 h at RT. Following HRP Ab incubation, the blots were washed 3 times and developed. Detection of immunoblots was by ECL kit (Amersham Bioscience, U.K.)
according to the manufacturer's instructions. ECL buffers were placed on the surface of the membrane for 1 minute, removed and placed between acetate sheets before being exposed to film (CP-BU new, Agfa, Belgium). Exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa-Genaert AG, Germany).

**His-Tag and other protein detection**

Non-specific sites on the membrane (PVDF or Nitrocellulose) were blocked with freshly prepared non-fat skimmed milk - Block Buffer II, pH7.4 - [20 mM Tris-Cl + 150 mM NaCl (TBS) + 2.5% (w/v) non-fat skimmed milk powder (Marvel, U.K.)] for 1 h at RT. The blot was then washed 3 times (5 min) with Wash Buffer II [20mM TBS] and incubated with primary Ab diluted in Block Buffer II (1/3000) for 1 h at RT or overnight at 4°C. The blot was then washed 3 times and incubated with relevant HRP- conjugated Ab in Block Buffer II (1/2000) for 1 hour at RT. Following HRP Ab incubation, the blots were washed 3 times and developed – blots were stored in TBS at 4°C if development could not be undertaken immediately. Detection of immunoblots was by ECL kit as described previously.

**Two Dimensional Electrophoresis**

**Isoelectric Focusing (IEF) – 1st dimension**

IEF was carried out using the Ettan IPGphor II system (Amersham Biosciences, U.K.) according to manufacturer instructions. Protein samples were solubilised in 2DE Rehydration Buffer, (Table 2.3), with gentle vortexing at RT between 30 min to 3 hours. Insoluble material was collected by centrifugation at 18,000 x g x 3 min. The sample was loaded into the centre of the strip holder and the Immobilised pH Gradient (IPG) strip (Amersham Biosciences, U.K.) was gently moved over the sample until sufficiently immersed. The strip was hydrated and focused accordingly (Table 2.4). Focused strips were stored at -20°C, in 10 ml pipettes, unless 2nd dimension separation was carried out immediately.
Table 2.3: 2DE Rehydration Buffer components

<table>
<thead>
<tr>
<th>Reagents</th>
<th>40% TFE</th>
<th>0.25% SDS</th>
<th>1% Zwittergent® 3-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>5 M</td>
<td>7 M</td>
<td>5.5 M</td>
</tr>
<tr>
<td>Thiourea</td>
<td>2 M</td>
<td>2 M</td>
<td>2 M</td>
</tr>
<tr>
<td>CHAPS</td>
<td>2.5% (w/v)</td>
<td>2% (w/v)</td>
<td>2% (w/v)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-</td>
<td>10% (v/v)</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Bromophenol Blue</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
<tr>
<td>Zwittergent® 3-14</td>
<td>-</td>
<td>-</td>
<td>1% (w/v)</td>
</tr>
<tr>
<td>SDS</td>
<td>-</td>
<td>0.25% (w/v)</td>
<td>-</td>
</tr>
<tr>
<td>TFE</td>
<td>40%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>*DTT</td>
<td>50 mM</td>
<td>50 mM</td>
<td>50 mM</td>
</tr>
<tr>
<td>*IPG Buffer</td>
<td>-</td>
<td>1% (v/v)</td>
<td>1% (v/v)</td>
</tr>
</tbody>
</table>

* DTT & IPG buffer are added fresh

Table 2.4: Program used to separate protein samples according to their isoelectric point

<table>
<thead>
<tr>
<th>Gradient Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rehydration</td>
</tr>
<tr>
<td>Gradient</td>
</tr>
<tr>
<td>Gradient</td>
</tr>
<tr>
<td>Hold</td>
</tr>
<tr>
<td>Hold</td>
</tr>
</tbody>
</table>

** 10 hours ≥ X ≤ 15 hours (adjust time for convenience)

***Adjust hours to give a final run total of 70,000 OR 100,083 volt hours
Size Separation – 2nd dimension

The IPG strip was incubated with 10 ml SDS Equilibrium Buffer pH 8.8 [50 mM Tris-Cl + 6 M Urea + 30% (v/v) Glycerol + 2% (w/v) SDS + trace Bromophenol Blue] containing DTT (10 mg/ml) for 15 min, in 10 ml sterile pipette, with gentle rocking followed by a 15 min incubation with 10ml fresh SDS Equilibrium Buffer containing iodoacetamide (25 mg/ml). The strip was placed on top of a resolving gel (see SDS-PAGE section). Electrophoresis was carried out at 15 mA/gel until the proteins entered the resolving gel and increased to 30 mA/gel thereafter.

Gel staining

Coomassie Blue

Gels were placed in Coomassie Blue (R-250) [0.025% R-250 (w/v) + 50% (v/v) methanol + 10% (v/v) glacial acetic acid] with gentle shaking for a minimum of 4 h, usually overnight, and destained with Destain I [50% (v/v) methanol + 10% (v/v) glacial acetic acid] to remove excess stain and further destained with Destain II [5% (v/v) methanol + 10% (v/v) glacial acetic acid] until the desired background intensity was achieved. The gel was stored in 10% (v/v) acetic acid solution at RT or 4°C.

Colloidal Coomassie Blue

Gels were fixed overnight in Colloidal Fixing Solution [50% (v/v) methanol + 2% (v/v) phosphoric acid] with gentle shaking. The gel was washed twice in sterile water for 5 min before incubating in Colloidal Incubation Solution [34% (v/v) methanol + 2% (v/v) phosphoric acid + 17% (w/v) ammonium sulphate] for 1 hr. The Incubation Solution was poured off and the gel was incubated in Coomassie Stain G-250 [Incubation solution + 0.025% (w/v) Coomassie Blue G-250] for 2-4 days and destained in 25% (v/v) methanol for 15 min (or until desired background intensity was achieved). The gel was stored in 10% (v/v) acetic acid solution at RT or 4°C.

Silver stain

Silver staining was performed as described by Ansorge (1985). Staining was carried out in a glass container with gentle shaking. Briefly, the gel was fixed with Silver Fix Solution I [50% (v/v) methanol + 12% (v/v) glacial acetic acid] for 10 min and Silver
Fix Solution II \([10\% (v/v) \text{ ethanol} + 5\% (v/v) \text{ glacial acetic acid}]\) for at least 10 min followed by incubations with 0.06\% (w/v) potassium permanganate for 5 min and 0.1\% (w/v) potassium carbonate for 5 min. After three 10 min washes in distilled water the gel was incubated with 0.1\% (w/v) silver nitrate for 10 min followed by two short rinses in distilled water. The gel was developed in Silver Develop Solution \([2\% (w/v) \text{ potassium carbonate} + 0.015\% (v/v) \text{ formaldehyde}]\) until the desired staining intensity was achieved and the reaction stopped by incubating with 1\% (v/v) glacial acetic acid solution. The gel was washed in distilled water for 10 min and stored in 1\% (v/v) glacial acetic acid in the dark at RT or 4°C.

**Blum silver stain – modified (BSS)**

Blum silver staining protocol was modified according to Mortz *et al.*, (2001). Staining was carried out in a stainless steel container with gentle shaking. The gel was fixed with BSS Fix Solution \([40\% (v/v) \text{ ethanol} + 10\% (v/v) \text{ glacial acetic acid}]\) for 1 hr and washed with 30\% (v/v) ethanol twice for 20 min followed by a final wash with distilled water for 20 min. The gel was incubated with cold 0.1\% (w/v) silver nitrate for 20 min and washed three times in distilled water for 20 sec. The gel was transferred to a new container and washed with distilled water for 1 min. The gel was then incubated in 0.02\% (w/v) Silver Fix Solution II \([10\% (v/v) \text{ ethanol} + 5\% (v/v) \text{ glacial acetic acid}]\) for at least 10 min followed by incubations with 0.06\% (w/v) potassium permanganate for 5 min and 0.1\% (w/v) potassium carbonate for 5 min. After three 10 min washes in distilled water the gel was incubated with 0.1\% (w/v) silver nitrate for 10 min followed by two short rinses in distilled water. The gel was developed in 3\% (w/v) sodium carbonate + 0.05\% (v/v) formaldehyde until the desired staining intensity was achieved and quickly washed with distilled water for 20 sec. The reaction was stopped by incubating with 5\% (v/v) glacial acetic acid solution. The gel was washed in distilled water for 10 min and stored in 1\% (v/v) glacial acetic acid in the dark at RT or 4°C.
iTRAQ™ labelling

Labelling of whole membrane samples was performed according to the manufacturer instructions (Applied Biosystems, U.K.). Briefly, membrane proteins, previously precipitated with acetone, underwent reducing, denaturing and cysteine block steps before being subjected to digestion with trypsin enzyme at 37°C for 16 h. The relevant iTRAQ™ reagent was added to the digested protein sample (Reagent 114 was used for proteins isolated from *H. pylori* grown under normal (N) growth conditions. Reagent 117 was used for proteins isolated from *H. pylori* grown under iron-reduced (IR) growth conditions). Each sample was incubated at RT for 1 h before both N and IR samples were combined into a single tube. The combined sample was concentrated by vacuum centrifugation – medium heat (DNA110, Savant, U.S.A.) and sent for MS analysis. Before MS analysis took place the sample was subjected to cation exchange chromatography to help isolate the digested peptides for analysis.

Labelling of surface exposed peptides, obtained by ‘shaving’ the membrane of *H. pylori*, was performed as described above except the trypsin digest step was omitted (as the peptides had been previously digested with trypsin during the preparation and collection of these from the membrane surface of *H. pylori*).
Affinity chromatography

Matrix Preparation

Cyanogen bromide-activated Sepharose

Laminin was coupled to CNBr-activated Sepharose 4B beads (Amersham Bioscience, UK) according to the manufacturer recommendations. 2 mg (1 mg/ml) laminin was dialysed in Coupling Buffer pH 8.3 [0.1 M sodium hydrogen carbonate + 0.5 M NaCl] at 4°C and incubated with 1.5 ml settled beads for 1h at RT or overnight at 4°C. The beads were washed 3 times with 3 volumes of ice-cold Coupling Buffer and blocked with 1 M ethanolamine pH 8.0 at RT. The beads were then washed with 4 cycles of cold 0.1 M acetate pH 4.0 containing 0.5 M NaCl and cold 0.1 M Tris-Cl pH 8.0 containing 0.5 M NaCl. The coated beads were stored at 4°C.

Activated immunoaffinity agarose - Affi-Gel®

Laminin was coupled to either Affi-Gel® 10 or Affi-Gel® 15 (BioRad, UK) according to the manufacturer recommendations. 2 mg (1 mg/ml) laminin was dialysed against 10 mM HEPES pH 7.5 at 4°C and incubated with 1.5 ml settled beads for 1h at RT or 4h at 4°C. The beads were washed twice with 3 volumes of HEPES and blocked with 1 M ethanolamine pH 8.0 at RT. The beads were then washed twice with HEPES. The coated beads were stored at 4°C.

Column preparation and use

The preparation and use of the columns was carried out at RT. Mini-columns (20-50 μl) were prepared using 1.5 ml sterile eppendorf tubes while larger columns (1ml volume) were made using a HR 5/5 column (Amersham Biosciences, UK). The mini-columns were manually operated and the larger columns were attached to and operated by an AKTA FPLC system (Amersham Biosciences, U.K.). The columns were washed with 10 mM TBS pH 7.5 (10 mM Tris-Cl + 150 mM NaCl) before and after use. Outer membrane fraction, diluted to 0.1% (v/v) Zwittergent® 3-14 were centrifuged at 20,000 x g x 20 min before being incubated on the column for 1h at RT. Unbound protein was washed away with 10 mM TBS. Non-specifically bound proteins were removed with 10 mM Tris-Cl, pH 7.5, containing 0.5 M NaCl. Bound proteins were removed from the beads using buffer containing 2% (v/v) SDS [62.5
mM Tris-Cl, pH 6.8, + 10% (v/v) Glycerol + 2% (w/v) SDS, trace Bromophenol Blue] at 100°C for 5 min.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISA analysis for laminin-binding was performed as described by Cameron (2003). 96 well Maxisorp plates (Nunclon, Denmark) were coated for 1.5 h in a moist environment at 37°C with 100 µl (0.5 µg) of either laminin, fibronectin or BSA at 5 µg/ml in 10 mM Phosphate Buffered saline (PBS) pH 7.2 [Gibco PBS tablet; Cat No: 18912-014]. Wells were washed three times with PBS Wash Buffer pH 7.2 [PBS + 0.05% (v/v) Tween-20®] and blocked for 30 min at 37°C with 200 µl PBS Block Buffer pH 7.2 [PBS + 1% (w/v) BSA + 0.05% (v/v) Tween-20®]. Following three washes 100 µl of protein was added and the plates incubated for 1.5 h at 37°C. Wells were washed six times and 100 µl of Mouse penta His-Tag antibody (diluted 1/3000 in Block Buffer) was added and incubated for 1 h at 37°C. Following six washes 100 µl of rabbit anti-mouse HRP (diluted 1/4000) was added and incubated for 1 h at 37°C. The wells were washed six times and bound antibody was detected using OPD tablets (DakoCytomation, Denmark) as per manufacturer instructions. The absorbance was measured at 490 nm on a Wallac Victor2 microplate reader. All determinations were carried out in duplicate.
Surface Plasmon Resonance analysis

All experiments were carried out at RT on Biacore 1000 & 3000 systems (Biacore, Sweden) using Biacore CM-5 research grade sensor chips. Buffers were autoclaved, filtered (0.2 μm) and degassed before use. Bacterial suspensions passed over the sensor chip surface were prepared under sterile conditions in 50 mM TBS pH 7.4 (50 mM Tris-Cl + 150 mM NaCl). Before, during and after use the chip surface was regenerated with fresh 20 mM NaOH -injected 5 μl at flow rate of 10 μl/min. The running buffer was 10mM HEPES Buffered Solution (HBS) pH 7.4 [10 mM HEPES + 166mM NaCl + 3.4 mM EDTA + 0.05 % (v/v) Tween-20®].

Preconcentration

Preconcentration was used to determine the optimum pH to immobilise the ligand to the channel surface of the chip. Each ligand (final concentration: 25 μg/ml) was diluted in fresh 10mM sodium acetate solution of increasing pH; from pH 3.8 – pH 5 in 0.2 pH unit increments. Ligand solution (5 μl (0.125 μg)) was injected at a flow rate of 5 μl/min over the channel surface.

Precondition

Following preconcentration the channel surface was cleaned twice with 5 μl of 50 mM NaOH containing 0.05% (w/v) SDS (flow rate of 10 μl/min) in preparation for ligand immobilisation.

Immobilisation

Ligands were immobilised to the channel surface at the optimum pH, determined during preconcentration, according to the manufacturer instructions. The chip surface was activated with fresh ethyl-N-(3diethylaminopropyl)carbodiimide (EDC) / N-hydroxysuccinimide (NHS) mix and 100 μl (10 μg) of ligand (100 μg/ml in fresh 10 mM sodium acetate) was passed over the channel at a flow rate of 5 μl/min. The channel surface was blocked with fresh 1 M ethanolamine and washed with 5 mM NaOH. The chips were stored at 4°C when not in use.

The immobilisation of hemin included some additional steps. Essentially, the carboxyl groups from both the chip surface and hemin were activated and the hemin
was immobilised to the chip surface using ethylene diamine (one amine binds to chip surface while the remaining amine binds to the hemin molecule). Following the activation of the chip surface with EDC/NHS it was capped with 50 μl 1M ethylene diamine. 100 μl (5 μg) of Hemin (50 μg/ml in fresh EDC/NHS mix) was passed over the channel at a flow rate of 5 μl/min. The channel surface was blocked with fresh 1 M ethanolamine and washed with 5 mM NaOH. [Note: the stock solution of hemin were solubilised in DMSO (2.5 mg/ml) in preparation for use in the immobilisation step].

**Preparation of bacteria**

Bacteria were collected by centrifugation at 500 x g x 10 min at RT, washed in 50 mM TBS pH 7.4 [50 mM Tris-Cl + 150 mM NaCl] before re-suspension in a final volume of 1 ml to an O.D. 600 nm of 0.4. This bacterial suspension (20 μl) was passed over the chip surface at a flow rate of 5 μl/min. The chip surface was regenerated with 20 mM NaOH. This cycle was repeated in duplicate or triplicate. Repeat assays were carried out on separate days.

All Biacore work was carried out in the School of Biotechnology at Dublin City University, Ireland under the guidance of Dr. Stephen Hearty and Professor Richard O’ Kennedy. Cultures of *H. pylori* were transported to DCU under microaerophilic conditions and stored at 30-37°C in a static incubator each day while the experiments were conducted.
Production of recombinant membrane proteins of H. pylori

DNA amplification by Polymerase Chain Reaction

Polymerase Chain Reactions (PCR) were performed in a PTC-200 thermal cycler (MJ Research, U.S.A.) with Pfu DNA Polymerase. Primers were from Operon Biotechnologies (Cologne, Germany) and Invitrogen (Paisley, UK).

H. pylori chromosomal DNA was amplified as follows. A single colony of bacteria was partially harvested by touching with a 200 μl pipette tip. The remaining colony was used to inoculate a plate for bacterial storage and recovery. The tip was immersed in 1 μl of sterile water to remove bacteria. This was mixed with 1.5x PCR Buffer (Promega, U.K.), primers (0.6 μM), dNTP (0.6 mM, Promega, U.K.), 1.5μl Pfu enzyme and brought to 50 μl with sterile water. The bacteria were lysed at 94°C for 5 min followed by DNA amplification: 35 cycles of DNA denaturing at 94°C for 30 s, primer annealing at 56°C for 1 min 20 s and primer extension at 72°C for 2 min 20 s. This method and PCR program was also used for screening transformed E.coli for recombinant plasmid DNA.

PCR amplified DNA was further amplified by PCR in a reaction volume of 70 μl as described above using the following PCR program: 25 cycles of DNA denaturing at 94°C for 30 s, primer annealing at 56°C for 1 min 20 s and primer extension at 72°C for 2 min.

Plasmid DNA and PCR amplified DNA was collected and purified using QIAPREP® Mini Prep Kit. QIAquick® and MiniElute® PCR Purification kits (Qiagen, U.K.) Plasmid DNA and PCR amplified DNA was stored in 10 mM TE pH 8 [10 mM Tris-Cl + 1mM EDTA] at RT, 4°C or −20°C.

DNA digest & DNA ligation

HindIII and EcoRI (Promega, U.K.) restriction enzymes were used to create cohesive ends on both the H. pylori genomic DNA and vector pET28 (a) DNA (Novagen, U.K.) as per the manufacturer instructions. HindIII digests, in a volume of 50 μl,
were carried out in a water bath at 37°C for at least 3 h. The gene/vector DNA was then purified using a PCR Purification Kit (Qiagen, U.K.) and subjected to an EcoRI digest, again in 50 µl volume in a 37°C water bath for at least 3 h. The double digested gene/vector DNA product was purified and ligated together using T4 DNA Ligase (Invitrogen, U.K.) as per manufacturer instructions. The reaction was carried out in 20 µl volume and incubated at 16°C overnight in a PCR machine. The end product was cloned into *E.coli*.

**DNA quality, concentration & sequence analysis**

The presence and approximate quality of DNA was determined by running samples on EtBr agarose gels (Table 2.5). DNA was mixed with 6x loading dye (Promega, U.K.) and electrophoresis was carried out at 120 volts using 40 mM TAE running buffer [40 mM Tris-Cl + 0.1% (v/v) acetic acid + 1 mM EDTA] until the dye front had reached the base of the gel. The quality and quantity of DNA was determined by spectrometry at 260nm, 280nm using a spectrophotometer (ND-1000, Nano Drop (Labtech, U.K.) or Gene Quant Pro (Amersham Bioscience, U.K.).

<table>
<thead>
<tr>
<th>Table 2.5: EtBr Agarose gel mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Agarose</td>
</tr>
<tr>
<td>TAE pH8</td>
</tr>
<tr>
<td>EtBr (10 mg/ml)</td>
</tr>
</tbody>
</table>

The identity of a cloned insert was confirmed by sequencing using the dideoxyribonucleotidure chain termination method performed by the Department of Biochemistry, University of Cambridge, U.K.
Preparation of competent *Escherichia coli* cells for transformation

**Chemically Competent Cells**

An overnight culture of *Escherichia coli* (*E. coli*) was used to inoculate 30ml LB Broth [5% (w/v) NaCl + 10% (w/v) Yeast Extract Powder (LabM, U.K.) + 20% (w/v) Tryptone Water (LabM, U.K.)] medium containing the appropriate selective antibiotic (Table 2.6). The bacteria were incubated at 37°C and rotated at 150 rpm to an O.D. 600 nm of 0.4 and harvested at 3,000 x g x 5 min at 4°C. The bacterial pellet was re-suspended in 30 ml ice-cold 100 mM CaCl₂ and incubated on ice for 45 min. The bacteria were collected, re-suspended in 0.5 ml ice-cold CaCl₂ and stored overnight at 4°C for use the following day. Alternatively the bacteria were re-suspended in 0.5 ml CaCl₂ with 10% (v/v) glycerol, and stored at -80°C for later use.

**Electroporetic Competent Cells**

An overnight culture of *E. coli* was used to inoculate 30 ml LB broth medium containing the appropriate selective antibiotic (Table 2.6). The bacteria were incubated at 37°C and rotated at 150 rpm to an O.D. 600 nm of 0.4 and harvested at 3,000 x g x 5 min at 4°C. The bacterial pellet was re-suspended in 10 ml sterile water. The bacteria were collected, re-suspended in 1 ml sterile water and used immediately for transformation.
Table 2.6: *E. coli* host strain characteristics

<table>
<thead>
<tr>
<th>Strain</th>
<th>Derivative/Supplier</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>K12/Novagen</td>
<td>F(^{+}) φ80d/lacZΔM15 recA1 endA1 gyrA26 thi-1 hsdR17 supE44 relA1 deoR Δ(lacZYA-argF)U169</td>
</tr>
<tr>
<td>M15[pREP4]</td>
<td>K-12/Qiagen</td>
<td>F(^{−}) lac ara gal mil, RecA(^{−})  uvr lon pRep4[lacI kan(^{R})]</td>
</tr>
<tr>
<td>Top10</td>
<td>K-12/Invitrogen</td>
<td>F(^{−}) mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str(^{R})) endA1 nupG</td>
</tr>
<tr>
<td>BL21pLysS</td>
<td>B/Novagen</td>
<td>F(^{−}) ompT hsdS(<em>{B})(r(</em>{B})m(_{B})) gal dem (DE3) pLysS (Cm(^{R}))</td>
</tr>
</tbody>
</table>

Transformation of *Escherichia coli* cells with plasmid DNA

Chemical Transformation

Chemically competent *E.coli* was transformed with plasmid DNA by incubating 100 µl of competent bacteria with between 5 – 100 ng of plasmid DNA on ice for 30 min. The bacteria were heat shocked for 2 min at 42°C and cooled on ice for 1 min. 200 µl of warm LB medium was added and the bacteria left at 37°C for 1 h. Transformed bacteria were plated onto selective LB agar plates [20% (w/v) LB Broth powder (Sigma, U.K.) + 14% agar] and left overnight at 37°C. Transformed colonies were screened by PCR. Single colony purification was performed on successful candidates where a single colony was selected and grown on selective LB agar plates overnight at 37°C. This was performed three times.

Electroporation Transformation

Electroporetic competent *E.coli* was transformed with plasmid DNA by incubating 45 µl of competent bacteria with between 5 – 100 ng of plasmid DNA on ice for 1 min.
The mixture was transferred to a 400 µl porator cuvette with a 2 mm gap (Eppendorf, Germany). Electroporation was carried out using an Eppendorf Multiporator Bacteria Module (Eppendorf, Germany) at 2,500 volts for 5 milliseconds. Warm LB (1 ml) medium was added immediately after electroporation and the bacteria left to incubate at 37°C for 1 h. Transformed bacteria were plated onto selective LB agar plates and left overnight at 37°C. Transformed colonies were screened by PCR. Single colony purification was performed on successful candidates where a single colony was selected and grown on selective LB agar plates overnight at 37°C. This was performed three times.

**Expression and purification of *H. pylori* recombinant proteins**

An overnight culture of *E. coli* was used to inoculate 30 ml LB medium containing the appropriate selective antibiotic (Table 2.6). The bacteria were incubated at 37°C and rotated at 150 rpm to an O.D. 600 nm of 0.4. Protein expression was induced over 3-4 h by adding 1 mM IPTG. Induced bacteria were collected at 3,000 x g x 4°C and stored at -20°C. Recombinant protein was purified using a Ni-NTA spin column (Qiagen, U.K.) under native conditions according to manufacturer instructions. *E.coli* was re-suspended in Lysis Buffer pH 8 [50 mM Na$_2$H$_2$PO$_4$ + 300 mM NaCl + 10 mM imidazole] with 5 mM PMSF and sonicated on ice using a Soniprep 150 (SanyoGallenkamp, U.K.) sonicator. Cellular debris was collected at 1,000 x g x 10 min at 4°C. The supernatant was filtered (0.45 µm) and loaded onto the Ni-NTA column. The column was washed 3 times with Wash Buffer pH 8 [50 mM Na$_2$H$_2$PO$_4$ + 300 mM NaCl + 40 mM imidazole] and bound protein was eluted with Elution Buffer pH 8 [50 mM Na$_2$H$_2$PO$_4$ + 300 mM NaCl + 250 mM imidazole].
Cloning, expression and purification of recombinant HpaA (HP0797)

A plasmid construct containing the hpaA gene (hp0797) - H. pylori strain 26695- was kindly provided by Dr. Petra Voland (Technical University of Munich, Germany) using the following primers:

Forward: ctttaggtgcagcggtggtg
Reverse: aatctctggcgcttttggga

The following primers were used to screen for positive plasmids.
Forward: gttattgtgtgtcatac
Reverse: atcgatctcagtggtatttgtga

The gene was inserted into pQE-TriSystem (Qiagen, U.K.) vector with a C-terminal His-tag (Figure 2.2). Cloning was carried out using chemically competent E. coli DH5α, while electroporetic competent E. coli M15 was used for expression (Table 2.7). Expression was induced with 1 mM IPTG and the expressed protein purified using Ni-NTA chromatography.
Figure 2.2: pQE-TriSystem vector and sequence. [www.qiagen.com]

pQE-TriSystem Vector

<table>
<thead>
<tr>
<th>Positions of elements in bases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector size (bp)</td>
</tr>
<tr>
<td>CMV immediate early enhancer region</td>
</tr>
<tr>
<td>Chicken actin promoter region</td>
</tr>
<tr>
<td>Transcription start of chicken actin promoter</td>
</tr>
<tr>
<td>T5 promoter/Tac operator element</td>
</tr>
<tr>
<td>Transcription start of T5 promoter</td>
</tr>
<tr>
<td>p10 promoter region</td>
</tr>
<tr>
<td>Transcription start of p10 promoter</td>
</tr>
<tr>
<td>Shine-Dalgarno sequence</td>
</tr>
<tr>
<td>Multiple cloning site (Ncol - Xhol)</td>
</tr>
<tr>
<td>6xHis tag coding sequence</td>
</tr>
<tr>
<td>Rabbit globin termination region</td>
</tr>
<tr>
<td>T7 transcriptional termination region</td>
</tr>
<tr>
<td>ORF 1629 (baculovirus recombination region)</td>
</tr>
<tr>
<td>pUC origin of replication</td>
</tr>
<tr>
<td>β-lactamase coding sequence</td>
</tr>
<tr>
<td>Orientation / ORF 603 (baculovirus recombination region)</td>
</tr>
</tbody>
</table>
Cloning and expression of recombinant HorF (HP0671)

Genomic DNA from *H. pylori* stain NCTC 11638 was PCR amplified using primes:

Forward: aaagaattcaagcagtgaagggacgagaaaaacgcttg
Reverse: aaaaagcttcaagcgttttaaatcagaaattg

The following primers were used to screen for positive plasmids:
Forward: aatacgactcactataagggaattgtg
Reverse: ttgctcagcggtggcagccagccaactc

The gene was inserted into the pET28 (a) vector (Novagen, U.K), containing a N-terminal His-tag, using *Hind*III and *EcoR*I restriction enzymes (Promega, U.K) and T4 DNA Ligase (Invitrogen, U.K.), Figure 2.3. Cloning was carried out using chemically competent *E. coli* DH5α and chemically competent *E. coli* BL21pLysS was used for expression (Table 2.7). Expression was induced with 1 mM IPTG.
Figure 2.3: pET28 (a) vector and sequence (image taken from pET28 (a) Information Leaflet TB074 12/98, www.novagen.com).
Cloning and expression of recombinant putative neuraminylactose-binding haemagglutinin homolog hpaA (HP0410)

A plasmid construct containing the putative NLBH hpaA gene (hp0410) - H. pylori strain P76 (mouse adapted streptomycin resistant strain derived from P49 (Dr. H. Kleanthous-OraVax) - was kindly provided by Dr. Dirk Bumann, (Max-Planck Institute for Infection Biology, Germany), using the following primers:

Forward: gaaaggaatcatatgaaaaaaggt
Reverse: gcgatatcgatccgactaatgatgatgatgatgatgctttcgttttttcatttcac

The gene was inserted into pET15 (b) vector (Novagen, U.K), containing a N-terminal His-tag, Figure 2.4. Cloning was carried out using chemically competent E. coli Top10, while electroporetic competent E. coli BL21pLysS was used for expression (Table 2.7). Expression was induced with 1 mM IPTG.
Figure 2.4: pET15 (b) vector and sequence (image taken from pET15(b) Information Leaflet TB045 5/99, www.novagen.com).
Table 2.7: *H. pylori* genes used to create recombinant proteins, the vectors used to carry the gene and the *E. coli* strains used to clone and express the recombinant proteins.

<table>
<thead>
<tr>
<th><em>H. pylori</em> gene</th>
<th>Protein</th>
<th><em>E. coli</em> (Antibiotic resistance)</th>
<th>Vector (Antibiotic resistance)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cloning</td>
<td>Expression</td>
</tr>
<tr>
<td><em>hp0797</em></td>
<td>HpaA</td>
<td>DH5α</td>
<td>M15[pREP4]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(none)</td>
<td>(kan&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>hp0671</em></td>
<td>HorF</td>
<td>DH5α</td>
<td>BL21pLysS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(none)</td>
<td>(cm&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
<tr>
<td><em>hp0410</em></td>
<td>Putative NLBH hpaA homologue</td>
<td>Top 10</td>
<td>BL21pLysS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(none)</td>
<td>(cm&lt;sup&gt;R&lt;/sup&gt;)</td>
</tr>
</tbody>
</table>

* plasmid kindly provided by Dr. Petra Voland, Technical University of Munich, Germany

** plasmid kindly provided by Dr. Dirk Bumann, Max-Planck Institute for Infection Biology, Germany.

amp = Ampicillin [Carbenicillin] (400 μg/ml)

kan = Kanamycin (25 μg/ml)

cm = Chloramphenicol (34 μg/ml)

**Mass Spectrometry analysis**

Protein samples were sent for mass spectrometry analysis to Dr. Len Packman (Protein & Nucleic Acid Chemistry Facility, Department of Biochemistry, University of Cambridge, U.K.) using an electro spray ionisation tandem MS based system and MASCOT (Matrix Science, U.K.) peptide database or to Dr. Catherine Botting (Centre for Biomolecular Sciences, School of Chemistry, St. Andrews University, Scotland) using a Liquid chromatography tandem MS based system and MASCOT or PROQUANT (Applied Biosystem, U.K.) peptide database.
CHAPTER III

ISOLATION AND CHARACTERISATION OF LAMININ-BINDING PROTEIN FROM HELICOBACTER PYLORI
Chapter III

Introduction

The ability of bacteria to attach to host tissue is considered a vital step in the pathogenesis of infection (Finlay and Cossart, 1997). Pathogenic bacteria have developed specific virulence determinants on their surface which allow them to recognise distinct proteins, lipid-like or carbohydrate structures expressed on host cells and, thereby, to colonise specific hosts and tissues (Falk et al., 1994). Adherence plays an important role in colonisation of mucosal surfaces in the respiratory (Wilson et al., 1996), the urogenital (Boris et al., 1998), and the gastrointestinal tract (Odenbreit et al., 2002), which are permanently subjected to clearance mechanisms, such as liquid flows, ciliary or peristaltic activities, or shedding of mucus layers (Odenbreit, 2005).

Adhesins found on pathogenic bacteria play an important role in the successful invasion of host tissue. For example, Gram-negative bacterium Yersinia pseudotuberculosis uses a 108 kDa outer membrane protein to attach to β1-integrin of mammalian cells to eventually become internalised (Isberg and Falkow, 1985, Isberg et al., 1987). The Gram-positive bacterium Listeria monocytogenes uses internalin (InlA) (Gaillard et al., 1991) to adhere to eukaryotic E-cadherin inducing uptake into the epithelial cell (Mengaud et al., 1996).

Pathogenic bacteria have also been shown to adhere to the Extracellular Matrix (ECM) of host tissue. Adhesins, or ligand binding proteins, that bind to ECM molecules have been termed microbial surface components recognising adhesive matrix molecules (MSCRAMM). Gram-positive Staphylococcus aureus uses a number of MSCRAMM to adhere to components of the ECM of host tissue to initiate colonisation. S. aureus have been shown to interact with collagen (Patti et al., 1992, Switalski et al., 1989), fibronectin (Jonsson et al., 1991a, Signas et al., 1989) and vitronectin (Liang et al., 1995). The collagen adhesin (Cna) (Patti et al., 1992) has been shown to be a virulence factor in septic arthritis (Patti et al., 1994). Other Staphylococcus species have also been shown to use MSCRAMM proteins to attach to host tissue. SdrI from S. saprophyticus is a collagen binding protein (Sakinc et al., 1994).
2006); *Streptococcus pyogenes* uses Cpa to bind collagen (Podbielski et al., 1999) and F1/Sfb I to bind fibronectin (Hanski and Caparon, 1992, Talay et al., 1992). Gram-positive nosocomial pathogens *Enterocci* have been shown to target ECM components for adhesion (Zareba et al., 1997, Xiao et al., 1998).

Gram-negative bacteria species *Aeromonas* contain many adhesion factors including lipopolysaccharide (LPS) O-antigen (Merino et al., 1996), flagellum (Rabaan et al., 2001, Gavin et al., 2002) and type-IV pili (Kirov et al., 1999). Adhesins for ECM components fibronectin, laminin and collagen have also been identified (Ascencio et al., 1990, Ascencio et al., 1991). Another Gram-negative bacterium, *Treponema pallidum* has been shown to bind ECM proteins, such as laminin, fibronectin and collagen IV (Hayes et al., 1977, Fitzgerald et al., 1984) and Cameron later identified a laminin-binding protein from *T. pallidum* (Cameron, 2003).

Attachment to laminin has been suggested to play a role in the pathogenesis of a number of microorganisms, including various species of bacteria (Fenno et al., 1996, Hytonen et al., 2001, Nallapareddy et al., 2000, Plotkowski et al., 1996, Tanskanen et al., 2001, Valkonen et al., 1997), parasites (Crouch and Alderete, 1999, Furtado et al., 1992, Ghosh et al., 1999, Li et al., 1995), and fungi (Cotter et al., 1998, Coulot et al., 1994, McMahon et al., 1995, Hamilton et al., 1998). Laminin-binding proteins (LBP) have been identified and isolated from a range of bacteria including *Treponema pallidum* (Cameron, 2003), *Staphylococcus aureus* (Carneiro et al., 2004), *Streptococcus anginosus* (Allen and Hook, 2002), *Streptococcus pyogenes* (Terao et al., 2002); the parasitic worm *Echinococcus granulosus* (Zhang et al., 1997) and the protozoan parasite *Leishmania donovani* (Ghosh et al., 1999).

Adhesion of *H. pylori* to the gastric mucosa has been described in both cell lines and animal models, as well as by ultrastructural examination of human gastric biopsy specimens (Logan, 1996, Petersen and Krogfelt, 2003). *H. pylori* adheres intimately to gastric epithelial cells with specific tissue tropism (Clyne and Drumm, 1993, Clyne et al., 2004). Adherence of *H. pylori* is essential for colonization to take place. *H. pylori* attach to and colonise gastric epithelial cells causing severe disruption of epithelial integrity - a significant increase in epithelial apoptosis in gastric and duodenal ulcer patients has been demonstrated (Houghton et al., 1999, Kohda et al.,
Different types of adhesins have been identified in *H. pylori*. Lewis b – Binding Adhesin (BabA) is a 78 kDa outer membrane protein that binds to the fuscosylated Lewis B blood group antigen (Ilver *et al.*, 1998). The O-specific chains of *H. pylori* LPS contain antigenic moieties identical to Lewis x or Lewis y human blood group antigens (Moran, 1996). Another Lewis antigen adhesin is the sialic acid-binding adhesin (SabA) (Mahdavi *et al.*, 2002). Evans *et al.* (1988) identified a Neuraminyl-lactose-binding haemagglutinin (NLBH) which they later identified as an adhesin subunit, *H. pylori* adhesin A (HpaA) (Evans *et al.*, 1993). HpaA also has antigenic properties (Voland *et al.*, 2003). Odenbreit *et al.* (1999) identified Adherence-associated lipoprotein A (AlpA) and AlpB which are necessary for specific adherence of *H. pylori* to human gastric tissue. Many of the adhesins identified are closely related. BabA, AlpA and AlpB belong to a large family of 32 related Outer Membrane Proteins (OMPs) (Tomb *et al.*, 1997). Other members of the family include porin proteins (Hops) and Hop related (Hor) proteins. HpaA also shares close similarities with this OMP family.

*H. pylori* has also been shown to adhere to extracellular matrix (ECM) proteins including type IV collagen (Trust *et al.*, 1991), laminin (Valkonen *et al.*, 1993) and heparan sulfate (Ascencio *et al.*, 1993). Microscopy studies have demonstrated the presence of *H. pylori* on the basal lamina of gastric biopsies obtained from infected patients as well as epithelial cell lines exposed to *H. pylori* (Petersen and Krogfelt, 2003). The role of *H. pylori*-ECM interactions in disease progression is unclear, however, potential receptors have been identified for heparan including the 58 kDa subunit of VacA (Utt *et al.*, 2001). Valkonen *et al.* describe an unidentified 25 kDa laminin-binding protein (LBP) mediated by lipopolysaccharide (LPS) (Valkonen *et al.*, 1994, 1997). Terres *et al.* (2003) found that soluble *H. pylori* extract induced a physiological change in T84 polarised epithelial cells resulting in fluid and electrolyte accumulation between the T84 monolayer and the underlying laminin substrate. The biological activity of the *H. pylori* extract was inhibited by pre-treatment with laminin, suggesting a possible role for a laminin-binding protein during infection by *H. pylori*. The accumulated data from the studies carried out by others indicate a potential role for *H. pylori*-ECM interactions, particularly laminin, in disease progression. A first step in analysing the role of laminin-binding by the bacterium is to identify and sequence laminin-binding protein candidates. In this chapter a method
to isolate native candidate LBPs from *H. pylori* was developed and selected recombinant proteins assessed for laminin-binding activity.

**Objectives**

- To develop a method to isolate and identify potential laminin-binding protein candidates from *H. pylori* in their native form.
- To identify *H. pylori* outer membrane proteins with laminin-binding properties.
- To create recombinant versions of identified laminin-binding proteins.
- To determine the laminin-binding properties of recombinant proteins.
Results

Affinity purification method development

CNBr-activated Sepharose™ 4B binds higher amounts of laminin than Affi-gel® beads

Three commercially available affinity matrices were evaluated for their suitability to form a laminin affinity matrix; CNBr-activated Sepharose™ 4B (CNBr), Affi-gel® 10 (Affi-10) and Affi-gel® 15 (Affi-15). Equal amounts of laminin were coupled to all matrices according to the manufacturers recommendations. The amount of laminin not bound (i.e., remaining in the supernatant) to the matrices after the coupling reaction was analysed by SDS-PAGE. The Affi-10 matrix bound the least amount of laminin while CNBr bound the most (Figure 3.1) and hence CNBr was chosen for developing the method further. Coupling of laminin to subsequent CNBr matrices was verified by SDS-PAGE.

0.1% Zwittergent does not affect Laminin binding to the CNBr matrix

As Zwittergent® 3-14 detergent was used to solubilise the outer membrane proteins from *H. pylori* it was important to evaluate the effect, if any, of this detergent on the CNBr-laminin matrix prior to attempting affinity purification. The CNBr-laminin matrix was incubated with 10 mM Tris-Cl buffer containing Zwittergent® 3-14 (0% or 1% (v/v)) for 30 min. The matrix was allowed to settle and the protein composition of the supernatant was analysed by SDS-PAGE. The remaining matrix was incubated with buffer containing 2% (w/v) SDS at 100°C. The supernatant was analysed by SDS-PAGE. The results demonstrate that laminin binding to the CNBr matrix was disrupted when the matrix was incubated at 100°C (Figure 3.2, A). However, the Zwittergent® 3-14 (1% (v/v)) containing buffer also severely disrupted the laminin-CNBr matrix interaction resulting in laminin elution from the CNBr matrix (Figure 3.2, B). Further analysis using serial dilutions of the detergent containing buffer revealed that the use of Zwittergent® 3-14 at 0.1% (v/v) resulted in minimal leaching of CNBr-bound laminin (Figure 3.2, C).
Figure 3.1: Comparison of capacity of various matrices to bind laminin. Equal amounts of laminin from Engelbreth-Holm-Swarm murine sarcoma were immobilised onto three commercially available affinity matrices - CNBr-activated Sepharose® 4B (CNBr), Affi-gel® 10 (Affi-10) and Affi-gel® 15 (Affi-15). The amount of laminin not bound (i.e., remaining in the supernatant) to the matrices after the coupling reaction was analysed by SDS-PAGE (7.5%). The gel was silver stained.
Figure 3.2: Effect of Zwittergent® 3-14 detergent on the laminin affinity matrix. The laminin affinity matrix was incubated with 10 mM Tris-Cl buffer containing (A) 0% or (B) 1% Zwittergent® 3-14 detergent for 30 min. The matrix was allowed to settle and the protein composition of the supernatant before (i) and after (ii) the matrix was incubated in buffer containing 2% (w/v) SDS at 100°C was analysed by SDS-PAGE (12.5%). The gel was stained with Coomassie blue. (C) As above except the effect of different amounts of Zwittergent® 3-14 (1% - 0.001%) on laminin-binding to the CNBr matrix was analysed by SDS-PAGE (7.5%). The gel was silver stained.
0.1% Zwittergent is sufficient to solubilise *H. pylori* membrane proteins

Zwittergent® 3-14 has been used to solubilise outer membrane proteins from *H. pylori* (Clegg *et al.*, 1988, Petersson *et al.*, 2000). In this study, membrane proteins were solubilised initially using 1% (v/v) Zwittergent® 3-14. However, due to the deleterious effect 1% detergent had on the CNBr-laminin interaction a lower percentage detergent buffer was required. Proteins initially solubilised in 1% (v/v) Zwittergent® 3-14 were diluted to 0.1% (v/v) detergent and left to stand for 30 min with regular mixing. Insoluble proteins were collected by centrifugation at 18,000 x g. The resulting pellet and supernatant were analysed by SDS-PAGE. A small number of proteins were not solubilised by the detergent at 0.1% (v/v) but it was sufficient to solubilise the majority of proteins present (Figure 3.3, A). The laminin-binding protein from *Treponema pallidum* (Tp0751), the protein used as a positive control throughout this study, was also solubilised by this detergent treatment (Figure 3.3, B).

*H. pylori* membrane proteins do not bind to unconjugated CNBr sepharose

To ensure that the *H. pylori* proteins binding to the CNBr-laminin matrix was specific and due to the presence of laminin, the unconjugated matrix (i.e. containing no laminin) was incubated with the membrane protein preparation from *H. pylori*. Following a 30 min incubation period with the unconjugated matrix the mixture was centrifuged and the supernatant was examined by SDS-PAGE. Additionally, the CNBr matrix was incubated with buffer containing 2% (w/v) SDS at 100°C for 5 min, centrifuged and the supernatant examined by SDS-PAGE. No protein fragments could be detected in the supernatants (Figure 3.4) indicating that none of the membrane proteins bound non-specifically to the unconjugated CNBr sepharose. Taken together, these data indicate that the proteins recovered from the laminin conjugated matrix were due to specific protein-laminin interactions.
Figure 3.3: Decreasing the concentration of Zwittergent® 3-14 detergent used for solubilisation causes minor protein precipitation. (A) *H. pylori* proteins initially solubilised in 1% Zwittergent® 3-14 were diluted to 0.1% detergent and left to stand for 30 min with regular mixing. Decreasing the detergent concentration caused minor protein precipitation (I) while most proteins remain in solution (S). (I) and (S) fractions were analysed by SDS-PAGE (12.5%). The gel was stained with Coomassie blue. (B) In the absence of detergent (0%), the laminin-binding protein from *T. pallidum* (Tp0751), partially precipitates out of solution (I) and part remains soluble (S). In the presence of 0.1% Zwittergent® 3-14 all of the protein is completely solubilised.
Figure 3.4: Binding of *H. pylori* membrane proteins to unconjugated CNBr matrix. *H. pylori* membrane proteins solubilised in Zwittergent® 3-14 detergent were incubated with unconjugated CNBr sepharose previously blocked with 1M ethanolamine. All of the proteins remained in the supernatant (SN) and none was bound to the unconjugated matrix (E). To determine if any protein bound non-specifically to the unconjugated matrix the matrix was washed and then treated with buffer containing 2% (w/v) SDS prior to heating at 100°C. The resulting supernatant (E) was analysed by SDS-PAGE (12.5%). The gel was stained with Coomassie blue.
Proteins bound to the CNBr-laminin matrix are eluted with buffer containing SDS

Laminin-binding proteins have previously been isolated and purified by affinity chromatography (Plotkowski et al., 1996, Pethe et al., 2001, Ghosh et al., 1999, Gee et al., 1993). To isolate all the potential laminin-binding protein candidates from *H. pylori*, membrane proteins were incubated with the CNBr-laminin matrix for 30 min at room temperature and washed in a high salt buffer (0.5-1M NaCl) to remove non-specifically bound proteins. After washing, the matrix was incubated with buffer containing 2% SDS (5 min at 100°C). The supernatant was analysed by Western blotting to probe for proteins bound to laminin (Laminin Blot). The control laminin-binding protein, Tp0751, from *T. pallidum*, was eluted from the CNBr-laminin matrix by buffer containing 2% (w/v) SDS (Figure 3.5). Not surprisingly, laminin was also released when the matrix was boiled in buffer containing 2% (w/v) SDS demonstrating the disruption of the CNBr-laminin interaction. The protein species at 36 kDa (Figure 3.5) was identified as a laminin β1 subunit fragment by mass spectrometry.

Two potential laminin-binding proteins isolated and identified from *H. pylori*

Membrane protein preparations from *H. pylori*, solubilised in Zwittergent® 3-14 (0.1% v/v), were incubated with the CNBr-laminin matrix and eluted with SDS (2% w/v) containing buffer. The eluted proteins were separated by SDS-PAGE (Figure 3.6, A), and subjected to Western Blotting also (Figure 3.6, B). The high molecular weight protein species present on the laminin blot correspond in size to laminin and were not processed further. However, the two proteins of 29 kDa and 36 kDa, both of which were visible on the laminin blot, were excised from the corresponding SDS-PAGE gel and subjected to MS analysis. The 36 kDa species was identified as a laminin β1 subunit fragment whereas the MS analysis of the 29 kDa species revealed the presence of two proteins; flagellar sheath adhesin HpaA (HpaA) [HP0797] and HorF [HP0671] (Table 3.1). The presence of these proteins was confirmed in repeat experiments (Figure 3.7). Another protein, porin protein HopE [HP0706] (Doig et al., 1995), was found in one MS experiment but not in repeat analyses.
Figure 3.5: Recovery of control LBP protein bound to the CNBr-laminin affinity matrix. Control LBP (Tp0751), a recombinant laminin-binding protein from *Treponema pallidum*, was incubated with the CNBR-laminin affinity matrix. After washing, the matrix was incubated with buffer containing 2% (w/v) SDS (5 min at 100°C). The supernatant was analysed by (A) SDS-PAGE (12.5%) stained with Coomassie blue and (B) Laminin Blot. The control protein, 19 kDa, is indicated by arrow ii. Arrow i indicates a laminin β1 subunit fragment (identified by MS). All remaining high molecular weight bands belong to laminin.
Figure 3.6: Recovery of *H. pylori* membrane proteins bound to the CNBr-laminin affinity matrix. *H. pylori* membrane proteins were incubated with the laminin affinity matrix. After washing, the matrix was incubated with buffer containing 2% (w/v) SDS (5 min at 100°C). The supernatant (E) was analysed by (A) SDS-PAGE (12.5%) stained with Coomassie blue and (B) Laminin Blot. Arrow (i) indicates a laminin β1 subunit fragment (identified by MS). Arrow (ii) indicates the protein species identified by MS as HpaA (HP0797) and HorF (HP0671).
Table 3.1: Potential laminin-binding proteins from the membrane of *H. pylori* isolated by laminin affinity chromatography and identified by mass spectrometry.

* Average sequence coverage from repeat experiments.

<table>
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<tr>
<th>Gene (strain 26695)</th>
<th>Name</th>
<th>Synonym</th>
<th>Functions</th>
<th>% Sequence coverage by MS*</th>
<th>Reference</th>
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<td>OMP 14</td>
<td><em>hor F</em></td>
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<td>11%</td>
<td>(Alm <em>et al.</em>, 2000, Baik <em>et al.</em>, 2004)</td>
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Figure 3.7: Identification of *H. pylori* membrane proteins bound to CNBr-laminin affinity matrix by mass spectrometry. The percentage sequence coverage, and peptides matched, is shown for repeat experiments. (A) HpaA (HP0797) and (B) HorF (HP0671).
Sequence analysis of potential laminin-binding proteins from *H. pylori*

The amino acid sequences (Figure 3.8) of laminin-binding proteins from *Treponema pallidum* (Cameron, 2003) and *Staphylococcus aureus* (Carneiro *et al.*, 2004) in addition to those identified from *H. pylori* in this study were subjected to the following sequence/motif analyses as follows:

(i) the local alignment tool, LALIGN, (www.ch.embnet.org) was used to determine if there were any amino acid motifs common to all the proteins

(ii) each *H. pylori* sequence was searched for the presence of known laminin-binding domains:
   a. LMWWML – epithelial laminin receptor (Castronovo *et al.*, 1991)
   b. VVGSPSAQDFASPL – epithelial elastin-binding protein (Hinek *et al.*, 1993)
   c. WSLLTPA and YPYIPTL – *Yersinia pestis* plasminogen activator (Benedek *et al.*, 2005)
   d. PVQT, LWIQ and TAIS – *Treponema pallidum* laminin-binding protein, Tp0751 (Cameron *et al.*, 2005)

(iii) the Protein Structure Prediction (PSIPRED) method (Bryson *et al.*, 2005, McGuffin *et al.*, 2000, Jones, 1999) and transmembrane topology prediction (MEMSAT) method (Jones *et al.*, 1994, Jones, 1998) were used to determine if there was any secondary structure or transmembrane topology types common to all proteins

During the course of this study *H. pylori* SabA was identified as a laminin-binding protein also (Walz *et al.*, 2005) and was thus incorporated into the sequence analysis data set.
**HP0797** Flagellar Sheath Adhesin HpaA (*H. pylori*)

MRANNHFDFAWKKCLLGASVVALLVGCSPHIIETNEVALKLNYHPASEKVQALDEKILLR
PAFQYSDNIAKEYENKVTALQLQNGQYKVISVDSDDKDFSFQKKEQYLVAMNGEIVLRDPDKRTIQKKEPGLLFSTGLDMEGLVLPAGFIKTILEPMGSELSDFTDMLSELDIQEKFLKTTHSSHSQGLVSTMKVGDNSDAIKSNKIFANIMEIDDKLTQKNLESYQKDKELKGKRR

**HP0671** Outer Membrane Protein 14 – HorF (*H. pylori*)

MKKFVVFKTLCLSVLGNSLVAEAGSTEVQKQLEKPKKEYAVGEEKNWYLGISYQVQGASQ
SVKNPPKSSEFNYPKFPGKTLYAVMQGGLGTVQKQTFGEKRFWGARYGFMYYGHAVF
ANALSNGGCVECQLHPCATKVGMNLSDMTFYVGIDTFNYINKEDASPGFQFGAQIAGNSGWGGTAGLTFKSKYPHTSYSIDLPAPAIIFQFLNMLGIRTHGHRQEDFGVKTINTVYYFNGNLSFTYRQYSFVYGRYNF

**Tp0751** Laminin Binding Adhesin (*T. pallidum*)

MNRPLOMLAVGFLAVAWALISCFQGHVPPRPQHDTFLAPLTAALPSNARIAAHPSD
TADNTSNGSSTTTTPRSHGNAPPAFVGQAQTHQPGPVTAAMRLAWRAHGEQGALQHLLA
GLNKTQIEISPNSDHIPLLLFFDRHAEEITFSRASVQIEFLDVDSDTHKRTVSFGTRNTAISSTIRRREFTFSEVHHRVADVARLKGSTSMWDGQYTRYHAGPAASAPSP

**O69174** Enolase [Laminin binding protein] (*S. aureus*)

MPIITDVYAREVLSRGNTPVEVEVLTSAGFGRALVPSGASTGEHEAVELRDGDKSRYLK
GVKTVAWNVEIAPIEIEEGFSLVDQVSDMIALDGPNKGLGANAILGVSAVARAA
ADLLGQPLYYLGGFNGKQLPVPMNIVNSGSHSDAPIAFQEMFLPVGATTFFKESLRWGETE
IFHNLKISLSQRGLETAVDEGGFPKAFKFGTDASVESITEIIAQIAEAGYKPEEVMFLGFDCASS
EFYENGYDYSKFEGEHHAKRTAEQVYDLEQYLKYPITIEGMDENDWDGKQLTERIG
DRVQVLGDGFVTNKTE1LAKGIENIGNISILIKVQITGLLETFTDIAEMQAQKAGYATAVYHR
SGETEATTIAIADAVTAQAQIKTGLSRTDRIRAKYQNOLLIRIEDLETAYDKGKSFYNLDK

**HP0725** Sialic acid binding adhesin – SabA (*H. pylori*)

MSAGYQITGEAVQVMKNTGELKNLKEYQELSLSQYLNQVAASLQKISQNANIELVNSLNLK
FSTNNYNSTTQIPFNAQAVITSVLGFWSLYAGNYTFFTGFVGKVDGQPSAVQGNPFFK
IIENCISQICMADQSPMDKSKLMEDAQLQAATGNSATKGNLCSGAATDSTSNPNSVT
SNALNLQALMLDQATKTMMKNIVISGSNTSGAISTTNYPTQAYFNNIKAMIPILQO
AVTLLSQHNLALSAQATGTQPKFADYIYFQKQVAQIQDFNLSPIAEQY
YLEKAYLKIPNAGSTPNTYRQVVNQLQNQEVQTIKNVSYGNRVDALSAVRDYNKLKNQA
EIVTAYNADKLTSEEISKLPNQVNTKDIVLTPYDKNAPAPAGQSNYQINPEQQSNLNLQALAA
MSNNPFLKVGMISSQNNNAGNGLQVQVQYKFQFFESKRWGLRYGFFDNHYGKISSFFN
SSDITYSWGGSDDLQVINDSTTRKNKLSVGLFGGIGLQAGTTLWNSQYVNTLAFNNPSYAK
VANATNFQLFLNGLRNLATARKDSEHSAQMHIQGIELGIKIPITTNNYSLFTGQYRRYLSSYLYNYVFAY

**Figure 3.8:** Amino acid sequences of known laminin-binding proteins and those identified by this study. Known laminin-binding proteins are Tp0751, O69174 and HP0725 [SabA (HP0725) was identified as a laminin-binding protein during the course of this study]. The laminin-binding domains identified on Tp0751 are highlighted in bold italics.
No common motif or domain was found following sequence analysis by LALIGN (see Appendix A for LALIGN sequence comparison data). None of the known laminin-binding domains was found in the H. pylori amino acid sequences analysed while each protein was found to possess distinct secondary (Figure 3.09, A-E) and transmembrane topology (Figure 3.10, A-E). This suggests that there is no common laminin-binding motif or secondary structure topology present among the known laminin-binding proteins and those identified in this study that might explain the laminin interaction or laminin-binding potential. It also suggests that the interactions of the various known laminin-binding proteins with laminin result from other types of interactions such as protein-sugar or sugar-sugar interactions.
Figure 3.09 - A: Predicted secondary structure of amino acid sequences of Laminin-binding protein (Tp0751) from *T. pallidum*. Secondary structure was predicted using PSIPRED.
Figure 3.09 - B: Predicted secondary structure of amino acid sequences of enolase (O69174) from S. aureus. Secondary structure was predicted using PSIPRED.
Figure 3.09 - B: continued
Figure 3.09 - C: Predicted secondary structure of amino acid sequences of HpaA (HP0797) from *H. pylori*. Secondary structure was predicted using PSIPRED.
Figure 3.09 - D: Predicted secondary structure of amino acid sequences of HorF (HP0671) from H. pylori. Secondary structure was predicted using PSIPRED.
Figure 3.09 - E: Predicted secondary structure of amino acid sequences of SabA (HP0725) from H. pylori. Secondary structure was predicted using PSIPRED.
Figure 3.09 - E: continued
Figure 3.09 - E: continued
Figure 3.10 - A: Predicted transmembrane topology of amino acid sequences of Laminin-binding protein (Tp0751) from *T. pallidum*. Transmembrane topology was predicted using MEMSAT.
Figure 3.10 - B: Predicted transmembrane topology of amino acid sequences of enolase (O69174) from S. aureus. Transmembrane topology was predicted using MEMSAT.
MRANNHFKDFAWKCLLGASVVALVGCSPIETNEVALKNYHPASEKVQALDEKILL

LRPAFQYSDNIAKEYENKFNQTALKVEQILQNYKVISVDSDKDDFSFAQKKEYLA

VAMNGEIVLRDPKRTIQKSEPGLLFSTGLDKMEGVIPAGFIKVILEPMSGESLDSF

TMDSELDIQEKFKTHSHHGGLVSTMVKGTDNSNDAIKSAHNKIFANIMQEIDKKT

QKNLESYQDKELRGRNR

Key:
S: Possible N-terminal signal peptide
+: Inside loop
-: Outside loop
O: Outside helix cap
X: Central transmembrane helix segment
I: Inside helix cap

Figure 3.10 – C: Predicted transmembrane topology of amino acid sequences of HpaA (HP0797) from H. pylori. Transmembrane topology was predicted using MEMSAT.
**Figure 3.10 – D: Predicted transmembrane topology of amino acid sequences of HorF (HP0671) from *H. pylori*.** Transmembrane topology was predicted using MEMSAT.

Key:

- **S**: Possible N-terminal signal peptide
- **+**: Inside loop
- **-**: Outside loop
- **0**: Outside helix cap
- **X**: Central transmembrane helix segment
- **I**: Inside helix cap

**MKKFVFKTLCLSLVVLGNLVAEGSTEVQKLEKKEYAKVGEKNAWYLGISYQVGQA**

**SQSVKNPPKSSFNFYPKFPVGKTPLYAVMQGLGLTVGYKQFFFGKEKRWFGARYYGFDYGHC**

**AVFGANALTSDNGVCCELHOPCATKVGMGNLSDFTYVGIDLYNVINKEDASFGFF**

**GAQIAGNSWGTGNTGLETKSPORTKHSYSLDPAIFQFLFNLRINGHCRMHEURDFGKV**

**TINVYYFNGNLSFYRQYSLEVGVY**

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**XXXOOOO----------**

TINVYYFNGNLSFYRQYSLEVGVY
Figure 3.10 – E: Predicted transmembrane topology of amino acid sequences of SabA (HP0725) from H. pylori. Transmembrane topology was predicted using MEMSAT.
Analysis of laminin-binding properties of recombinant laminin-binding proteins from *H. pylori*

Recombinant HpaA (HP0797) protein expression and purification

A plasmid construct (pQE-TriSystem vector, Qiagen, U.K.) containing the *hpaA* (*hp0797*) gene from *H. pylori* strain 26695 (Figure 3.11) – was kindly provided by Dr. Petra Voland (Technical University of Munich, Germany). The plasmid was transformed into *E. coli* DH5α (Novagen, U.K.) and the identity of the cloned insert was confirmed by sequencing using the dideoxynucleotide chain termination method (Department of Biochemistry, University of Cambridge, U.K.). The plasmid construct was then transformed into *E. coli* M15 (Qiagen, U.K.). Expression was induced with 1 mM IPTG over 4 h. Expression of the protein was monitored by Western blotting as described in the legend to Figure 3.12. Both soluble and insoluble forms of recombinant HP0797 protein were expressed following a 4 h induction with IPTG (Figure 3.12, A). Induced bacteria were collected and recombinant HP0797 protein was purified, under native conditions, using nickel chelate chromatography (Figure 3.12, B). Column eluates were combined and concentrated using centrifugal filtration.

Laminin-binding characteristics of recombinant HpaA and putative NLBH homologue

HpaA (HP0797) was initially described as a neuraminyl-lactose-binding haemagglutinin (NLBH), i.e. a sialic acid binding haemagglutinin (Evans et al., 1988). Another *H. pylori* protein, with a different amino acid sequence, was also described as a putative NLBH homologue hpaA (HP0410) (Tomb et al., 1997). Valkonen et al. suggested that a sialic acid binding motif was involved in *H. pylori* binding to laminin (Valkonen et al., 1997). A comparison of the laminin-binding characteristics of HpaA and the putative NLBH homologue hpaA could help determine the potential laminin-binding domain of HpaA. As the plasmid construct for the putative NLBH homolog HpaA was available it was expressed and analysed by Laminin Blot in this study.
Figure 3.11: Gene and protein sequences of *H. pylori* hpaA [protein HpaA]. A plasmid construct (kindly provided by Dr. Petra Voland -Technical University of Munich, Germany) was amplified by PCR with primers highlighted with underlined bold italics. The amplified gene sequence (A) and corresponding protein sequence (B) are highlighted in bold.
Figure 3.12: Expression and purification of recombinant HpaA (HP0797). E. coli M15 (Qiagen, U.K.) was induced with IPTG to express HP0797, collected by centrifugation and sonicated to release soluble and insoluble recombinant protein. (A) both soluble (Sol) and insoluble (Insol) proteins were analysed by His-tag Western blot. (B) following IPTG induction total bacteria were collected and the recombinant protein was purified, under native conditions, using nickel affinity chromatography. The wash (Wash) and elution (E) fractions were analysed by His-tag western blot. Column eluates were later combined and concentrated using a centrifugal concentration device.
The ability of the recombinant HpaA (rHP0797) protein to bind laminin was examined by Laminin Blot and ELISA. Recombinant HpaA did not bind laminin as determined using either method (Figure 3.13). The positive control laminin-binding protein, Tp0751, demonstrated an affinity for fibronectin as well as laminin (Figure 3.13, B).

A plasmid construct (pET 15(b) vector, Novagen, U.K.) containing the hp0410 gene from H. pylori strain P76 (Figure 3.14) [mouse adapted streptomycin resistant strain derived from P49 (Dr. H Kleanthous-Ora Vax)]-- was kindly provided by Dr. Dirk Bumann (Max-Planck Institute for Infection Biology, Germany). The plasmid was transformed into E. coli Top10 (Invitrogen, U.K.) and expressed in E. coli BL21pLysS (Novagen, U.K.). Expression was induced with 1 mM IPTG over 3 h. Both soluble and insoluble proteins were analysed by SDS-PAGE and Western blotting. Both soluble and insoluble forms of the recombinant HP0410 protein were generated following a 3 h induction with IPTG (Figure 3.15). The presence of a doublet band may have resulted from partial degradation of the protein product. Total bacteria harvested after a 3 h induction were solubilised in SDS-PAGE Sample Buffer and analysed by Laminin Blot. No binding to laminin was observed for the expressed protein.
Figure 3.13: Binding of recombinant HpaA (HP0797) protein to laminin. The ability of the purified recombinant HpaA protein to bind laminin was analysed by (A) laminin blot and (B) ELISA. LBP\(^+\) indicates the positive control laminin-binding protein Tp0751 from *T. pallidum*. The (Ab) antibody used in both methods was Mouse Anti-penta-His tag (Qiagen, U.K.).
Figure 3.14: Gene and protein sequences of *H. pylori* *hp0410*. A plasmid construct (kindly provided by Dr. Dirk Bumann - Max-Planck Institute for Infection Biology, Germany) was generated by PCR with primers highlighted with underlined bold italics. The amplified gene sequence (A) corresponds to the entire HP0410 protein (B).
Figure 3.15: Expression of recombinant protein HP0410. *E. coli* BL21pLysS (Novagen, U.K.) was induced to express with IPTG, collected by centrifugation and sonicated to release soluble and insoluble recombinant protein. Total bacteria previously induced with IPTG were solubilised in Sample buffer and analysed by His-tag Western blot.
Construction of hp0671 expression plasmid

Little information is available on the final potential laminin-binding protein, HorF (HP0671), identified in this study. Thus, to evaluate the laminin-binding properties of this candidate it was necessary to construct an expression plasmid. The gene sequence was obtained from the published genome for *H. pylori* 26695 (Tomb *et al.*, 1997). Figure 3.8. The sequence was analysed for the presence of N-terminal signal sequences using the SignalP tool. Two potential signal cleavage sites were located between amino acids 22-23 and 23-24 (Figure 3.16). Primers (with HindIII and EcoRI restriction sites included) were designed to amplify the hp0671 gene without the N-terminal signal sequences (Figure 3.17). Genomic DNA encoding hp0671 was amplified by PCR from *H. pylori* strain NCTC 11638. The size and quality of the amplified gene product was analysed on an agarose gel. DNA of the expected size (711bp) was amplified (Figure 3.18, A). pET 28(a) vector DNA (Novagen, U.K.) and hp0671 DNA were digested separately with HindIII and EcoRI restriction enzyme; the vector and gene DNA were joined by cohesive end ligation and the resulting plasmid was transformed into *E. coli* DH5α (Novagen, U.K.). Colonies were screened by PCR (using vector-derived primers). The plasmid construct DNA was analysed on an agarose gel. A plasmid of expected size (1011bp) was obtained (Figure 3.18, B) and its identity confirmed by sequencing using the dideoxynucleotide chain termination method (Department of Biochemistry, University of Cambridge, U.K.)
SignalP-NN result:

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<tr>
<td>D</td>
<td>1-22</td>
<td>0.797</td>
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# Most likely cleavage site between pos. 22 and 23: LVA-AE

SignalP-HMM result:

Prediction: Signal peptide
Signal peptide probability: 0.999
Max cleavage site probability: 0.667 between pos. 23 and 24

Figure 3.16: Predicted N-terminal signal sequence on *H. pylori* HorF (HP0671) protein according to signal prediction tool SignalP.
Figure 3.17: Gene (A) and protein sequence (B) of *H. pylori* hp0671 [HorF]. The entire gene is highlighted in bold. The primers used to amplify the gene by PCR are highlighted with underlined bold italics. The expected amino acid sequence encoded by the cloned gene is highlighted in bold.
Figure 3.18: Amplification of gene \textit{hp0671} from genomic DNA and insertion into plasmid vector. (A) \textit{H. pylori} gene \textit{hp0671} was amplified by PCR from genomic DNA and the product from selected reactions (i-iii) analysed by agarose gel (1.5%) stained with ethidium bromide. (B) Amplified gene \textit{hp0671} was ligated into vector pET28(a) (Novagen, U.K.) and transformed into \textit{E. coli} DH5\textalpha{} (Novagen, U.K.). Colonies were screened by PCR (using vector derived primers) and plasmid DNA both with and without the insert was analysed by agarose gel (1.5%) stained with ethidium bromide. In both cases a band of expected size was obtained.
Recombinant HP0671 protein expressed but at a lower molecular mass than expected: no evidence of laminin-binding

The *hp0671* plasmid was transformed into *E. coli* BL21pLysS (Novagen, U.K.). Expression was induced with 1 mM IPTG over 3 h and expression levels were monitored by SDS-PAGE and Western blotting. The Western blot showed that a protein with an approximate molecular mass of 18 kDa was expressed after the 3 h induction (Figure 3.19, A-B). A protein of an approximate molecular mass of 30 kDa was expected (26 kDa HP0671, + 4 kDa vector his-tag). Transformed bacteria were again induced to express with 1 mM IPTG but in the presence of 0.5% (v/v) PMSF and 1 mM DTT to determine if the expressed protein was being degraded by proteases resulting in a smaller protein. The addition of the protease inhibitor, PMSF, improved protein expression. In addition to the 18 kDa protein a faint band corresponding to a protein of approximate molecular mass 29 kDa can be seen (Figure 3.19, C). A Laminin blot of the same material showed no evidence of laminin-binding by the 18 kDa or 29 kDa species. The plasmid sequence was assessed for the presence of rare codons, which might affect the ability of *E. coli* to successfully express the recombinant protein. The presence of rare codons was determined from the Codon Usage Table for *E. coli* B and *E. coli* K12 obtained from Merck Bioscience (www.merckbiosciences.co.uk). The number and type of rare codons present in gene *hp0671* was compared with *hp0797* (successful expression in *E. coli*). Gene *hp0671* contains single copies of three rare codons, CUA, CCC, GGA, and 2 copies of rare codon AGA; gene *hp0797* contains multiple copies of four rare codons, AGG, AUA, CCC, GGA, and a single copy of codon CUA (Table 3.2).
Figure 3.19: Expression of recombinant protein HorF (HP0671). *E. coli* BL21pLysS (Novagen, U.K.) were induced with IPTG for 3 h, collected by centrifugation, solubilised in Sample buffer and analysed by (A) His-tag Western blot and (B) SDS-PAGE (15%) stained with Coomassie blue. (C) As above except the protease inhibitor PMSF was added prior to induction of protein expression. Total bacteria were solubilised in Sample buffer and analysed by His-tag Western blot. The arrow points to a faint band at 29 kDa.
Table 3.2: Presence of codons rare to *E. coli* in the gene sequence of the laminin binding candidate proteins identified in *H. pylori*.

<table>
<thead>
<tr>
<th>Codon</th>
<th>E.coli (K12) (freq/thousand)</th>
<th>E.coli (B) (freq/thousand)</th>
<th>H.pylori (26995) (freq/thousand)</th>
<th>Gene hp0797 plasmid (freq/thousand)</th>
<th>Gene hp671 plasmid (freq/thousand)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGG</td>
<td>1.2</td>
<td>2.1</td>
<td>8.5</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>AGA</td>
<td>2.1</td>
<td>2.4</td>
<td>9.2</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>AUA</td>
<td>4.3</td>
<td>5</td>
<td>9.3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>CUA</td>
<td>3.9</td>
<td>3.4</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CCC</td>
<td>5.5</td>
<td>2.4</td>
<td>8.7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>GGA</td>
<td>7.9</td>
<td>8.2</td>
<td>6.2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>CCU</td>
<td>7</td>
<td>5.8</td>
<td>16</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Discussion

Laminin is a major component of the extracellular matrix (ECM) that underlies epithelial and endothelial cells and surrounds connective tissue cells. Attachment to laminin has been suggested to play a role in the pathogenesis of a variety of bacterial infections (Fenno et al., 1996, Hytonen et al., 2001, Nallapareddy et al., 2000, Plotkowski et al., 1996, Tanskanen et al., 2001, Valkonen et al., 1997). Although Valkonen et al. (Valkonen et al., 1997) identified a 25 kDa laminin-binding protein in *H. pylori* none has been isolated and sequenced to date. In this study two potential laminin-binding proteins from *H. pylori* were isolated by affinity chromatography and their sequences identified by mass spectrometry. The laminin-binding characteristics of the corresponding recombinant versions were also evaluated. During the course of this study another group showed that the adherence of *H. pylori* to laminin is mediated in part by the 70 kDa sialic acid-binding adhesin, SabA, HP0725 (Walz et al., 2005).

Affinity chromatography has been used to isolate laminin-binding proteins from different organisms by attaching laminin to a variety of matrices including CNBr-activated Sepharose (CNBr) and Immunoaffinity supports (Plotkowski et al., 1996, Pethe et al., 2001, Ghosh et al., 1999, Gee et al., 1993, Sengupta et al., 1991, Douville et al., 1988). Similarly, this study evaluated three different matrices for use in the affinity purification step and found that CNBr bound the most amount of laminin and, as such, would have the highest number of laminin-binding sites available to isolate potential laminin-binding proteins. Additionally, optimal amounts of detergent for use with the affinity purification step were identified. The inclusion of a detergent would help minimise non-specific binding of proteins to the laminin affinity matrix.

A total elution strategy (buffer containing 2% (w/v) SDS at 100°C) was employed in this study to obtain a range of potential laminin-binding proteins from *H. pylori* on the assumption that potential proteins could remain undetected if they bound tightly to the affinity matrix. Candidate proteins were selected for further study based on the following criteria: (i) they bound to laminin affinity matrix, (ii) they were positive for laminin-binding in a Laminin Blot and (iii) they were clearly separate and visible
bands on Coomassie stained SDS-PAGE (due to the sensitivity of mass spectrometry tools used for this study). Two *H. pylori* proteins [HpaA (HP0797) and HorF (HP0671)] were isolated and their sequences identified following this shotgun strategy. A similar strategy was employed in another study to identify the total proteins binding to fetuin from *H. pylori* (Bennett and Roberts, 2005).

HopE (HP0706) was also identified in one MS analysis, although, the reasons why this molecule was not identified in subsequent analysis is not clear, it is most likely due to protein abundance. Either the concentration of a protein is too low to be detected by MS analysis or high abundant proteins can mask the MS signal of lower abundant proteins found in a single gel piece containing protein. HpaA, HorF and HopE, are of similar molecule mass (29 kDa, 30 kDa and 30 kDa, respectively) and, not surprisingly, can co-migrate as a single band on a SDS-PAGE gel. Protein abundance may have resulted in the porin protein HopE being identified in a single MS analysis but being masked by the more abundant HpaA and HorF proteins in repeat analysis. The proteins identified and used in this study had good sequence coverage (i.e., peptide fragments corresponding to different sections of the protein sequence) and high probability scores in repeat mass spectrometry analyses.

In early studies, *H. pylori* was found to bind laminin with high affinity (Trust *et al.*, 1991), involving a lectin-like interaction (Valkonen *et al.*, 1993) and mediated by lipopolysaccharide (LPS) (Valkonen *et al.*, 1994). Valkonen *et al.* later identified a 25 kDa laminin-binding protein which was inhibited by 3′ sialyllactose (Valkonen *et al.*, 1997). More recently another protein, SabA (70 kDa), was shown to be involved in laminin binding (Walz *et al.*, 2005). Here, two additional potential laminin-binding proteins have been identified, HpaA (HP0797) and HorF (HP0671).

The unsequenced 25 kDa protein described by Valkonen *et al.* (1997) may well be the HpaA protein found in this study. Valkonen’s protein has an approximate molecular mass of 25 kDa, was isolated from *H. pylori* strain 11638 and 3′ sialyllactose (sialic acid) partially inhibited laminin-binding. In this study HpaA has a molecular mass of 29 kDa, was isolated from *H. pylori* strain 11638 and contains a sialic acid binding motif, kxxxxxkk, which interestingly, is not found in the putative NLBH homologue hpaA (HP0410). The discrepancies in molecular weight between the 25 kDa protein
and HpaA may be due to the use of different percentage SDS-PAGE gels (10% Vs 12.5%) and molecular weight markers with different kDa ranges (97-14 Vs 205–20)

used in the studies.

HpaA displays antigenic properties (Voland et al., 2003) and has been shown to be essential for *H. pylori* colonization in mice (Carlsohn et al., 2006a). However, the identification and location of HpaA is controversial. Evans et al. (1993) described a 20 kDa protein located in the outer membrane while O’Toole et al. (1995) identified a 26-29 kDa protein located in the cytoplasm. Jones et al. (1997) found the 29 kDa flagella protein identified by Luke and Penn (1995) to be identical to HpaA. However, Lundström et al. (2001) noted that HpaA was located both on the flagella and outer membrane. Jones et al. (1997) demonstrated that the flagellar sheath protein was not an adhesin for erythrocytes or AGS cells. Evans et al. (1993) have demonstrated that HpaA is a haemagglutinin and has a binding specificity for the same isomeric form of sialyllactose as the 25 kDa LBP identified by Valkonen et al. (1997). These discrepancies may be explained by strain differences. Evans et al. used strain 8826, O’Toole et al., Jones et al. and Valkonen et al. used strain NCTC 11637 while Lundström et al. used a variety of strains including strains NCTC 11637 and NCTC 11638. Lundström et al. (2001) also found that the expression level of HpaA was growth phase dependent.

Little is known about HorF (HP0671) encoded by the gene horF. It belongs to a group of genes that have sequence homology to the Helicobacter Outer Membrane Porin (HOP) Family of proteins, Hor (Hop related) (Alm et al., 2000). It also belongs to a 32 member family of sequence related outer membrane proteins (Tomb et al., 1997). Other family members include the adhesins BabA, AlpA and AlpB. Baik et al. (2004) found HorF to be an immunoreactive protein in the sarcosine insoluble fraction of *H. pylori*. Bacterial porins are specific or non-specific channel forming transmembrane proteins found in the outer membrane of Gram-negative bacteria and function as molecular sieves to allow diffusion of molecules into the periplasmic space (Jeanteur et al., 1991, Nikaido, 1992). *H. pylori* porins have been shown to induce interleukin – 3, 4, 6 and 8 along with tumour necrosis factor α and interferon γ from human monocytes and lymphocytes (Tufano et al., 1994). Keenan et al. (2000a)
also demonstrated the potential of membrane vesicles from \textit{H. pylori} to deliver porin proteins to the gastric mucosa.

Laminin is a large glycoprotein and receptors from a variety of sources have been found to bind laminin, ranging from ECM proteins themselves to gastric cells and pathogenic bacteria, such as \textit{Treponema pallidum}, \textit{Staphylococcus aureus} and \textit{H. pylori} (Rao \textit{et al.}, 1985, Cameron, 2003, Eckstein and Shur, 1989, Allen and Hook, 2002, Walz \textit{et al.}, 2005, Carneiro \textit{et al.}, 2004). In addition, the amino acids responsible for binding to laminin have been identified for epithelial surface proteins, elastin binding protein and laminin receptor as well as laminin-binding proteins from \textit{Yersinia pestis} and \textit{Treponema pallidum} (Hinek \textit{et al.}, 1993, Cameron \textit{et al.}, 2005, Benedek \textit{et al.}, 2005, Castronovo \textit{et al.}, 1991).

None of the known laminin-binding domains was found on any of the proteins analysed in this study. This is not so unusual due in part to the large number of binding sites available on laminin but also the increased potential binding arrangements present in secondary and tertiary protein structures. Although no apparent laminin-binding motif was found on either HpaA or HorF they both possess a sialic acid binding motif kkxxxxxk (also found in \textit{E. coli} (Jacobs \textit{et al.}, 1986) and \textit{Vibrio cholera} (Ludwig \textit{et al.}, 1985)). Valkonen \textit{et al.} (1997) found that 3'sialyllactose (sialic acid) inhibited binding of \textit{H. pylori} to laminin. The presence of a sialic acid binding motif on HpaA and HorF may account for some of their binding to laminin. However, it should be noted that the basic kkxxxxk motif can be found in a number of other \textit{H. pylori} proteins (e.g., HP0033, HP0036, HP0264 and HP0280) and as such is not a specific marker for laminin-binding. This observation was also noted by Bennett and Roberts while characterising a new sialic acid binding protein in \textit{H. pylori} (Bennett and Roberts, 2005). Interestingly, the kkxxxxxk motif is not found on SabA, suggesting the involvement of another domain in laminin binding for this protein.

It is difficult to predict that a given protein will possess laminin-binding properties. For example, the laminin binding protein of \textit{S. aureus} (O69174) belongs to the enolase family of proteins (Carneiro \textit{et al.}, 2004); the SpeB virulence factor of \textit{Streptococcus pyogenes} is a strepadhesin, exhibits cysteine protease activity as well
as binding to laminin (Hytonen et al., 2001); the LamB porin protein from *E. coli* also binds laminin (Valkonen et al., 1991). *T. pallidum's* laminin binding protein (Tp0751), used as a control throughout this study, was shown to bind both laminin and fibronectin (Figure 3.13). Therefore an examination of the protein structure of laminin-binding proteins may provide evidence of common structural features that interact with laminin.

The structure of a protein is determined on four levels. The most basic level comprises the linear sequence of amino acids, referred to as the primary structure. Secondary structure refers to the spatial, or conformational, relationships of amino acids to those nearby in a sequence; they fall predominantly into two regular patterns: helix and strand. The remaining amino acids are grouped together as coils or turns. Tertiary structure refers to the three dimensional relationship of these elements of secondary structure within a single polypeptide chain, or monomer. Many proteins are parts of larger complexes that contain several constituent polypeptide chains; the relationship among these chains comprises the quaternary structure (Kretsinger et al., 2004). In a study of five membrane protein topology prediction algorithms MEMSAT and TMHMM demonstrated the highest reliability scores (Melen et al., 2003). The MEMSAT algorithm is available on the Protein Structure Prediction Server (PSIPRED).

PSIPRED was used during this study for membrane topology along with secondary structure prediction. Comparison of the secondary and membrane structures of the laminin-binding proteins analysed in this study demonstrated no apparent similarities in the location or pattern of helix, strand or coil structures. This reflects the lack of sequence homology between them. The tertiary structure of laminin-binding proteins is probably more important than their primary and secondary structures because potential binding domains are created when linearly separated amino acid sequences come closer to each other. As previously mentioned, the laminin-binding domains found in *Y. pestis* (Benedek et al., 2005) and *T. pallidum* (Cameron et al., 2005) are located along the length of their respective proteins suggesting they bind to laminin at multiple locations when in their native form.
The possible similarity of HpaA (HP0797) to the 25 kDa laminin-binding protein, described by Valkonen et al. (1997), led to the evaluation of a recombinant version of the protein to assess its laminin-binding properties. A plasmid containing the $hp0797$ gene (missing the N-terminal sequence) was kindly provided by Dr. Petra Voland (Technical University of Munich, Germany). Even though the recombinant protein was successfully sequenced, expressed and purified it failed to demonstrate any level of binding to laminin in its denatured form (Laminin Blot) or non-denatured form (ELISA). While it is commonly accepted that not all recombinant proteins prove to be biologically functional previous studies by Voland et al. (2003) used this same plasmid construct to demonstrate the antigenic properties of HpaA. The recombinant protein was expressed in the human kidney cell line HK293 and was found to react with sera from patients infected with $H. pylori$. It also induced secretion of interleukin-12 (IL-12) and IL-10 from peripheral blood mononuclear cells as well as stimulating major histocompatibility class II and CD83 expression on dendritic cells. Together, these observations suggest that the recombinant product was at least antigenic.

The absence of the N-terminal sequence from the recombinant protein (because of the presence of a signal sequence (Voland et al., 2002)) used in this study might account for its inability to bind laminin. Lundström et al. (2003) compared the biological properties of native HpaA with recombinant HpaA using a plasmid containing the entire gene sequence of $hp0797$. Purified recombinant and native HpaA bound similarly to sialylated fetuin and non-sialylated fetuin in addition to inducing similar amounts of specific antibodies in the serum from immunised mice. Binding of HpaA to laminin possibly requires the entire protein and future studies should focus on either purifying native HpaA or creating a full-length recombinant protein.

The planned comparison of the laminin-binding properties between HpaA and putative NLBH homologue HpaA (HP0410) was not possible during this study because of the failure of the recombinant version of HpaA protein to bind laminin.

During this study, initial conditions have been determined for the expression of HorF (HP0671). Two potential signal cleavage sites are located between amino acids 22-23 and 23-24 therefore a plasmid containing the $hp0671$ gene without the signal
sequence was created. Proteases present in the expression host, *E. coli*, appear to interfere with the production of full length recombinant HorF protein. Recombinant HorF of reduced molecular mass (18 kDa as opposed to 30 kDa) was initially produced in *E. coli*. Although the host *E. coli* strain is deficient for some proteases the addition of PMSF (a serine and papain (cysteine) protease inhibitor) during the induction stage yielded a small amount of recombinant HorF of 29 kDa along with the smaller 18 kDa protein. It is possible the recombinant protein was being degraded as it was being produced. Any deleterious effects rare codon usage might have on the *E. coli* expression host can possibly be ruled out because the *horF* plasmid contains less rare codons than the gene encoding the successfully expressed HpaA. No laminin binding was detected by Laminin blot but the anomalous expression of HorF renders the result void.

Production of recombinant porin proteins by others has yielded varied results (Papavinasasundaram *et al.*, 1997, Blanco *et al.*, 1996, Champion *et al.*, 1997, Bina *et al.*, 2000, Gotschlich *et al.*, 1987). Blanco *et al.* (1996) found including the signal sequence for the recombinant porin protein Tromp1 (from *T. pallidum*) proved lethal to the *E. coli* expression host but no such difficulty was experienced when expressing another protein Tromp2 in *E. coli* (Champion *et al.*, 1997). Bina *et al.* (2000) were able to express the *H. pylori* porin HopE in *E. coli* without difficulty while Gotschlich *et al.* (1987) were unable to express *Neisseria gonorrhoeae* porin protein (PI). A change in cloning strategy for HorF may prove more successful. Fischer *et al.* (1999) developed a new plasmid-based shuttle vector system for the cloning and expression of *H. pylori* genes encoding outer membrane proteins. SOMPES (Shuttle vector-based Outer Membrane Protein Expression System) allows an active gene to be reconstituted by recombination in *H. pylori* from partial gene sequences cloned on an *E. coli-H. pylori* shuttle vector. This technique has been used to successfully express recombinant VacA and AlpA (1999, Fischer *et al.*, 2001a).

During the course of this study another group demonstrated the laminin-binding properties of sialic acid-binding protein A (SabA) (Walz *et al.*, 2005). However, SabA was not identified by this study. A combination of protein abundance and solubility may have contributed to the failure to detect SabA protein by affinity chromatography and subsequent laminin Western blot. Various detergents and
organic solvents have been used by research groups to study membrane proteins, each detergent or solvent will favour the solubilisation of certain protein species (Rabilloud et al., 1999, Deshusses et al., 2003, Molloy et al., 1999, Jungblut et al., 2000). Detergents are also used to minimise non-specific interactions between proteins. Therefore, a balance between protein solubility and functional activity is required to maximise the quality and quantity of data obtained from a specific experiment. In addition, the identification of a low abundance protein by MS can be masked by the presence of more abundant proteins. Furthermore, the presence of proteins with multiple functional properties can interfere with the function of other proteins and compete for binding sites by blocking or inhibiting their interaction with specific ligands, as may happen during affinity chromatography. For example, Dallo et al. (2002) demonstrated an adhesive role for the cytoplasmic protein EF-Tu from *Mycoplasma influenzae*. EF-Tu is translocated to the surface where it was shown to bind to fibronectin. Cytoplasmic proteins have also been found on the surface of *H. pylori* membrane, and they too, may perform additional functions unrelated to their cytoplasmic roles, which may mask/interfere with the function of other membrane proteins.

The greater abundance of other proteins, such as HpaA, may have blocked SabA from interacting with laminin. Alternatively, the SabA-laminin interaction may have been too strong to be eluted by the SDS containing buffer; however, this is highly unlikely, as the elution method also included a heating step of 100°C, which destroyed even the interaction between laminin and the affinity matrix. Re-probing the affinity Western blots for SabA would help answer some of these questions. The antibodies could be used to determine if SabA was present in the initial protein sample incubated with the laminin matrix as well as being present in the eluted protein sample, albeit, at a lower abundance. SabA antibodies are not commercially available but antibodies to a recombinant version of SabA have recently been raised in rabbits by Aspholm et al. (2006). However, a SabA deletion mutant of *H. pylori* was further characterised in this thesis with respect to laminin-binding, and this is described in the following chapter.

In summary, *H. pylori* has previously been shown to bind ECM proteins, including laminin. However, the role of laminin binding during infection with *H. pylori* is
uncertain. In this study, two potential laminin-binding proteins were identified by affinity chromatography, namely, HpaA and HorF. However, their characterisation proved challenging. The difficulty establishing functional recombinant versions limited the characterisation of their laminin-binding properties. No common amino acid motif/domain, secondary structure or transmembrane topology feature was found when the amino acid sequences of known laminin-binding proteins, from other bacteria, and the potential laminin-binding proteins identified by this study were examined. Improved isolation of the candidate laminin-binding proteins in their native form may help to overcome the difficulties of working with the recombinant versions. This could be achieved by creating antibodies to the proteins that could be used to isolate the proteins in their native form by immuno-affinity techniques.
CHAPTER IV

EFFECT OF IRON AVAILABILITY ON THE ADHESIVE PROPERTIES OF *HELICOBACTER PYLORI* TO ECM AND IRON-BINDING PROTEINS/MOLECULES
Chapter IV

Introduction

Iron is an essential nutrient for all living organisms and its acquisition from the environment is critical to bacteria. It plays an essential role in cellular metabolism, participating in respiration, enzyme catalysis and protein structure stabilisation (Wooldridge and Williams, 1993). It also acts as an environmental stimulus for differential gene expression in bacterial pathogens; factors encoded by iron-regulated genes include iron acquisition systems, haemolysins, toxins and oxidative stress defence (Litwin and Calderwood, 1993b, Grifantini et al., 2003, Kadner, 2005, Fassbinder et al., 2000). In aerobic environments, iron exists primarily in the oxidised ferric form, Fe(III), which at pH 7, has low solubility (Chipperfield and Ratledge, 2000) but is also found in the more soluble ferrous form, Fe(II). Tight control of free iron by the host not only reduces oxidative stress [caused by the iron ions catalysing the formation of reactive oxygen radicals (Miller and Britigan, 1997)] but also acts as an important host bacterial-defence system (Payne, 1993, Weinberg, 1993). However, pathogenic bacteria can often circumvent this defence system by developing mechanisms to acquire iron from the host.

In humans iron-binding glycoproteins such as transferrin, in serum, and lactoferrin, on mucosal surfaces (Barclay, 1985, Graham et al., 1992, Griffiths and Williams, 1999) are used to control the availability of free iron. Pathogenic bacteria have adapted to this iron-limiting strategy by developing highly specific and effective iron assimilation systems. A common iron acquisition system present in many pathogens is the secretion of low molecular weight, high-affinity iron chelators (siderophores) which are able to remove iron from transferrin or lactoferrin (Baig et al., 1986, Crosa, 1989). Another acquisition mechanism used by bacteria is to take iron directly from transferrin and lactoferrin through direct binding to a specific receptor on the outer membrane (Otto et al., 1992, McKenna et al., 1988, Neilands, 1982). Iron-binding protein receptors have been found in pathogenic species of the genus Neisseria (Lee and Schryvers, 1988, Mickelsen et al., 1982), Haemophilus influenzae (Schryvers, 1989), Pasteurella haemolytica (Ogunnariwo and Schryvers, 1990), Actinobacillus pleuropneumoniae (Gerlach et al., 1992), and Bordetella pertussis (Menozzi et al., 1989).
A large number of bacteria also use haem compounds as a source of iron (Law et al., 1992, Perry, 1993, Stoebner and Payne, 1988, Stull, 1987) and specific haem receptors have been identified in some species (Worst et al., 1995, Lee, 1992, Stojiljkovic and Hantke, 1992).

Expression of iron acquisition, transport and storage systems in bacteria is controlled by the transcriptional regulator ferric uptake regulator (Fur), with ferrous iron acting as a co-repressor (Hantke, 2001, Andrews et al., 2003). Fur was first identified in Salmonella typhimurium (Ernst et al., 1978) and has been most extensively characterised in Escherichia coli (Hantke, 1984, Schaffer et al., 1985). Fur homologues have been identified in several other Gram-negative bacteria, including Pseudomonas species (Prince et al., 1993, Venturi et al., 1995), Vibrio species (Litwin et al., 1992, Litwin and Calderwood, 1993a, Tolmasky et al., 1994), Neisseria species (Berish et al., 1993, Karkhoff-Schweizer et al., 1994, Thomas and Sparling, 1994), Yersinia pestis (Staggs and Perry, 1991) and H. pylori (Bereswill et al., 1998a). Besides its role in iron homeostasis FUR is also involved in the regulation of additional metabolic processes including oxidative stress defence and acid resistance (Escolar et al., 1999, Bijlsma et al., 2002, Hall and Foster, 1996).

Other, Fur independent, bacterial systems are affected by iron depleted conditions. The expression of adherence factors by several species is influenced by an iron-depleted environment. Iron-depleted growth medium promotes slime production by Staphylococcus aureus (Baldassarri et al., 2001) and Staphylococcus epidermidis (Deighton and Borland, 1993), production of mucin-binding adhesins by Pseudomonas aeruginosa (Scharfman et al., 1996) and adherence to HEp-2 cells by Vibrio parahaemolyticus (Dai et al., 1992). De Oliveira Moreira et al. found that Corynebacterium diphtheriae showed enhanced adherence to erythrocytes and HEp-2 cells but inhibited adherence to glass surfaces (Moreira Lde et al., 2003) under iron-depleted conditions. Iron-depleted conditions also increased adhesion of S. aureus to plastic (Morrissey et al., 2002) while an increase in expression of fibronectin binding protein (Cj1478c) and Haemin-uptake system outer membrane receptor (Cj1614) was observed for Campylobacter jejuni (Holmes et al., 2005) grown under iron-depleted conditions. Gene expression study of Neisseria meningitidis found an increase in
expression of adherence genes such as *frpC* and *frpA/C*-related genes (Grifantini *et al.*, 2003).

Although the production of siderophores by *H. pylori* is uncertain (Husson *et al.*, 1993, Illingworth *et al.*, 1993) receptors to iron-binding proteins have been identified. These include lactoferrin binding protein (Husson *et al.*, 1993, Dhaenens *et al.*, 1997) and haem binding proteins (Worst *et al.*, 1995, Worst *et al.*, 1996, Worst *et al.*, 1999) which showed enhanced expression in iron-depleted conditions. Gene and protein expression studies have identified a variety of components controlled by iron availability. Some of these are under FUR control such as ferric iron transporters *fecA1/A2*, high affinity ferrous iron transporter (*feoB*) and iron storage ferritin *Pfr* (Ernst *et al.*, 2005, Fassbinder *et al.*, 2000, van Vliet *et al.*, 2002, Gancz *et al.*, 2006). Addition of human lactoferrin to the iron-deficient growth medium of *feoB* mutants reversed their restricted growth demonstrating the ability of *H. pylori* to use lactoferrin as a source of iron (Velayudhan *et al.*, 2000). Fur-mediated regulation is also required for gastric colonisation by *H. pylori*, as demonstrated in a mouse model of infection (Bury-Mone *et al.*, 2004).


The gene and protein expression profiles of many other iron-responsive genes/proteins have been determined in *H. pylori* but their roles have not been well defined (Merrell *et al.*, 2003b, Ernst *et al.*, 2005, Fassbinder *et al.*, 2000, van Vliet *et al.*, 2002, Gancz *et al.*, 2006). No studies have been carried out to date on the effect iron has on the adherence properties of *H. pylori*. Previous studies have demonstrated the ability of *H. pylori* to adhere to extracellular matrix (ECM) proteins with potential receptors being identified for heparan (Utt *et al.*, 2001) and laminin (Valkonen *et al.*, 1997, Walz *et al.*, 2005). Walz *et al.* used a combination of a *sabA* mutant and sialidase treated glycoconjugate arrays to demonstrate that the adherence of *H. pylori* to laminin is mediated by the sialic acid-binding adhesin, SabA. In another study,
Terrés et al. (2003) found that laminin protected the barrier function of intact T84 epithelial cell monolayers from disruption by H. pylori extracts.

Much of the current literature on H. pylori adherence contains information on ligand binding, which is qualitative but not fully quantitative. The use of surface plasmon resonance analysis will provide more accurate real-time quantitative information than is currently available. This is the first study providing real-time data of whole cell H. pylori binding to ECM and iron-binding proteins/molecules under normal and iron-depleted conditions. No published data exist on the effect iron has on the adherence of H. pylori to ECM ligands along with iron-binding protein, lactoferrin and iron-binding molecule, hemin. These characteristics were examined during this study.

**Objectives**

- Determine and compare the effect of iron availability on *H. pylori* adherence to ECM ligands.
- Determine and compare the effect of iron availability on *H. pylori* adherence to iron-binding proteins/molecules
- Determine the effect ferric uptake regulator protein has on *H. pylori* adherence to ECM and iron-binding proteins/molecules.
Technology Overview - Surface Plasmon Resonance

The principle of SPR is summarised in Figure 4.a. SPR detects binding interactions by monitoring the reflection of a beam of light off the interface between an aqueous solution of potential binding molecules (prey) and a biosensor surface [containing a thin layer of gold] carrying immobilised ligand (bait) protein. The energy from a reflected light interacts with the cloud of electrons in the gold film, generating a plasmon – an oscillation of the electrons at right angles to the plane of the gold surface. The plasmon generates an electric field above and below the gold surface. Any change in the composition of the environment within the range of the electric field causes a measurable change in the resonance angle. Real-time changes in the refractive index, measured as response units, correspond to a bait-prey interaction. The surface of the sensor chip can be regenerated for further experiments (Alberts et al., 2002).

The measurement of the interaction between proteins/molecules and whole cell microorganisms is an infrequent use of surface plasmon resonance technology. Although bacteria have been immobilised to the sensor chip surface instead of the ligand (Medina and Fratamico, 1998), the usual experimental strategy allows bacteria to flow freely over the surface of the chip (Clyne et al., 2004, Oli et al., 2006). The physical dimensions of the flow cells are l = 2.4 mm, w = 0.5 mm, h = 0.05 mm, allowing large particles to be analysed. The average H. pylori bacterial cell has a diameter of 0.5 to 0.9 µm, (Young et al., 2001) around 1/50 to 1/100th the size of a flow cell (Christensen, 1997). For the purpose of this thesis, the ligands of interest were immobilised to the chip surface and viable H. pylori were passed over the surface of the chip under a constant flow rate of 5 µl per min. Equilibrium was established between association/disassociation of the H. pylori-ligand interaction, which was measured over a short time period (Figure 4.b). By utilising SPR in this manner some of the conditions H. pylori experiences in vivo, particularly the flow of liquid along the epithelial lining of the intestine, can be mimicked and the interactions recorded as they occur.
Figure 4.a. Surface plasmon resonance. SPR measures the interaction between a ligand immobilised to the sensor chip surface (red – bait) and a solution containing the ligand of interest (green – prey). Association and disassociation interactions result in a change to the angle of reflection of a beam of light off the interface between the interacting ligands and is monitored in real-time. Using SPR in this manner facilitates studies to be carried out on *H. pylori* which more closely mimic the conditions experienced by the bacterium in the gut, such as the continuous flow of liquid along the epithelial lining of the gut. Figure taken from Alberts (2002).
Figure 4.b. Illustration of the real-time measurement of the interaction between *H. pylori* and a ligand immobilised to the sensor chip surface. A ligand is attached to the sensor chip. Any association between *H. pylori* and the ligand changes the optical properties of the chip surface and these are recorded as response units (RU). Equilibrium is established between association/dissociation of the *H. pylori*-ligand interaction. The chip surface can be regenerated for further use by removing any ligand-bound bacteria with fresh medium. Figure adapted from Biacore Instruction presentation (Biacore, Sweden).
Results

*H. pylori* strain NCTC 11638 (WT) and isogenic strain mutants *sabA* (Δ*sabA*) & *ferric uptake regulator* (Δ*fur*), were grown in normal or iron-depleted liquid medium. Bacteria were harvested and re-suspended in 50 mM Tris buffer. *H. pylori* binding to laminin (Lam), fibronectin (Fib), collagen IV (Col), lactoferrin (Laf) and hemin (Hem) was measured by surface plasmon resonance using a BIAcore® system. Each ligand was covalently attached to a sensor chip and the bacteria were passed over the ligand under controlled flow conditions. A change in the refractive index corresponding to bacteria-ligand interaction was recorded. Data were combined from repeat experiments and analysed by mean comparison and Student’s (paired) t-test. The data can be found in Table 4.1 located at the end of the results section.

**H. pylori** does not grow in the presence of desferal but growth was restored when medium was supplemented with FeSO₄

To demonstrate that iron was the limiting factor for bacterial growth, *H. pylori* was grown in normal medium, medium depleted of iron (using desferal), and medium previously depleted of iron but supplemented with additional iron (using FeSO₄). The optical density at 600 nm was measured over a period of 72 h. *H. pylori* growth was severely reduced in the presence of desferal and growth was restored when FeSO₄ was added back to the medium (Figure 4.1). The protein profiles of the bacteria grown under the 3 conditions were compared by SDS-PAGE after 72 h of growth. The protein profiles for *H. pylori* grown in normal and FeSO₄ supplemented medium are similar to one another (Figure 4.2). However, when these profiles are compared with those obtained from *H. pylori* grown under iron-depleted conditions clear differences are apparent (Figure 4.2); for example, additional proteins at 55 – 66 kDa and approx. 150 kDa are evident in the bacteria grown under iron-reduced conditions. Also, reduced expression of other proteins is a consequence of growth in the absence of iron. For example, a series of proteins between 84 kDa and 116 kDa appear to be under expressed compared with the adjacent profiles of bacteria grown under iron-sufficient conditions.
Figure 4.1: Growth of *H. pylori* in iron sufficient and iron reduced conditions. *H. pylori* was grown in normal medium, medium depleted of iron (using desferal – iron chelator) and medium previously depleted of iron but supplemented with additional iron (using FeSO₄). The optical density at 600 nm was taken over a period of 72 h.
Figure 4.2: Protein profile of *H. pylori* grown in iron sufficient and iron depleted conditions. *H. pylori* was grown in normal medium (N), medium depleted of iron (IR) and medium previously depleted of iron but supplemented with additional iron (IS) for 3 days. Equal amounts of total bacteria were harvested, re-suspended in Sample Buffer and analysed by SDS-PAGE (7.5%). The gel was stained with Coomassie blue. [MW Molecular Mass Markers].
*H. pylori* protein profile effected by iron-depleted conditions after 24 hours

Previous research groups have reported changes in the protein profile of *H. pylori* grown under normal and iron-depleted conditions. As shown earlier in this study, the growth of *H. pylori* is retarded in iron-depleted conditions. To facilitate the surface plasmon resonance experiments the minimum time required for the protein profile to adopt the profile seen under iron-reduced conditions was determined. *H. pylori* was grown under iron-depleted conditions over a 3-day period. Each day, total bacteria were harvested and analysed by SDS-PAGE. The altered protein profile becomes apparent after 24 h of growth with little additional changes observed at 48 and 72 h (Figure 4.3).

**Optimisation of conditions for surface plasmon resonance measurements**

Tween-20® is often added to buffers when passing solutions over the sensor chip surface during surface plasmon resonance analysis to reduce non-specific binding of molecules to the chip surface and/or attached ligand. To determine the effect Tween-20® might have on *H. pylori* viability, liquid grown *H. pylori* were harvested and re-suspended in Tris buffered saline (TBS) with and without Tween-20® (0.05% v/v). Columbia blood agar plates were inoculated with *H. pylori* from these suspensions over a period of 2.5 h. Colony counts were carried out after 4 days of growth. Few colonies were observed when bacteria were treated with Tween-20® for 30 min, and no colonies were observed after bacteria were treated for 1 h. No deterioration in viability was observed for *H. pylori* suspended in TBS only (Figure 4.4). Thus Tween-20® was excluded from all experiments with the SPR instrument, as it clearly had a deleterious effect on *H. pylori* viability.
Figure 4.3: Protein profile of *H. pylori* grown in iron depleted conditions. *H. pylori* was grown in iron-depleted conditions over a period of 3 days. Each day total bacteria were harvested, re-suspended in Sample Buffer and analysed by SDS-PAGE (7.5%). The gel was stained with Coomassie blue. [MW = Molecular Mass Markers].
Figure 4.4: Viability of \textit{H. pylori} in TBS with and without Tween-20\textsuperscript{®}. Liquid grown \textit{H. pylori} were harvested and re-suspended in 50 mM Tris Buffered Saline (pH 7.4) with and without Tween-20\textsuperscript{®} (0.05\% v/v). Columbia Blood Agar plates were inoculated with \textit{H. pylori} from these suspensions over a period of 2.5 h. Colony counts were carried out after 4 days of growth.
Under iron-depleted conditions H. pylori displays increased adhesion to laminin

In the following sections, the units used to define the extent of adhesion are ‘response units’ derived from the SPR instrument and directly reflect binding events. *H. pylori* wild-type (WT) grown under iron-depleted conditions showed an increase in binding to laminin \[80 +/- 8 \text{ SEM} \text{ vs. } 136 +/- 19 \text{ SEM}, p<0.009\] when compared with bacteria grown in the presence of iron (Figure 4.5, A & Figure 4.6). An increase in binding to laminin was also observed for \(\Delta\text{fur}\) \[57 +/- 6 \text{ SEM} \text{ vs. } 113 +/- 27 \text{ SEM}, p<0.038\]. This indicates that the increased binding to laminin occurred independent of Fur. Laminin binding by \(\Delta\text{sabA}\) under normal conditions was reduced when compared to the WT with no further change seen when the cells were grown under iron-depleted conditions \[42 +/- 2 \text{ SEM} \text{ vs. } 47 +/- 2 \text{ SEM}, p<0.085\].

Under iron-depleted conditions H. pylori displays increased adhesion to fibronectin

*H. pylori* WT grown under iron-depleted conditions showed an increase in binding to fibronectin \[85 +/- 11 \text{ SEM} \text{ vs. } 102 +/- 12 \text{ SEM}, p<0.013\] when compared with bacteria grown in the presence of iron (Figure 4.5, B & Figure 4.6). An increase in binding to fibronectin was also observed for \(\Delta\text{fur}\) \[83 +/- 12 \text{ SEM} \text{ vs. } 95 +/- 13 \text{ SEM}, p<0.029\]. This indicates that the increased binding to fibronectin occurred independent of Fur also, similar to laminin. The binding of \(\Delta\text{sabA}\) to fibronectin showed a slight increase when grown under iron-depleted conditions \[61 +/- 2 \text{ SEM} \text{ vs. } 65 +/- 2 \text{ SEM}, p<0.038\] when compared with bacteria grown in the presence of iron. However, the level of binding for \(\Delta\text{sabA}\), under normal conditions, was reduced when compared to the WT.
Figure 4.5: Binding of *H. pylori* strains to specific ECM ligands. *H. pylori* strain NCTC 11638 (WT), *sabA* mutant (∆*sabA*) & *ferric uptake regulator* mutant (∆*fur*) were grown in normal or iron-depleted liquid media. Bacteria were harvested and re-suspended in 50 mM Tris buffer. *H. pylori* binding to (A) Laminin, (B) Fibronectin and (C) Collagen was measured by surface plasmon resonance. Data were combined from repeat experiments and analysed by mean comparison and Student’s (paired) t-test, - p-value < 0.05, n=3
Figure 4.6: Binding of specific *H. pylori* strains to ECM ligands. *H. pylori* strain NCTC 11638 (WT), *sabA* mutant (Δ*sabA*) & ferric uptake regulator mutant (Δ*fur*) were grown in normal or iron-depleted liquid media. Bacteria were harvested and re-suspended in 50 mM Tris buffer. *H. pylori* WT (A), Δ*fur* (B) and Δ*sabA* (C) binding to ECM ligands was measured by surface plasmon resonance. Data were combined from repeat experiments and analysed by mean comparison and Student’s (paired) t-test, - p-value < 0.05, n=3

Note: No Blank data exists for Δ*fur*.
**H. pylori** showed an increase in adhesion to collagen IV under iron-depleted conditions

*H. pylori* WT grown under iron-depleted conditions showed an increase in binding to collagen IV [25 +/- 2 SEM vs. 41 +/- 2 SEM, p<0.023] when compared with bacteria grown in the presence of iron (Figure 4.5, C & Figure 4.6). The binding of ΔsabA to collagen IV showed an increase when grown under iron-depleted conditions [20 +/- 1 SEM vs. 33 +/- 2 SEM, p<0.038]. The level of binding for ΔsabA, under normal conditions, was reduced when compared to the WT.

**H. pylori** showed a Fur independent increase in adhesion to lactoferrin under iron-depleted conditions

*H. pylori* WT grown under iron-depleted conditions showed an increase in binding to lactoferrin [89 +/- 3 SEM vs. 112 +/- 5 SEM, p<0.001] when compared with bacteria grown in the presence of iron (Figure 4.7, A & Figure 4.8). An increase in binding to lactoferrin was also observed for Δfur [81 +/- 4 SEM vs. 100 +/- 6 SEM, p<0.001]. This indicates that the increased binding to lactoferrin occurred independent of fur. The binding of ΔsabA to lactoferrin showed a decrease when grown under iron-depleted conditions [146 +/- 17 SEM vs. 120 +/- 13 SEM, p<0.013]. The level of binding for ΔsabA, under normal conditions, was increased when compared to the WT.

**H. pylori** showed no change in adhesion to haemin under iron-depleted conditions

*H. pylori* WT grown under iron-depleted conditions showed no change in binding to haemin [24 +/- 1 SEM vs. 22 +/- 1 SEM, p<0.081] (Figure 4.7, B & Figure 4.8). No change in binding to haemin was observed either for Δfur [21 +/- 1 SEM vs. 18 +/- 1 SEM, p<0.290]. The binding of ΔsabA to haemin showed no change when grown under iron-depleted conditions [26 +/- 2 SEM vs. 22 +/- 2 SEM, p<0.120]. The level of binding for ΔsabA, under normal conditions, was slightly increased when compared to the WT.
Figure 4.7: Binding of *H. pylori* strains to lactoferrin and haemin. *H. pylori* strain NCTC 11638 (WT), *sab*A mutant (∆*sab*A) & ferric uptake regulator mutant (∆*fur*) were grown in normal or iron-depleted liquid media. Bacteria were harvested and re-suspended in 50 mM Tris buffer. *H. pylori* binding to (A) lactoferrin, (B) haemin was measured by surface plasmon resonance. Data were combined from repeat experiments and analysed by mean comparison and Student’s (paired) t-test, *p*-value < 0.05, *n*=3
Figure 4.8: Binding of specific H. pylori strains to iron binding protein/molecule. H. pylori strain NCTC 11638 (WT), sabA mutant (ΔsabA) & ferric uptake regulator mutant (Δfur) were grown in normal or iron-depleted liquid media. Bacteria were harvested and re-suspended in 50 mM Tris buffer. H. pylori WT (A), Δfur (B) and ΔsabA (C) binding to iron binding protein/molecule was measured by surface plasmon resonance. Data were combined from repeat experiments and analysed by mean comparison and Student’s (paired) t-test, - p-value < 0.05, n=3
### Table 4.1: Data used to generate Figures 4.5 to 4.8.

*H. pylori* strain NCTC 11638 (WT), *sabA* mutant (Δ*sabA*) & *ferric uptake regulator* mutant (Δ*fur*) were grown in normal or iron-depleted liquid medium. Bacteria were harvested and re-suspended in 50 mM Tris buffer. *H. pylori* binding to ECM and iron binding proteins/molecule was measured by surface plasmon resonance. Data was combined from repeat experiments and analysed by mean comparison and Student’s (paired) t-test: - p-value < 0.05, n=3

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**Discussion**

The level of free iron in the immediate environment of bacteria has profound effects on their survival and viability – too much is toxic while too little leads to severely reduced growth. Pathogenic bacteria have developed various mechanisms to maintain iron homeostasis to increase their survival and colonisation abilities in the host. These include modulation of adhesin/receptor proteins, development of iron acquisition/uptake systems and alteration to virulence proteins. Some of these changes are under the control of the Ferric Uptake Regulator (Fur) protein while others are controlled directly by iron concentrations. This study has demonstrated an increase in adherence of *H. pylori* to ECM proteins and the iron-binding protein lactoferrin when the bacteria were grown under iron-reduced conditions. This increased adhesion occurred independently of Fur regulation.

The protein profile of *H. pylori* is altered when grown under iron-reduced conditions. Similar data were obtained by Dhaenens *et al.* (1997) for *H. pylori* grown for 72 h, however, this current study also demonstrates that the altered protein profile occurs after 24 h with little additional changes observed at 48 and 72 h. The binding analysis for this study was carried out using bacteria grown for 48 h under iron-reduced conditions.

The BIAcore® system is based on the principle of surface plasmon resonance, which can detect and measure, in real-time, binding interactions occurring in solution between different ligands (Jonsson *et al.*, 1991b, Alberts *et al.*, 2002). Binding characteristics obtained by surface plasmon resonance have been successfully obtained for *H. pylori*, *E. coli*, *S. aureus*, *Streptococcus pneumoniae* and *S. mutans* (Clyne *et al.*, 2004, Oli *et al.*, 2006, Berube *et al.*, 1999, Holmes *et al.*, 1997).

In a study of 14 different strains of *H. pylori* Trust *et al.* (1991) demonstrated that *H. pylori* binding to radiolabelled laminin in liquid medium varied across the different strains. Another similar study demonstrated that *H. pylori* strain NCTC 11637 binds more radiolabelled laminin than strain NCTC 11638 (Valkonen *et al.*, 1997) which was used for the studies described in this chapter. Here, this study confirms the ability of *H. pylori* to bind laminin. Additionally, this study demonstrates a greatly
enhanced (71%) ability of *H. pylori* to bind laminin under iron-reduced conditions. The increased adherence to laminin does not appear to be under the influence of the FUR protein as a similar substantial increase is observed in the *fur* mutant when grown under iron-reduced conditions. These data suggest the protein(s) responsible for binding to laminin are up regulated.

Walz *et al.* (2005) identified SabA as a laminin binding protein. This current study reinforces the important role *H. pylori* SabA plays in binding to laminin. A considerable decrease (48%) in *H. pylori* ability to bind laminin was seen for the *sabA* mutant. The residual binding of ΔsabA indicates that other adhesins must be involved in binding to laminin possibly including the 25 kDa laminin binding protein isolated by Valkonen *et al.* (1997) and lipopolysaccharide (Valkonen *et al.*, 1994). The observed iron-responsive increase in adhesion to laminin in WT cells may be due to SabA given the lack of increase in adhesion by the *sabA* mutant grown under iron-reduced conditions. However, it is not known if the increase in adhesion is due to an increase in SabA protein expression or a change in ligand-bacteria interaction due to the presence of iron. Gene array studies carried out on *H. pylori* grown under iron-reduced conditions show no change in expression of the *sabA* gene (Merrell *et al.*, 2003b, Ernst *et al.*, 2005, Gancz *et al.*, 2006).

Binding of various strains of *H. pylori* to fibronectin was previously shown to be much weaker than to laminin (Trust *et al.*, 1991, Ringner *et al.*, 1994) however this current study shows a level of binding not much different compared to laminin. These discrepancies may be explained by a combination of difference in source of fibronectin and bacterial strains. Ringner *et al.* (1994) do not describe the type of fibronectin they used but Trust *et al.* (1991) used fibronectin derived from porcine plasma while the fibronectin used in this study was derived from human tissue. Plasma fibronectin is more soluble than tissue fibronectin and is structurally and biologically different (Mosher and Furcht, 1981). Walsh *et al.* (1995) found that eosinophils preferentially survive on tissue as opposed to plasma fibronectin which they attribute to an increased presence of integrin binding sites on tissue fibronectin. Both Trust *et al.* (1991) and Ringner *et al.* (1994) included strain NCTC 11637 in their studies but strain NCTC 11638 was used for this study. There was a decrease of 28% in binding to fibronectin for the *sabA* mutant suggesting a possible role for SabA
binding to fibronectin also. Walz et al. (2005) previously demonstrated using overlay assays that the *H. pylori* strain J99 *babA/sabA* mutant could still bind to fibronectin.

Both WT and Δfur show a similar level of increase in binding to fibronectin under iron-reduced conditions suggesting a Fur-independent mechanism. Although VacA is normally viewed as a secreted toxin, the surface-associated toxin (Telford et al., 1994) has been shown to be biologically active (Ilver et al., 2004, Pelicic et al., 1999) and purified VacA was shown to bind fibronectin (Hennig et al., 2005). Other studies have shown its expression is up regulated under iron-reduced conditions independent of Fur control (Merrell et al., 2003b, Szczepanek et al., 1999, Ernst et al., 2005). VacA may have some role in the increased adherence observed under iron-reduced conditions but SabA also plays a part. The binding of ΔsabA to fibronectin showed an increase when grown under iron-reduced conditions of 6% down from 19% observed for WT bacteria grown in iron-reduced conditions.

Few studies have analysed *H. pylori* binding to collagens and to date no receptor has been identified (Trust et al., 1991, Ringner et al., 1994). As with laminin and fibronectin, Trust et al. (1991) demonstrated that *H. pylori* binding to radiolabelled collagen in liquid medium varied across different strains. In this current study, both WT and ΔsabA were found to adhere to collagen IV albeit at a lower level than for laminin and fibronectin. As with fibronectin, SabA may play a role in this interaction as the level of binding to collagen for ΔsabA, under normal conditions, was reduced by 20% when compared to the WT. However, the binding of WT and ΔsabA to collagen IV showed an increase when grown under iron-depleted conditions of 65% and 66%, respectively, demonstrating the limited role SabA might have for collagen IV binding.

Dhaenens et al. (1997) demonstrated the presence of a lactoferrin binding protein in *H. pylori* but only when the bacteria were grown in iron-depleted conditions. Both Walz et al. (2005) and Khin et al. (1996) found that WT J99 and the J99 *sabA/babA* mutant, and strains NCTC 11637 and CCUG 17875 (equivalent to NCTC 11638) all bound lactoferrin under normal conditions. This study confirms these previous observations in addition to demonstrating an increased (25%) level of binding of the WT to lactoferrin under iron-depleted conditions. A similar level of increased (24%)
adhesion was observed for Δfur suggesting the increase is independent of Fur regulation. Interestingly, the level of binding of the ΔsabA, under normal conditions, was increased by 64% when compared to the WT. Although not reported, this can also be seen in the bacterial overlay assays of Walz et al. (2005)—the spot intensity of bound bacteria is less intense for WT J99 than for the babA/sabA mutant (their study was qualitative rather than quantitative). This substantial level of increase is not seen when WT and ΔsabA binding to lactoferrin under iron-depleted conditions are compared; the levels of binding differ by 7%. Few, if any, studies have been carried out on the binding characteristics of SabA to lactoferrin. The results presented here suggest that SabA may interfere with the binding of H. pylori to lactoferrin under normal conditions and this interference is removed under iron-depleted conditions. From the current data available there is no obvious reason for this to occur.

Husson et al. (1993) demonstrated that iron-depleted growth medium supplemented with haemin was able to stimulate growth of H. pylori. Later Worst et al. (1999, 1996, 1995) identified an immunogenic haemin binding protein in H. pylori strain ATCC 43504 (equivalent to NCTC 11637) and a corresponding protein in strain NCTC 11638. The receptor was only present in bacteria grown in iron-depleted conditions. This current study observed low levels of haemin binding across all three strains with no additional enhanced binding when grown under iron-depleted conditions. Worst et al. (1999) found that a haem-uptake system in H. pylori (NCTC 11638) was constitutively active; iron concentrations in the growth medium had no effect. The data from this current study lack information on binding affinities. Perhaps the affinity for binding haemin increases under iron-depleted conditions, which would not require additional receptors. Another possible explanation for the data is that the receptor-binding site is blocked by an aberrant structural conformation of haemin when immobilised to the surface of the sensor chip. For this study the amine groups from the ECM proteins and lactoferrin were attached to the carboxyl group on the chip surface. However, the haemin molecule (Figure 4.c, below) does not contain an amine group therefore an amine linker molecule was required for the ligand attachment step.
To minimise the disruption of the entire aromatic ring structure of the haemin molecule and reduce the number of chemical groups to be altered the carboxyl group was targeted. Haemin contains two carboxyl groups that have the additional advantage of being furthest from the aromatic ring thus minimising the possible disruption of the complex carbon ring. Ethylene-diamine was used as a linker molecule – the amine of one arm attached to the chip surface while the remaining amine group attached to the altered haemin carboxyl groups. This immobilisation procedure may have blocked the access of \textit{H. pylori} to interact with the iron containing haemin molecule.

In conclusion, this study provides the first direct evidence that the adhesion of \textit{H. pylori} to ECM proteins and iron-binding protein, lactoferrin, increases when grown under iron-reduced conditions. The reduced iron availability created by this study most likely mimics the \textit{in vivo} environment experienced by \textit{H. pylori} which makes the observations all the more relevant. The ability to bind to the ECM of the infected host may provide an advantage to \textit{H. pylori} by enabling it to continue to colonise and persist in the gastric mucosa, eventually leading to the development of gastric ulcers or cancers. Furthermore, the presence of a lactoferrin binding on \textit{H. pylori} has previously been described. The increased binding of \textit{H. pylori} to lactoferrin when grown under iron-reduced conditions may provide \textit{H. pylori} with vital access to a source of iron and possibly increase its pathogenic potential in the infected host.
CHAPTER V
EFFECT OF IRON AVAILABILITY ON THE DIFFERENTIAL EXPRESSION OF MEMBRANE PROTEINS FROM
HELCIOBACTER PYLORI
Chapter V

Introduction

The bacterial membrane represents the vital interface between the microorganism and its environment. For pathogenic bacteria, membrane proteins, particularly surface exposed proteins, directly interact with components of the host and can act as adhesins or receptors to facilitate colonization and mediate acquisition of nutrients and macromolecules from their milieu (Cullen et al., 2004, Odenbreit, 2005, Albertson et al., 1998). The membrane protein expression profile modifies as the bacteria adapt to their ever-shifting surroundings. Analysis of these changing membrane profiles will help to improve our understanding of pathogenic bacteria and may help to identify potential therapeutic targets for vaccines and other antimicrobial agents.

_H. pylori_ is known to be associated with various gastric diseases including chronic gastritis, peptic ulcer, and gastric cancer (Dunn et al., 1997). More recently, _H. pylori_ has been linked to iron-deficiency anaemia (IDA), particularly in adolescents and pre-menopausal women (Barabino, 2002, DuBois and Kearney, 2005). Choe et al. (Choe et al., 2001, Choe et al., 1999, Choe et al., 2000) demonstrated that IDA in _H. pylori_ infected patients was refractory to iron therapy and was reversed only after eradication of _H. pylori_ infection, suggesting a possible association between _H. pylori_ infection and IDA in adolescents. Epidemiological studies provide further evidence supporting a link between refractory IDA and _H. pylori_ infection (Milman et al., 1998, Seo et al., 2002).

Several microarray studies have provided extensive data on the effect iron availability has on gene expression in _Helicobacter pylori_ (Merrell et al., 2003b, Ernst et al., 2005, Szczebara et al., 1999, van Vliet et al., 2002, Gancz et al., 2006). A wide variety of gene classes have been shown to be affected; ranging from genes encoding proteins for virulence factors and iron acquisition to energy metabolism and motility. However, gene expression does not always directly correlate with protein expression (Anderson and Seilhamer, 1997, Gygi et al., 1999). Additional post-translational
mechanisms, including protein translation, post-translational modification, and degradation, also influence the level of protein present in a given cell or tissue.

Various proteomic approaches have been successfully used to examine protein expression in bacteria (Rabilloud et al., 1999, Raulston, 1997, Hall and Foster, 1996, Radosevich et al., 2007, Musser, 2006, Rodriguez-Ortega et al., 2006). These include two-dimensional electrophoresis (2DE), proteolytic enzyme ‘shaving’ of surface exposed proteins and the labelling technique ‘iTRAQ™’ (isobaric Tag for Relative and Absolute Quantitation (Applied Biosystems)). McAtee et al. (2001) used 2DE to identify differentially regulated cellular proteins in metronidazole resistant *H. pylori*. In a more recent study Park et al. used 2DE to compare the total protein profiles of *H. pylori* strains obtained from IDA and non-IDA patients (Park et al., 2006). Their study indicated that the non-IDA and IDA strains could be distinguished by their protein profiles, suggesting that the polymorphism of *H. pylori* strains may be one of the factors determining the occurrence of *H. pylori*-associated IDA.

Few iron-responsive membrane proteins have been identified on *H. pylori*. Worst et al. (1995, 1996, 1999) describe three iron-responsive haem-binding proteins while Dhaenens et al. (1997) describe a lactoferrin binding protein on the membrane of *H. pylori*. Little work has been carried out to determine the alterations to the membrane protein expression profile, particularly adhesins, of *H. pylori* in response to iron availability. In this chapter the effect of iron availability on the membrane enriched proteome of *H. pylori* was examined by 2DE and iTRAQ™ labelling in order to determine whether or not potential adhesins and other membrane proteins were modulated by the different growth conditions.

**Objectives**

- To determine and compare the effect of iron availability on the expression of *H. pylori* membrane proteins.
- To determine and compare the effect of iron availability on the expression of *H. pylori* surface exposed proteins.
Technology Overview - iTRAQ™ labelling

A number of techniques exist to compare protein expression levels between two biological states. Two dimensional gel electrophoresis (2DE) is the most commonly used and best characterised (Lopez, 2007). Another more recent technique is iTRAQ™ labelling (Ross et al., 2004) using isobaric reagents, i.e. same mass (Figure 5.a). The reagents react with each lysine side chain and N-terminal group of a peptide in a sample thus enhancing peptide coverage for any given protein, while allowing retention of structural information such as post-translational modifications.

During tandem mass spectrometry (MS/MS), the isobaric reagents are fragmented and the reporter group ions are generated displaying diagnostic ions in the region between m/z 114-117. Quantifying the peak areas of these resultant ions represents the relative amount of a given peptide in the respective sample. An additional series of mass spectrometry signals are generated (y- and b-ions) and used for protein identification (AppliedBiosystems, 2004) (Figure 5.b).

The incumbent comparative technique used by this laboratory is 2DE. Since iTRAQ™ labelling is only a recent development, it was employed following a 2DE strategy to enhance the data obtained for the overall comparative study.
**Figure 5.a. Structure of iTRAQ™ reagent.** The complete molecule consists of a reporter group, a mass balance group, and a peptide-reactive group. Figure taken from Ross *et al.* (2004).
Figure 5.b. MS/MS spectra from a multiplex sample (TPHPALTEAK) labelled with 4 iTRAQ™ reagents (1:1:1:1 ratio). Components of the spectra illustrated are (i) isotopic distribution of the precursor (parent) ion - TPHPALTEAK, (ii) low mass region showing the diagnostic reporter ions, (iii) & (iv) peptide fragment ions for identification. Figure taken from Ross et al. (2004).
Results

Comparison of membrane proteins isolated from *H. pylori* grown under normal and iron-depleted conditions by 1D SDS-PAGE

A number of studies (using various membrane isolation methods) have demonstrated differential expression of membrane proteins from *H. pylori* when grown under normal or iron-depleted conditions. To ensure differences in protein profiles can be observed for the membrane preparations used in this study, *H. pylori*, (strain NCTC 11638), membrane proteins were analysed by one-dimensional SDS-PAGE. The membrane proteins were isolated as outlined in Methods & Materials. Briefly, *H. pylori* was sonicated and the crude membrane fraction collected by centrifugation. The membrane fraction was further separated using Zwittergent® 3-14 detergent; outer membrane proteins are more soluble in Zwittergent® 3-14 while inner membrane proteins are less soluble. However, for this analysis the total membrane was required therefore the Zwittergent® 3-14 soluble and insoluble fractions were combined and the protein precipitated with acetone before being solubilised in SDS-PAGE sample buffer. Several proteins displayed differential expression when grown under iron-reduced conditions (Figure 5.1). For example, nine proteins, ranging from approximately 18 kDa to 200 kDa, displayed increased expression while four proteins, ranging from approximately 15 kDa to 116 kDa displayed reduced expression.
Figure 5.1: *H. pylori* membrane protein profile from bacteria grown in normal (N) and iron-reduced (IR) media. Zwittergent® 3-14 soluble and insoluble membrane fractions, from *H. pylori* strain NCTC 11638, were combined, the proteins acetone precipitated and solubilised in SDS-PAGE sample buffer. The protein profiles were compared on a Coomassie stained SDS-PAGE gel (12.5%). Solid arrows indicate proteins displaying increased protein expression under iron-reduced growth conditions while broken arrows indicate proteins displaying reduced protein expression.
Optimisation of conditions for 2 dimensional electrophoresis

Although the one-dimensional separation of membrane proteins isolated from *H. pylori* grown in normal and iron-depleted medium demonstrated differential expression, their separation was incomplete. To further separate the proteins two-dimensional electrophoresis (2DE) was employed. To achieve maximum membrane protein separation by 2DE different combinations of (a) *H. pylori* membrane enrichment preparations and (b) 2DE solubilising buffers were analysed.

*H. pylori* (strain NCTC 11638), membrane proteins were collected and fractionated with Zwittergent® 3-14 detergent. Detergent insoluble and soluble membrane fractions were precipitated with acetone, solubilised in 2DE Rehydration Buffer containing SDS (Figure 5.2), Zwittergent® 3-14 (Figure 5.3) or Trifluoroethanol (TFE) (Figure 5.4) and analysed by 2DE. More proteins were resolved when rehydration buffer containing TFE was used but many proteins were unresolved beyond pH 6. There was also increased horizontal and vertical streaking at the acidic end of the gel when detergent containing rehydration buffer was used.

*H. pylori* (strain NCTC 11638), membrane proteins were collected and fractionated by TFE-chloroform phase separation. The TFE soluble (Figure 5.5, A) and TFE insoluble (Figure 5.5, B) fractions were solubilised in rehydration buffer containing TFE and analysed by 2DE. The resolution and separation of proteins was much improved when compared to all previous 2DE conditions. Basic proteins were represented in both gels while horizontal and vertical streaking was reduced. The complement of TFE-soluble proteins was consistent in repeat experiments (Figure 5.6, A) while the TFE-insoluble proteins demonstrated variable resolution (Figure 5.6, B).
Figure 5.2: 2D protein profile of detergent fractionated *H. pylori* membrane proteins using rehydration buffer containing SDS. 100μg of Zwittergent® 3-14 (A) insoluble or (B) soluble membrane proteins, from *H. pylori* strain NCTC 11638, were acetone precipitated and solubilised in rehydration buffer containing SDS (0.25% v/v). The proteins were separated by IEF, pH 3-10 and SDS-PAGE (12.5%). The gels were stained with Colloidal Coomassie Blue (CCB) and then silver stained (BSS) as described by Moertz *et al.* (2001).
Figure 5.3: 2D protein profile of detergent fractionated *H. pylori* membrane proteins using rehydration buffer containing Zwittergent® 3-14. 100μg of Zwittergent® 3-14 soluble membrane proteins, from *H. pylori* strain NCTC 11638, were acetone precipitated and solubilised in rehydration buffer containing Zwittergent® 3-14 (1% v/v). The proteins were separated by IEF, pH 3-10 and SDS-PAGE (12.5%). The gels were stained with Colloidal Coomassie Blue (CCB) and then silver stained (BSS) as described by Moertz *et al.* (2001).
Figure 5.4: 2D protein profile of detergent fractionated *H. pylori* membrane proteins using rehydration buffer containing TFE. 100μg of Zwittergent® 3-14 (A) insoluble or (B) soluble membrane proteins, from *H. pylori* strain NCTC 11638, were acetone precipitated and solubilised in rehydration buffer containing TFE (40% v/v). The proteins were separated by IEF, pH 3-10 and SDS-PAGE (12.5%). The gels were stained with Colloidal Coomassie Blue (CCB) and then silver stained (BSS) as described by Moertz *et al.* (2001).
Figure 5.5: 2D protein profile of TFE/CHCl₃ fractionated *H. pylori* membrane proteins. Whole cell bacteria, from *H. pylori* strain NCTC 11638, were sonicated and the membrane fraction collected by centrifugation. The membrane proteins were phase separated using TFE/CHCl₃. The TFE soluble (A) and insoluble (B) proteins were solubilised in rehydration buffer containing TFE (40% v/v). The proteins were separated by IEF, pH 3-10 and SDS-PAGE (12.5%). The gels were stained with Colloidal Coomassie Blue (CCB).
Figure 5.6: Repeat 2D protein profiles of TFE/CHCl₃ fractionated *H. pylori* membrane proteins. Whole cell bacteria, from *H. pylori* strain NCTC 11638, were sonicated and the membrane fraction collected by centrifugation. The membrane proteins were phase separated using TFE/CHCl₃. The TFE soluble (A) and insoluble (B) proteins were solubilised in rehydration buffer containing TFE (40% v/v). The proteins were separated by IEF, pH 3-10 and SDS-PAGE (12.5%). The gels were stained with Colloidal Coomassie Blue (CCB).
**2 dimensional gel comparison of the membrane sub-proteome from *H. pylori* grown under normal and iron-depleted conditions**

Having established conditions that appeared to yield good resolution of membrane proteins *H. pylori*, (strain NCTC 11638), was grown under normal and iron-reduced conditions. Membrane enriched fractions were fractionated by TFE-chloroform phase separation. The TFE soluble fractions were solubilised in rehydration buffer containing TFE and compared by 2DE. Several proteins demonstrated altered expression patterns (Figure 5.7). For example, five proteins were unregulated under iron-reduced conditions (Figure 5.7, spots 1-5). Spots 1 and 2 were excised from the gel and identified by mass spectrometry. Spot 1 was identified as cell division protein (FtsH – HP1069) while spot 2 was identified as alkyl hydroperoxide reductase (AhpC – HP1563). Interestingly, AhpC is classified as a cytoplasmic protein yet it was associated with the *H. pylori* membrane fraction. Although the resolution of proteins was adequate it was going to be prohibitively expensive to identify all proteins of interest using the 2DE approach. Hence an alternative strategy was employed and this is presented in the following section.
Figure 5.7: 2D protein profiles of TFE/CHCl₃ fractionated *H. pylori* membrane proteins from *H. pylori* grown in normal and iron-reduced conditions. Whole cell bacteria, from *H. pylori* strain (NCTC 11638), grown in normal (N) or iron-reduced (IR) conditions were sonicated and the membrane fraction collected by centrifugation. The membrane proteins were phase separated using TFE/CHCl₃. The TFE soluble proteins were solubilised in rehydration buffer containing TFE (40% v/v). Proteins were separated by IEF, pH 3-10 and SDS-PAGE (12.5%). The gels were stained with Colloidal Coomassie Blue. Equal load was obtained by bacterial concentration determined by optical density (600 nm).
Quantitative analysis of the effect of iron availability on the differential expression of membrane proteins by *H. pylori*.

The cost implications associated with identifying protein spots highlighted by 2DE coupled to the exclusion of the TFE insoluble protein pool (because of inconsistent resolution), limited the scope of 2DE comparative analyses.

To overcome these limitations as well as increasing the sensitivity of the screening iTRAQ™ was employed. iTRAQ™ labelling, developed by Ross et al. (2004) at Applied Biosystems, uses isobaric reagents. iTRAQ™ labelling is an emerging technology with recent studies demonstrating its application for accurate identification and quantification of protein expression (Aggarwal et al., 2006). In addition, the cost of processing a complex protein sample labelled with iTRAQ™ reagents was similar to a single protein spot obtained by 2DE.

Stringent parameters were used to analyse the qualitative and quantitative data obtained from the iTRAQ™ analysis described in this chapter. Protein identification was obtained with 99% confidence while differential expression was deemed significant at a p-value < 0.05. However, the analysis was based on a single experiment.

**Comparison of iTRAQ™ labelled membrane sub-proteome from *H. pylori* grown under normal and iron-depleted conditions**

The differential expression of *H. pylori* membrane proteins obtained from bacteria grown under normal and iron-reduced conditions, was determined by iTRAQ™ labelling. Zwittergent® 3-14 soluble and insoluble membrane fractions, from *H. pylori*, (strain NCTC 11638), grown under either condition, were combined and the protein precipitated with acetone. The membranes were labelled with iTRAQ™ reagents 114 (Normal growth conditions) and 117 (Iron-reduced conditions), as recommended by the manufacturer, prior to analysis by mass spectrometry. Sixty two proteins were identified, many of which were either membrane, secreted (Bumann et al., 2002) or membrane-associated proteins (Backert et al., 2005, Sabarth et al., 2002), (Table 5.1). Many of the proteins identified have previously been shown to be
immunogenic (Baik et al., 2004, Haas et al., 2002, Jungblut et al., 2000, Kimmel et al., 2000). Of the sixty-two proteins, twenty-nine (47%) proteins displayed differential expression, including virulence factors (e.g. CagA and NapA), adhesins (e.g. HpaA and AlpA) and proteins involved in cellular processes, such as chaperone (e.g. SlyD), storage (e.g. Pfr) and transporter (e.g. GlnH) functions (Table 5.2). Of these, just under 50% displayed low level changes (fold: +/- 1.1 -1.3) while over 75% displayed low to moderate level changes (fold: +/- 1.1-1.6). The remainder displayed high fold changes between +/- 1.7 and 2.2.

Identification of membrane proteins with surface-exposed domains from \textit{H. pylori} grown under normal and iron-depleted conditions

The membrane enriched preparation used in the studies described above contained proteins from the three components of the \textit{H. pylori} membrane fraction (inner, periplasmic and outer). Therefore, it was of interest to focus specifically on those proteins that interact directly with the extracellular environment in an attempt to reduce sample complexity and to identify potential adhesins. Furthermore, surface exposed proteins are also the most attractive anti-microbial and vaccine candidates. To help reveal differentially expressed surface exposed adhesins, live \textit{H. pylori}, (strain NCTC 11638), bacteria were subjected to a short trypsin digest (shaving) to cleave peptides protruding from the bacterial membrane before being labelled with iTRAQ™ reagents. Similar ‘shaving’ procedures were carried out by others to identify surface proteins from bacteria such as Group A \textit{Streptococcus} and \textit{Borrelia burgdorferi} (Bunikis and Barbour, 1999, Barbour et al., 1984, Rodriguez-Ortega et al., 2006). Rodriguez-Ortega et al. (2006) described a ‘shaving’ protocol to recover trypsin-digested fragments of surface exposed epitopes for the purpose of identifying vaccine candidates.

Normally, when protein is digested by a protease many peptides are generated covering the entire sequence of the protein. However, in the case of ‘shaving’ fewer peptides will be generated which cover only a fraction of the entire protein sequence. This reduced peptide coverage makes identification less robust however. Thus, before iTRAQ™ labelling was carried out, repeat digests were performed to determine the consistency of protein identification following trypsin cleavage.
The expression of surface exposed proteins of *H. pylori* was determined by subjecting live bacteria to a short trypsin digest to cleave surface exposed peptides (shaving). The cleaved proteins from *H. pylori* grown in normal (N) and iron-reduced (IR) conditions were identified by mass spectrometry (Table 5.3). Forty-three proteins were identified between repeat experiments but only eighteen were found either in all experiments or at least in both a normal and iron-reduced preparation. A range of different proteins was found including virulence factors, VacA and CagA, and adhesins HpaA, AlpB and BabB. Of the 18 proteins consistently identified by repeat experiments, with the exception of NADPH flavin-oxidoreductase (FrxA), Cis-trans isomerase (SlyD) and hypothetical protein (HP1588), all are outer membrane proteins or have previously been shown to be associated with the *H. pylori* membrane (Backert *et al.*, 2005, Sabarth *et al.*, 2002) while almost 50% have demonstrated immunogenic properties (Baik *et al.*, 2004, Haas *et al.*, 2002, Jungblut *et al.*, 2000, Kimmel *et al.*, 2000). Thus, this shaving method is also suitable for identifying components of the membrane from *H. pylori* that are exposed to the extracellular environment.

To determine differential expression of these proteins, recovered surface exposed proteins were labelled with iTRAQ™ reagents 114 (N) and 117 (IR) before being analysed by mass spectrometry. Five proteins in total were identified representing a sharp decrease when compared to the forty-three identified without iTRAQ™ labelling. The five proteins identified were alkyl hydroperoxide reductase (AhpC – HP1563), outer membrane protein 6 (HopA – HP0229), outer membrane protein 5/29 (HopM/N – HP0277/HP1342), HopQ (HP1177) and probable outer membrane protein (HP0710). Two of these displayed differential expression, outer membrane protein 6 (HopA – HP0229) expression decreased by 1.6-fold while outer membrane protein 5/29 (HopM/N) displayed a 2-fold decrease in expression when grown under iron-reduced conditions.
Table 5.1: *H. pylori* proteins from a membrane enriched fraction labelled with iTRAQ™ reagents and identified by mass spectrometry. *H. pylori* was grown in normal and iron-depleted medium. Zwittergent 3-14® soluble and insoluble membrane fractions were combined and the proteins precipitated and labelled with iTRAQ™ reagents.

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<th>Protein</th>
<th>Location</th>
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<th>TIGR M.</th>
<th>Synonym</th>
<th>hp locus</th>
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Table 5.1: *H. pylori* proteins from a membrane enriched fraction labelled with iTRAQ™ reagents and identified by mass spectrometry. *H. pylori* was grown in normal and iron-depleted medium. Zwittergent 3-14® soluble and insoluble membrane fractions were combined and the proteins precipitated and labelled with iTRAQ™ reagents.
SB = surface bound (Backert et al., 2005, Sabarth et al., 2002); sec = secreted protein (Bumann et al., 2002); SI = sarcosine insoluble (Baik et al., 2004); OMV = outer membrane vesicle (laboratory data – Erica Mullaney); OMP = outer membrane protein (TIGR designation). Immunogenicity classified according to Jungblut et al., 2000, Baik et al., 2004, Haas et al., 2002, Kimmel et al., 2000. Virulence factors are highlighted in bold.
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<th>hp locus</th>
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Table 5.2: Differentially expressed *H. pylori* membrane proteins identified by iTRAQ labelling. *H. pylori* was grown in normal and iron-depleted medium. Zwittergent 3-14® soluble and insoluble membrane fractions were combined and the proteins precipitated and labelled with iTRAQ™ reagents. The ratios have been normalised to show fold changes. SB = surface bound (Backert et al., 2005, Sabarth et al., 2002); sec = secreted protein (Bumann et al., 2002); SI= sarcosine insoluble (Baik et al., 2004); OMV = outer membrane vesicle (laboratory data – Erica Mullaney); OMP = outer membrane protein (TIGR designation). Immunogenicity classified according to Jungblut et al., 2000, Baik et al., 2004, Haas et al., 2002, Kimmel et al., 2000. Virulence factors are highlighted in bold.
Table 5.3: *H. pylori* surface exposed proteins, ‘shaved’ with trypsin and identified by mass spectrometry. *H. pylori* was grown in normal (N) and iron-reduced (IR) medium. Live bacteria were subjected to trypsin digestion and the cleaved peptides were identified by mass spectrometry. Proteins above the double line are those that were found either in both a normal and iron-reduced preparation while proteins below the double line were not found in repeat experiments.

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<tr>
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<th>Location</th>
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<td>OMP</td>
<td>OMP</td>
<td>29.04</td>
<td>hpaA</td>
<td>hpp0797</td>
<td>Adhesin</td>
<td>x</td>
<td>x</td>
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<td>x</td>
</tr>
<tr>
<td>amylase</td>
<td>SB</td>
<td>SB</td>
<td>49.65</td>
<td>amyl</td>
<td>hpp0810</td>
<td>Protein synthesis</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>hydrogenase expression/formation protein</td>
<td>SB</td>
<td>hydrogenase</td>
<td>SB</td>
<td>77.21</td>
<td>hpdB</td>
<td>hpp0900</td>
<td>Metabolism</td>
<td>x</td>
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</tr>
<tr>
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<td>SB, SL, OMY</td>
<td>SB</td>
<td>24.068</td>
<td>nda</td>
<td>hpp0954</td>
<td>Energy metabolism</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>NADPH nitroreductase</td>
<td>SB, SL, OMY</td>
<td>SB</td>
<td>69.74</td>
<td>flav</td>
<td>hpp1060</td>
<td>Cell division</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>nitroreductase</td>
<td>SB</td>
<td>SB</td>
<td>19.978</td>
<td>omp18</td>
<td>hpp1120</td>
<td>Membrane structure</td>
<td>x</td>
<td>x</td>
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<td>x</td>
</tr>
<tr>
<td>outer membrane protein</td>
<td>OMY, OMP</td>
<td>OMY, OMP</td>
<td>76.346</td>
<td>omp25</td>
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<td>Outer membrane structure</td>
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<td>Flavodoxin</td>
<td>sec</td>
<td>Y</td>
<td>17.492</td>
<td>flav</td>
<td>hpp1161</td>
<td>Energy metabolism</td>
<td>x</td>
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<tr>
<td>Adhesin-binding fucosylated histo-blood group antigen</td>
<td>OMP</td>
<td>OMP</td>
<td>79.128</td>
<td>fbbA</td>
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<td>Adhesin</td>
<td>x</td>
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<td>x</td>
<td>x</td>
</tr>
</tbody>
</table>

SB = surface bound (Bacskert et al., 2005, Sabarth et al., 2002); sec = secreted protein (Bumann et al., 2002); SI= sarcosine insoluble (Baik et al., 2004); OMV = outer membrane vesicle (laboratory data – Erica Mullaney); OMP = outer membrane protein (TIGR designation). Immunogenicity classified according to Jungblut et al., 2000, Baik et al., 2004, Haas et al., 2002, Kimmel et al., 2000. Virulence factors are highlighted in bold.
Discussion

The membrane proteins of pathogenic bacteria play a central role in their survival as well as the colonisation of their host. The compliment of membrane proteins continuously change as they adapt to their external host environment. Iron is an essential element in this process and acts as a stimulant for differential gene and protein expression in *H. pylori* (Merrell *et al.*, 2003b, Ernst *et al.*, 2005, Dhaenens *et al.*, 1999, Dhaenens *et al.*, 1997, Worst *et al.*, 1995). In this chapter, differentially expressed proteins from the membrane proteome of *H. pylori* grown in normal and iron-reduced conditions were identified and sequenced.

Comparison of *H. pylori* membrane preparations at the 1D level demonstrated several protein species displaying differential expression in response to iron availability. Of these, nine protein bands displayed increased expression under iron-reduced conditions and at least four species appeared to be down regulated. Similar differences in protein profiles have been demonstrated for membrane proteins by others using different *H. pylori* strains and membrane enrichment protocols (Dhaenens *et al.*, 1997, Dhaenens *et al.*, 1999, Worst *et al.*, 1995, Szczepańska *et al.*, 1999).

2DE is widely used to further increase the separation of complex protein mixtures offering more accurate qualitative and quantitative data; the first dimension separates constituents by isoelectric point (*pI*) while the second dimension separates by size. Membrane proteins tend to be hydrophobic and can be difficult to solubilise in preparation for 2DE using standard protocols. In this study, a selection of membrane protein solubilisation procedures were analysed to maximise the number of proteins that could be subjected to comparative analysis by 2DE. The inclusion of zwitterionic detergent (Taylor and Pfeiffer, 2003, Backert *et al.*, 2005) or organic solvents (Deshusses *et al.*, 2003) in the rehydration buffer have been shown to improve solubility and resolution of membrane proteins. SDS can also be added to enhance protein solubility (AmershamBioscience, 2004). In this study, the addition of either SDS (0.25%, v/v) or Zwittergent® 3-14 (1%, v/v) detergents to the 2DE rehydration buffer reduced the number of protein spots visible as well as increasing vertical and horizontal streaking when compared to TFE-containing rehydration buffer. Streaking,
among other factors, can be caused by reduced protein solubility (AmershamBioscience, 2004, Gorg and Weiss, 2000). Merten et al. (2005) used 1% Zwittergent® 3-14 in their rehydration buffer but their starting sample was homogenised heart muscle which probably accounts for the high solubility of the proteins examined. Membrane proteins have also been isolated by solvent extraction using a mixture of chloroform and methanol (Molloy et al., 1999) or chloroform and TFE (Deshusses et al., 2003). In this chapter, the combination of TFE solvent membrane extraction and use of rehydration buffer containing TFE yielded the greater amount of proteins resolvable by 2DE. The presence of cytoplasmic proteins in the membrane-enriched fractions became apparent when some of the spots were subjected to MS analysis. However, this is not unusual and other groups working with \textit{H. pylori} membrane have demonstrated the dual location of cytoplasmic proteins (Backert et al., 2005, Baik et al., 2004).

Consistency of protein expression patterns is critical when comparing gels to determine differential expression patterns of proteins by 2DE. A consistent protein profile was obtained for membrane proteins extracted with, and soluble in, TFE in repeat experiments but was not achieved for TFE insoluble proteins. A number of areas on the gel displayed poor resolution making comparisons between gels difficult. Hence only TFE soluble membrane enriched protein samples were used for the final comparison.

The initial focus of the 2DE comparison of \textit{H. pylori} membrane enriched proteome was to determine those proteins that were up regulated under iron-reduced conditions. Five proteins displayed increased expression, two of which were identified by tandem mass spectrometry (MS). They were cell division protein (FtsH – HP1069) and alkyl hydroperoxide reductase (AhpC – HP1563).

Alkyl hydroperoxide reductase (AhpC) is a member of the thiol-dependent peroxiredoxin family (Wood et al., 2003), which is the most abundant antioxidant protein in \textit{H. pylori} (Jungblut et al., 2000). Increased AhpC expression is also associated with metronidazole resistant \textit{H. pylori} (McAtee et al., 2001) and can change its role from a peroxide reductase to a molecular chaperone for prevention of protein mis-folding under oxidative stress (Chuang et al., 2006). The cell division
protein (FtsH) is a member of the ATPases Associated to a variety of cellular Activities (AAA) protein family (Confalonieri and Duguet, 1995). It is located in the membrane of H. pylori and plays an essential role in its growth, since disruption of this gene leads to a loss in viability of H. pylori (Carlsohn et al., 2006b). However, its functional role at present is not well understood.

The sequence identification of each protein in the 2DE gels obtained from this study would have been prohibitively expensive. In addition, many proteins were excluded from the comparative study because of their failure to resolve sufficiently during the 2DE process. iTRAQ™ labelling was employed instead to help overcome these limitations and to broaden the range of proteins which could be included in the comparative analysis. iTRAQ™ labelling identified sixty-two proteins in total, of which twenty-nine displayed differential expression.

The role of iron in the function/regulation of the proteins identified by iTRAQ™ labelling, along with the implications in terms of bacterial pathogenicity, is not yet clear. The virulence factor CagA was marginally down regulated in this study (-1.3-fold). CagA, part of H. pylori's Type IV secretion system, is injected into the host, disrupting cell function (Odenbreit et al., 2000). Szczebara et al. (1999) demonstrated that cagA gene expression varied from culture to culture in iron-depleted medium suggesting a more complex relationship between iron and CagA. UreA and UreB were up regulated by 1.2 and 1.6-fold, respectively. Urease is an important colonisation factor. The H. pylori urease complex consists of two subunits, UreA and UreB, that are acid resistant under physiological conditions (Dalio et al., 2002). Much of the enzyme associates with the cell surface of intact cells due to either specific secretion (Vanet and Labigne, 1998) or absorption of the urease released upon lysis of neighbouring bacteria (Phadnis et al., 1996). Catalase (KatA) showed a 1.5-fold decrease under iron restriction. KatA is responsible for the removal of hydrogen peroxide, which, in the presence of iron, acts as a catalyst to generate reactive oxygen species. Previous studies have shown a 2.5-fold reduction in gene expression (Ernst et al., 2005) in addition to reduced protein activity (Harris et al., 2002) in an iron-reduced environment.
Neutrophil activating protein (NapA) is an important virulence factor that promotes neutrophil adhesion to endothelial cells (Evans et al., 1995) and plays a role in protection against oxidative stress damage (Cooksley et al., 2003). NapA displayed a 2.2-fold increase in expression, the highest recorded in this study. Neutrophil activation, coupled with moderate levels of inflammation induced by \textit{H. pylori}, might promote cellular damage that results in leakage of nutrients from host cells (Blaser, 1993). NapA was shown to bind iron (Tonello et al., 1999) and an increase in expression might serve as a means of releasing iron from host cells. However, Cooksley et al. (2003) using the same \textit{H. pylori} strain and similar experimental conditions to those described in this study, found NapA expression significantly reduced under iron-depleted conditions. However, the reason(s) for this discrepancy is not yet clear. NapA may also play a role in \textit{H. pylori} adhesion to salivary mucin molecules (Namavar et al., 1998).

Many of the proteins identified by this study are involved in a variety of cellular processes. The increased expression of AhpC (1.6-fold) was also observed in the 2DE analysis discussed earlier in the chapter. Cis-trans isomerase (SlyD) functions as a metallochaperone in \textit{E. coli} and interacts with HydB, a [Ni-Fe] hydrogenase enzyme (Zhang et al., 2005) and probably has a similar role in \textit{H. pylori}. Both SlyD & HydB were down regulated in response to reduced iron. Nitrogenase cofactor synthesis protein (NifS) displayed a 1.5-fold increase in expression. Interestingly, NifS also displayed increased expression in \textit{H. pylori} strains taken from iron-deficient anaemia patients (Park et al., 2006) but its role is not fully understood. Ferritin (Pfr) is an iron storage protein previously shown to be highly down regulated in the absence of iron (Bereswill et al., 1998b) which is also the case in this study with a 2-fold reduction. Clearly, the cell has no requirement for iron storage under conditions of depleted iron. The glutamine transporter (GlnH) and glutamine synthetase (GlnA) are associated with ammonia production and their increased expression (1.8 - 1.5 fold, respectively) is probably related to the increased UreA and UreB expression. ATP synthase (AtpA), along with HspA & HspB, were found to be surrogate markers for the detection of gastric carcinoma (Krah et al., 2004). Elongation factor Tu (TufA) is found in abundance in \textit{H. pylori} (Jungblut et al., 2000). A recent study showed that the EF-Tu in \textit{Mycoplasma pneumoniae}, in addition to its biosynthetic role in the
cytoplasm, is translocated to the surface where it was shown to bind fibronectin (Dallo et al., 2002).

Bacterial adhesion is affected by iron-reduced conditions (Scharfman et al., 1996, Holmes et al., 2005, Grifantini et al., 2003). This study demonstrated a marginal decrease (1.2-fold) in expression for adhesin AlpA while adhesin HpaA and the NLBH HpaA homologue (HP0410) displayed marginal increases (1.1 and 1.2-fold, respectively) but their role in iron regulated adhesion is unproven. Heat shock protein 70 (DnaK/Hsp70) showed a marginal increase in expression (1.3-fold) while HspB expression increased 1.6-fold. Huesca et al. (1996, 1998) provide evidence suggesting that DnaK and HspB may be stress-induced surface adhesins capable of binding to sulphated glycolipid receptors on gastric epithelial cells.

Surface exposed membrane proteins were prepared by ‘shaving’ live bacteria with trypsin. During the optimisation forty-three proteins were initially identified, of which, eighteen were consistently identified following repeat experiments. Of these, AhpC, HopA, TufA, SlyD and HSP70/DnaK were also found in the membrane enriched preparations labelled with iTRAQ™ reagents discussed earlier. However, following iTRAQ™ labelling of the trypsin ‘shaved’ surface proteins only five proteins in total were identified of which two displayed differential expression. These were OMP6 (HopA) and OMP5/29 (HopM/N). HopA is a member of a closely related family of porins (HopA to HopD) whose precise function is unknown (Exner et al., 1995). Allan et al. (2001) showed that hopA gene expression was down regulated when H. pylori was exposed to a low pH environment. In this thesis, HopA was found to be down regulated 1.6-fold when membrane protein expression was studied. However, HopA displayed a marginal increase, of 1.1-fold, when the surface exposed membrane preparations (i.e. obtained by trypsin shaving) were examined by iTRAQ™. Porins possess transmembrane loops some of which are surface exposed. The structure of porin protein changes according to their environment as they carry out their function. The E. coli porin protein, FepA, contains a ligand binding surface exposed loop that undergoes structural changes in response to iron (Jiang et al., 1997). A similar structural change may occur in HopA, which changes its surface exposure without any significant change to its overall membrane expression. HopM/N is a putative porin protein belonging to the same super family as HopA.
(Alm et al., 2000) and displays antigenic properties (Sumie et al., 2001). However, little is known about its role in *H. pylori*.

The low number of overall proteins found in iTRAQ™ labelled ‘shavings’ when compared to those obtained by the ‘shaving’ optimisation preparations may be explained by two key processing differences. The optimisation preparations were solubilised in organic solvents prior to MS analysis whereas the iTRAQ™ preparations were solubilised in a different proprietary solvent buffer, as well as undergoing reducing, blocking and labelling steps before MS analysis. Bearing in mind that ‘shaving’ produces peptides covering only a fraction of a particular protein the combination of different solubilisation buffer and additional processing steps required for iTRAQ™ labelling may have led to losses of a portion of the small peptides. In addition, different MS spectrum analysis software algorithms were used for the identification steps resulting in differences with peptide spectrum interpretations; MASCOT (MatrixScience) was used for the optimisation preparations while PROQUANT (Applied Biosystems) was used for the iTRAQ preparations.

Although, the overlap of all identified proteins between all of the different membrane preparations employed during this chapter is approximately 14% (Figure 5.c), this is not unexpected.

**Figure 5.c. Venn diagram showing the overlap of proteins identified during this chapter.** *H. pylori* membrane proteins were either labelled with iTRAQ™ reagents, ‘shaved’ with trypsin or ‘shaved’ with trypsin and labelled with iTRAQ™ reagents, before being identified by MS. The total number of proteins identified for each membrane preparation is indicated in brackets while the Venn diagram shows the overlap of protein numbers between all membrane preparation types. * = indicates the subset where the proteins identified from the 2DE gel would belong if they were included in the Venn diagram data set.
The membrane-enriched preparation contained a complex mix of proteins of varying abundance. During MS analysis abundant proteins can mask the presence of less abundant species. The 'shaved' preparations were enriched for domains from surface exposed proteins, thereby increasing their relative abundance, which may have been lower in the membrane enriched preparations. In addition, the use of different MS spectrum analysis software algorithms, described earlier, might lead to differences in peptide identification.

As with all bacteria, *H. pylori* populations go through different phases of growth as they adapt to their surroundings. An initial lag phase of little growth is seen when the bacteria are introduced to a new environment. This is followed by exponential or logarithmic (log) growth phase as the population expands in number. As nutrients become depleted, the population size becomes static and eventually, the population size will decrease as bacteria die. Throughout the various growth phases, different genes are expressed. For example, Karita *et al.* (1996) found that *cagA* gene expression was up regulated during the stationary growth phase while Lundstrom *et al.* (2001) determined that transcription of the adhesin *hpaA* was maximal at 3-4 days growth in liquid culture. Recent DNA array studies on *H. pylori* demonstrate the growth phase dependent expression of various genes, including those encoding virulence factors (Thompson *et al.*, 2003, Merrell *et al.*, 2003b). This, however, is not an unexpected finding, as cellular processes and membrane functions need to adapt to changing environmental conditions, which will be influenced by the size of the bacterial population. Indeed, sub-populations of infecting *H. pylori* are most likely going through different growth phases in response to differing local mucosal environments.

Similar to many studies, the work described in this chapter necessitated the use of single time points to analyse the membrane protein expression profiles from *H. pylori* when cultured under different conditions. The analyses of *H. pylori* membrane protein expression were performed using bacteria grown under normal and iron-reduced conditions. Although the starting culture for both conditions was taken from the same stock, the growth curves for normal and iron-reduced cultures differed significantly. Under iron-reduced conditions the *H. pylori* population displayed reduced growth and little population expansion, suggesting an almost stationary phase.
of growth at harvest time, while the *H. pylori* population grown under normal conditions was expanding in a log phase-like manner when harvested for analysis.

The differential expression of membrane proteins observed for *H. pylori* when grown under iron-reduced conditions may be due, in part, to the different growth phases at which the bacteria were harvested. Merrell *et al.* (2003b) examined the response of *H. pylori* genes to growth phase and iron availability and identified a number of outer membrane proteins with altered expression. However, direct comparison with the Merrell *et al.* study was not possible. Apart from the established complications of post-translational mechanism that can modulate protein expression levels (Anderson and Seilhamer, 1997, Gygi *et al.*, 1999), the gene array study examined gene expression at log and stationary phase growth after only 90–120 minutes exposure to iron-reduced conditions, whereas, the expression levels of proteins described in this study were determined after the bacteria had been exposed to 2 days of iron-reduced conditions. Interestingly, Cooksley *et al.* (2003) found the protein NapA to be maximally expressed at the stationary growth phase; but when *H. pylori* were grown under iron-reduced conditions, NapA protein expression was reduced. However, as mentioned earlier, using similar experimental conditions, this present study found that NapA expression levels increased under iron-reduced conditions. The growth phases were not determined for this thesis but any potential effects growth phase variation may have on protein expression profiles could be overcome in future work by ensuring *H. pylori* are grown to the same growth phase before harvesting.

In this study, the proteomic tools, 2DE and iTRAQ™ labelling, were used to analyse differentially expressed membrane proteins from *H. pylori* grown in normal and iron-reduced conditions. Although the 2DE gels produced numerous protein spots with many displaying differential expression the identification of these would have required additional labour/cost intensive steps. In contrast, the data generated by iTRAQ™ labelling, while producing less overall protein coverage, simultaneously provided both protein identification and quantification using much less material and in less time than required by 2DE. Under conditions of low iron availability a number of *H. pylori* virulence factors, such as NapA, Urease, and CagA, displayed differential expression. In addition, the expression of adhesins, such as AlpA and HpaA, increased under reduced iron conditions along with other membrane proteins.
Furthermore, the increase in adhesin expression identified here under iron-restricted conditions supports the data from Chapter IV demonstrating increased adhesion of *H. pylori* to ECM ligands. Adhesion by *H. pylori* is important for colonisation of the host gastric mucosa. The increased expression of adhesins and other membrane proteins, under iron-reduced conditions, may enhance the pathogenic nature of infection. This suggests a possible role for the development of vaccines against adhesins and other membrane proteins to prevent initial *H. pylori* colonisation of the host.
CHAPTER VI

GENERAL DISCUSSION
Chapter VI

_H. pylori_ is a human pathogen infecting half of the world's population. Although most infected individuals are asymptomatic, a proportion develop gastrointestinal pathologies including severe gastritis, ulcers, and cancers (Blaser and Berg, 2001, Ernst and Gold, 2000). _H. pylori_ has also been linked to other diseases such as iron-deficiency anaemia (DuBois and Kearney, 2005). The membrane of _H. pylori_ plays a pivotal role in the survival, persistence, and overall pathogenicity of infection. As well as providing structural integrity, the membrane also acts as a platform for (1) deploying virulence factors such VacA (by secretion), and CagA (by injection); (2) modulating the host immune response such as that provided by Le antigen switching of LPS; (3) adhesins such as BabA and SabA that are used to colonise and infect the gastric epithelial cells; and (4) acquiring micronutrients from the external environment such as nickel, copper and iron.

_H. pylori_-host interactions are clearly important in disease progression, however, the molecular mechanisms are not yet fully understood. A primary aim of this thesis was to study the interaction between _H. pylori_ and the host with a focus on _H. pylori_-ECM interactions. The rationale for this study was provided by a variety of studies demonstrating the ability of _H. pylori_ to interact with the ECM (Ascencio et al., 1993, Valkonen et al., 1993, Trust et al., 1991), the description of potential receptors for the ECM proteins heparan and laminin (Utt et al., 2001, Valkonen et al., 1997) and research carried out at our own laboratory which identified a potentially ulcerogenic interaction between _H. pylori_ extract and polarised T84 epithelial monolayer. This interaction induced a deleterious physiological change in the monolayer resulting in the accumulation of fluid and electrolytes between the T84 monolayer and the underlying laminin substrate and loss of barrier function. When the _H. pylori_ extract was pre-treated with laminin, this biological activity of the _H. pylori_ extract was inhibited, indicating a role for laminin-binding to components of the _H. pylori_ extract in this process (Terres et al., 2003).

Furthermore, microscopy studies have shown a close association between _H. pylori_ and intercellular epithelial cell tight junctions as well as between _H. pylori_ and the ECM-rich basal lamina of gastric biopsies (Petersen and Krogfelt, 2003). Taken
together, these studies suggest that the interaction between *H. pylori* and laminin may have physiological relevance in terms of disease progression.

Secondly, it was of interest to evaluate the influence of environmental conditions on the interaction of *H. pylori* with the ECM. In particular, the effect of iron on *H. pylori*-ECM interactions was studied, as iron availability is a limiting factor *in vivo*, due to the ability of infected individuals to sequester free iron as a primary defence mechanism against infecting pathogens. Adhesion studies, carried out by others, have shown that pathogenic bacteria, such as *Pseudomonas aeruginosa* and *Corynebacterium diphtheriae*, display increased levels of adhesion to epithelial cells when grown under low iron conditions (Scharfman *et al.*, 1996, Moreira Lde *et al.*, 2003, Dai *et al.*, 1992). Furthermore, iron availability is known to modulate the expression profile of *H. pylori* genes and proteins (Ernst *et al.*, 2005, Worst *et al.*, 1995, Dhaenens *et al.*, 1997, Keenan and Allardyce, 2000). Thus, a third aspect of this study focused specifically on the protein expression profile of membrane proteins under iron-replete and iron-reduced conditions.

This study resulted in the identification of two potential laminin-binding proteins, HpaA (HP0797) and HorF/OMP14 (HP0671). No common amino acid motif/domain or protein structure topology was found between the identified proteins and those from known laminin-binding proteins that might explain the laminin interactions, however. Additionally, the characterisation of the laminin-binding properties of these potential laminin-binding proteins was hampered by the difficulties experienced with creating functional recombinant versions of these proteins. Alternative strategies to circumvent these issues are considered in the future work section.

Significantly, however, a clear impact of the environmental growth conditions on *H. pylori*-ECM interactions was observed. This study demonstrated the increased adherence of *H. pylori* to the ECM ligands laminin, fibronectin and collagen IV as well as the iron-binding protein, lactoferrin, when the pathogen was cultured under iron-reduced conditions. The involvement of SabA in binding to laminin observed by Walz *et al.* (2005) was also confirmed. In addition, the increased adhesion of *H. pylori* to the ECM and lactoferrin observed was found to be independent of the ferric uptake regulator protein (FUR). Furthermore, this study demonstrated the differential
expression of membrane proteins, including adhesins HpaA, AlpA and virulence factors, such as NapA, CagA and urease when *H. pylori* was grown under conditions of reduced iron availability.

The identification of two potential laminin-binding proteins in combination with reduced laminin-binding observed with *sabA*-deficient *H. pylori*, under normal growth conditions, highlights the potential multifactorial interaction between *H. pylori* and the ECM, particularly laminin. As mentioned earlier, laminin is a large molecule with multiple binding sites and three different *H. pylori* proteins have already been described to date that demonstrate laminin-binding capabilities. These are SabA, LPS and an unidentified 25 kDa protein (Walz et al., 2005, Valkonen et al., 1993, 1994, 1997). Significantly, the work described in this thesis identified phenotypic differences with respect to *H. pylori* adhesion to ECM proteins, when studied under conditions of low iron availability. More specifically, increased adhesion to laminin, fibronectin and collagen IV was observed under iron-reduced conditions.

Although the identification of ECM receptors from *H. pylori* is incomplete, the combination of the increased adherence of *H. pylori* to ECM proteins under iron-reduced conditions and the increased expression of the adhesins HpaA and DnaK/Hsp70 (and possibly other adhesins not yet described) suggests that colonisation, and thus the pathogenicity of *H. pylori*, would be enhanced under iron-reduced conditions. Indeed, the ability to acquire iron would also enhance the pathogenicity of *H. pylori* and the observations of increased adhesion to the iron-binding protein, lactoferrin, coupled to that of increased expression of NapA, which can bind 500 atoms of iron per molecule (Tonello et al., 1999), as well as mucin binding characteristics, further supports the body of evidence linking the importance of *H. pylori* adhesins/receptors to disease progression.

While *H. pylori* interaction with the basal lamina is not required for initial colonisation, the ability of *H. pylori* to interact with and adhere to ECM proteins, such as laminin, fibronectin and collagen IV may be an important virulence mechanism that promotes enhanced colonisation due to exposure of ECM proteins in the basal lamina as a result of lesions caused by the initial *H. pylori* infection (Trust et al., 187).
Indeed, damage and loss of gastric epithelial cells (microlesions) caused by ingested substances such as ethanol, aspirin and some spicy foods (Willems, 1988, Hawkey, 1994, Robert et al., 1983) may also exacerbate this process. This ability to adhere to exposed ECM ligands, particularly in conditions most likely experienced in vivo, such as low iron availability, would facilitate the persistent colonisation of the gastric mucosa by \textit{H. pylori} and further promote the continued inflammation and potential progression to gastric ulcers or gastric cancers in the infected host.

The variation of expression in membrane proteins by \textit{H. pylori}, such as virulence factors AlpA, CagA, NapA and urease or adhesins HpaA, DnaK/Hsp70 under conditions of low iron availability might affect the type of immune response employed by the infected host against \textit{H. pylori}. For example, NapA is a potent immune polarising agent and can promote a shift from a Th2 to a Th1 immune response (D'Elios et al., 2007). It would be of interest to evaluate and compare the immune polarizing ability of \textit{H. pylori} when cultured under normal and iron-reduced conditions, at least \textit{in vitro} and determine the cytokine/chemokine profiles from T-cells exposed to \textit{H. pylori}. The changes to \textit{H. pylori} membrane protein expression under iron-reduced conditions might drive the host immune response in a direction that allows \textit{H. pylori} to avoid complete clearance and thus increase its virulent potential and pathogenicity.

The enhanced adherence of \textit{H. pylori} to ECM ligands and the iron-binding protein, lactoferrin, coupled to the differentially expressed membrane proteins from \textit{H. pylori} observed under iron-reduced conditions (which may mimic the \textit{in vivo} situation) suggests that membrane proteins, particularly those involved in \textit{H. pylori}-host interactions, are prime targets for vaccines and other anti-microbial agents. Indeed, membrane associated proteins, such as urease, VacA and CagA have already been shown to provide some protection against \textit{Helicobacter} infection (Del Giudice et al., 2001, Kabir, 2007). Perhaps, the inclusion of adhesins to ECM or iron-binding ligands in future anti-microbial agents will help prevent or treat \textit{H. pylori} infection.
**Future Work**

The breadth of data obtained throughout this thesis demonstrates the potential of the experimental approaches employed. Extending the scope of research will provide further information on the characteristics of *H. pylori* membrane proteins summarised in Figure 6.1, particularly in relation to their role in colonisation. The search for laminin-binding proteins could be extended to look for other receptors using affinity chromatography with other ECM or iron-binding proteins/molecules attached. The sample preparations incubated with the affinity matrices could be further fractionated to help reduce sample complexity thereby avoiding the possible complications of high/low abundance proteins.

The observation that *H. pylori* shows increased adhesion to ECM ligands and lactoferrin under iron-reduced conditions, described in this thesis, could be exploited, in the first instance, to identify receptors/adhesins for these ligands. The receptor activity-directed affinity tagging (Retagging) method described by Ilver *et al.* (1998) and Mahdavi *et al.* (2002) to purify the BabA and SabA adhesins could be usefully employed in such studies. Once receptors have been identified recombinant versions of the protein could be expressed and used to generate antibodies; a fully functional recombinant protein is not required for this. The antibodies could then be used to purify the receptors in their native form for further analyses.
Figure 6.1. Summary of future work:

Effects of pH, oxidation, nutrient depletion on:
- membrane protein expression
- adhesion to ECM and other ligands

Effects of strain variation on:
- membrane protein expression
- adhesion to ECM and other ligands

Enhance protein sample preparation:
- use of different detergents & solvents
- use of multiple digestive enzymes
- use of soluble phase IEF
- use of antibody capture

Characterisation of binding interactions by SPR:
- competitive inhibition assays
- blocking assays
- properties of purified native adhesin

Additional use of affinity chromatography:
- screen for other ECM/iron-binding protein adhesins
- purify individual adhesins using antibodies raised against recombinant versions

Effect of chemokine/cytokine expression from T-cells exposed to *H. pylori* grown under normal and iron-reduced conditions.
*H. pylori* possesses a gene (*hp1392*) that encodes a putative fibronectin/fibrinogen binding protein (Tomb *et al.*, 1997). Little information is available on the function or properties of this gene and its protein product. Recently, Danielli *et al.* (2006) suggested that the expression of *hp1392* is under the control of the FUR regulon. However, an earlier study failed to show this property (Ernst *et al.*, 2005). Gene array studies conducted on *H. pylori* exposed to iron and acid stress conditions did not detect any changes to *hp1392* transcription (Ernst *et al.*, 2005, Merrell *et al.*, 2003a). Furthermore, the expression of *hp1392* did not appear to be affected by different growth phases (Thompson *et al.*, 2003). Perhaps examining the interaction of either a mutant *H. pylori* deficient in the *hpI392* gene or a recombinant protein version, with fibronectin may help to determine the adhesive properties, if any, of the protein product.

The protein samples used to determine the effect of iron availability on membrane protein expression would also benefit from further refinement. By using different detergents and solvents a range of 2DE studies could be carried out to compare smaller subsets of proteins than were examined in this thesis. The detergent sacosine has been used to isolate membrane proteins by various groups analysing the membrane of *H. pylori* by 1DE and 2DE (Carlsohn *et al.*, 2006b, Baik *et al.*, 2004, Backert *et al.*, 2005). Molloy *et al.* (2000) used a carbonate incubation method to isolate outer membrane from *E. coli*. Minimising the membrane isolation steps should also improve the pool of proteins available for analysis. The membrane-enriched preparation used in this study included an acetone precipitation step, which most likely resulted in loss of some protein species. A more recent proteomic and differential expression analysis using iTRAQ™ labelling used a membrane fraction collected directly from sonicated *Mycobacterium avium subsp. paratuberculosis* cells (Radosevich *et al.*, 2007). They obtained expression data for 550 proteins from the membrane fractions, of which 37 proteins were expressed at higher abundance.

Although the use of detergents and solvents in the preparation of samples for iTRAQ™ labelling is limited by the current proprietary solubilisation buffer, the inclusion of additional proteolytic enzymes should increase the number of protein species identified by MS. Trypsin was used in this study to prepare proteins for MS analysis and to isolate surface exposed proteins from the membrane of *H. pylori*, due
to the limitation of the peptide spectral analysis software, ProQUANT, which only recognises peptides cleaved by trypsin. Other proteases such as proteinase K and endoproteinase lycine-C/ glutamic-C have been used to increase the range of proteins identified by MS (Zybailov et al., 2007, Elenitoba-Johnson et al., 2006). Recently Zybailov et al. (2006, 2007) used trypsin and proteinase K to digest membrane enriched fractions from Saccharomyces cerevisiae before MS identification. Of the proteins identified by proteinase K digestion, 25% were not identified in the trypsin digested samples, demonstrating the benefit of using additional proteases to analyse protein mixtures. Furthermore, the next software release of MASCOT, which can identify peptides cleaved by a range of proteases, will include the capability to interpret iTRAQ™ labelled preparations. The various proteases could be used at the membrane isolation stage (e.g. ‘shaving’) or at the MS sample preparation stage. The inclusion of membrane proteins from different H. pylori strains, particularly virulent and non-virulent strains, may help to identify vaccine targets to prevent infection by the more pathogenic strains. In addition, the effect of other stress factors, (e.g. pH, oxidation and micronutrient availability), on membrane protein expression could also be assessed as these stressors have in vivo relevance.

The value of surface plasmon resonance (SPR) in examining the binding characteristics of H. pylori under iron-reduced conditions has been highlighted by this study. A range of experiments could be included to provide further information on the binding characteristics of H. pylori adhesins. Isogenic knock-out mutants could be created to characterise the properties of adhesins for ECM or iron-binding proteins isolated and identified by affinity chromatography. Varying the concentration of immobilised ligand and bacterial load across the chip surface would also provide additional binding/affinity data. The adhesin-ligand interactions could be further characterised through competitive inhibition and blocking experiments, for example, to determine the effect on laminin-binding when H. pylori is pre-incubated with fibronectin or visa-versa. In addition, the binding characteristics of the purified protein could be assessed using SPR. Furthermore, similar to the analysis of membrane protein expression, the effects on adhesion caused by other stress factors could be examined. Strain variation may also have an effect on adhesion and is worthy of further investigation.
To conclude, a greater understanding of the role played by membrane proteins, particularly adhesins and other receptors, in the pathogenicity of *H. pylori* infection will enable the development of more effective therapeutic and prevention strategies. Further refinement and expansion of the molecular and proteomic approaches used throughout this thesis may help to provide vaccine candidates which target specific adhesins/receptors on *H. pylori* thereby preventing colonisation.
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APPENDIX A

Local alignment of amino acid sequences from laminin-binding proteins using LALIGN
lalign output for 67kDa Laminin receptor (LMWWML) vs. HP0671

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

33.3% identity in 3 aa overlap; score: 20 E(10,000): 1.8e+03

67kDa WWM
   :
HP0671 WYL
   50

66.7% identity in 3 aa overlap; score: 20 E(10,000): 1.8e+03

67kDa WML
   :
HP0671 WYL
   50

50.0% identity in 2 aa overlap; score: 16 E(10,000): 3.6e+03

67kDa WW
   :
HP0671 WF

239
lalign output for 67kDa Laminin receptor (LMWWML) vs. HP0725

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 25 E(10,000):
1.6e+03

67kDa LMW
.:.
HP0725 MMW

66.7% identity in 3 aa overlap; score: 21 E(10,000):
3.3e+03

67kDa MWW
::
HP0725 MMW

50.0% identity in 6 aa overlap; score: 21 E(10,000):
3.3e+03

67kDa LMWWML
:::
HP0725 LGFWSL
90
lalign output for 67kDa Laminin receptor (LMWWML) vs. HP0797

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

100.0% identity in 1 aa overlap; score: 15 E(10,000):
4.1e+03

67kDa W
:
HP0797 W

100.0% identity in 1 aa overlap; score: 15 E(10,000):
4.1e+03

67kDa W
:
HP0797 W

33.3% identity in 3 aa overlap; score: 9 E(10,000):
8.4e+03

67kDa LMW
:
HP0797 LLF
lalign output for 67kDa Laminin receptor (LMWWML) vs. O69174

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 18 E(10,000): 3.9e+03

67kDa LMW
: :
O69174 LRW

50.0% identity in 4 aa overlap; score: 17 E(10,000): 4.6e+03

67kDa WWML
: :.
O69174 WKQL

100.0% identity in 1 aa overlap; score: 15 E(10,000): 6e+03

67kDa W
 :
O69174 W
lalign output for 67kDa Laminin receptor (LMWWML) vs. Tp0751

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

100.0% identity in 2 aa overlap; score: 22 E(10,000):
1.1e+03

67kDa MW
:::
Tp0751 MW
220

66.7% identity in 3 aa overlap; score: 19 E(10,000):
1.9e+03

67kDa WML
:::
Tp0751 WAL

50.0% identity in 2 aa overlap; score: 18 E(10,000):
2.3e+03

67kDa MW
:::
Tp0751 LW
Align output for Elastin (VVGSPSAQDFASP) vs. HP0671

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

30.8% identity in 13 aa overlap; score: 29 E(10,000): 3e+02

10
Elasti  VVGSPSAQDFASP
  :. :.....: :
HP0671  VKNPPKSEFNP
  70

33.3% identity in 9 aa overlap; score: 20 E(10,000): 1.8e+03

10
Elasti  VGSPSAQDF
  .: . ::
HP0671  IGRHQEFD
  230

41.7% identity in 12 aa overlap; score: 19 E(10,000): 2.1e+03

10
Elasti  VGSPSAQDFASP
  :: : :: .:
HP0671  VQGAS-QSVKNP
  60
lalign output for Elastin (VVGSPSAQDFASPL) vs. HP0725

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

33.3% identity in 12 aa overlap; score: 25 E(10,000):
1.6e+03

10
Elasti GSPSAQDFASPL
:: . ::::
HP0725 GSQTNPKFAKDI
270 280

50.0% identity in 8 aa overlap; score: 23 E(10,000):
2.3e+03

Elasti VGSPSAQD
:: ::::
HP0725 VGMISSQN
450

25.0% identity in 12 aa overlap; score: 22 E(10,000):
2.8e+03

10
Elasti GSPSAQDFASPL
:::: . ::::
HP0725 GQPASVQGNPPF
120
lalign output for Elastin (VVGSPSAQDFASPL) vs. HP0797

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

40.0% identity in 10 aa overlap; score: 24 \(E(10,000): 7.9e+02\)

10
Elasti VVGSPSAQDF
: : : : : : :
HP0797 VDSSDKDDFS
110

75.0% identity in 4 aa overlap; score: 23 \(E(10,000): 9.6e+02\)

10
Elasti QDFA
HP0797 KDFA
10

33.3% identity in 9 aa overlap; score: 21 \(E(10,000): 1.4e+03\)

Elasti VVGSPSAQD
HP0797 VKGTDNSND
210
lalign output for Elastin (VVGSPSAQDFASPL) vs. O69174

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

33.3% identity in 6 aa overlap; score: 23 E(10,000): 1.6e+03

Elasti GSPSAQ
  :
  
O69174 GNPTVE
  20

30.8% identity in 13 aa overlap; score: 21 E(10,000): 2.4e+03

  10
Elasti VGSPSAQDFASPL
  :
  
O69174 VARAAADLLGQPL
  120  130

50.0% identity in 4 aa overlap; score: 21 E(10,000): 2.4e+03

Elasti GSPS
  :
  
O69174 GTPN
lalign output for Elastin (VVGSPSAQDFASPL) vs. Tp0751

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

50.0% identity in 8 aa overlap; score: 27 E(10,000):
3.9e+02

Elasti VGSPSAQD
   .:: : : :
Tp0751 IGSTSMWD
      220

50.0% identity in 6 aa overlap; score: 23 E(10,000):
8.7e+02

Elasti SPSAQD
   ::... ::
Tp0751 SPNSGD

42.9% identity in 7 aa overlap; score: 21 E(10,000):
1.3e+03

Elasti GSPSAQD
   :: .. ::
Tp0751 GSSTTTD
      70

248
lalign output for Plasminogen 1 (WSLLTPA) vs. HP0671

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

75.0% identity in 4 aa overlap; score: 19 E(10,000):
2.2e+03

Plasmi LTPA
  : ::
HP0671 LDPA
  210

66.7% identity in 3 aa overlap; score: 18 E(10,000):
2.6e+03

Plasmi WSL
  : :  
HP0671 WYL
  50

42.9% identity in 7 aa overlap; score: 18 E(10,000):
2.6e+03

Plasmi WSLLTPA
  :: ::
HP0671 WGNTTGA
  190
lalign output for Plasminogen 1 (WSLLTPA) vs. HP0725

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

100.0% identity in 3 aa overlap; score: 25 E(10,000): 1.6e+03

Plasmi WSL
:::
HP0725 WSL

66.7% identity in 3 aa overlap; score: 20 E(10,000): 3.9e+03

Plasmi WSL
:::
HP0725 WGL

50.0% identity in 2 aa overlap; score: 17 E(10,000): 6e+03

Plasmi WS
::
HP0725 WT
lalign output for Plasminogen 1 (WSLLTPA) vs. HP0797

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

80.0% identity in 5 aa overlap; score: 24 E(10,000):
8e+02

Plasmi LLTPA
  :: ::
HP0797 LLRPA
  60

60.0% identity in 5 aa overlap; score: 20 E(10,000):
1.7e+03

Plasmi LLTPA
  .: ::
HP0797 VLIPA
  160

40.0% identity in 5 aa overlap; score: 18 E(10,000):
2.5e+03

Plasmi SLLTP
  ..: :
HP0797 TILEP
  170
lalign output for Plasminogen 1 (WSLLTPA) vs. 069174

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

60.0% identity in 5 aa overlap; score: 23 E(10,000):
1.6e+03

Plasmi WSLLT
::: ::
069174 WKQLT

50.0% identity in 4 aa overlap; score: 16 E(10,000):
5.3e+03

Plasmi LTPA
:::
069174 LVPS

100.0% identity in 1 aa overlap; score: 15 E(10,000):
6e+03

Plasmi W
:
069174 W
lalign output for Plasminogen 1 (WSLLTPA) vs. Tp0751

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 21 E(10,000):
1.3e+03

Plasmi WSL
:::
Tp0751 WAL

50.0% identity in 2 aa overlap; score: 16 E(10,000):
3.2e+03

Plasmi WS
::
Tp0751 WN

75.0% identity in 4 aa overlap; score: 16 E(10,000):
3.2e+03

Plasmi SLLT
:::
Tp0751 SFLT
lalign output for Plasminogen 2 (YPYIPTL) vs. HP0671

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

60.0% identity in 5 aa overlap; score: 26 E(10,000):
5.6e+02

Plasmi YPYIP
::  .:
HP0671 YPKFP

75.0% identity in 4 aa overlap; score: 22 E(10,000):
1.2e+03

Plasmi IPTL
::  .:
HP0671 IPTI
240

100.0% identity in 2 aa overlap; score: 18 E(10,000):
2.6e+03

Plasmi PY
::
HP0671 PY

254
lalign output for Plasminogen 2 (YPYIPTL) vs. HP0725

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

75.0% identity in 4 aa overlap; score: 22 E(10,000):
2.8e+03

Plasmi IPTL
:::
HP0725 IPTI
600

75.0% identity in 4 aa overlap; score: 19 E(10,000):
4.5e+03

Plasmi IPTL
:::
HP0725 IPIL

100.0% identity in 2 aa overlap; score: 18 E(10,000):
5.2e+03

Plasmi PY
::
HP0725 PY
lalign output for Plasminogen 2 (YPYIPTL) vs. HP0797

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

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lalign output for Plasminogen 2 (YPYIPTL) vs. 069174

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

75.0% identity in 4 aa overlap; score: 22 E(10,000):
2e+03

Plasmi YPYI
:::
O69174 YPII

50.0% identity in 4 aa overlap; score: 17 E(10,000):
4.6e+03

Plasmi YPYI
::
O69174 YKYL

33.3% identity in 3 aa overlap; score: 16 E(10,000):
5.3e+03

Plasmi IPT
:::
O69174 VPS
align output for Plasminogen 2 (YPYIPTL) vs. Tp0751

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 17 E(10,000):
2.7e+03

Plasmi IPT

Tp0751 LPT

33.3% identity in 3 aa overlap; score: 16 E(10,000):
3.2e+03

Plasmi YIP

Tp0751 HVP
30

50.0% identity in 4 aa overlap; score: 16 E(10,000):
3.2e+03

Plasmi PYIP

Tp0751 PPAP
lalign output for Tp 1 (PVQT) vs. HP0671

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

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100.0% identity in 2 aa overlap; score: 15 E(10,000): 4.3e+03

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50.0% identity in 4 aa overlap; score: 13 E(10,000): 5.7e+03

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100.0% identity in 2 aa overlap; score: 12 E(10,000): 6.4e+03
lalign output for Tp 1 (PVQT) vs. HP0725

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

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100.0% identity in 3 aa overlap; score: 17 \(E(10,000)\): \(6e+03\)

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66.7% identity in 3 aa overlap; score: 17 \(E(10,000)\): \(6e+03\)

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50.0% identity in 4 aa overlap; score: 16 \(E(10,000)\): \(6.7e+03\)
lalign output for Tp 1 (PVQT) vs. HP0797

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

50.0% identity in 4 aa overlap; score: 13 E(10,000):
5.5e+03

Tp    PVQT
      :..:
HP0797 PKRT

100.0% identity in 2 aa overlap; score: 12 E(10,000):
6.3e+03

Tp    QT
      ::
HP0797 QT

100.0% identity in 2 aa overlap; score: 12 E(10,000):
6.3e+03

Tp    VQ
      ::
HP0797 VQ
lalign output for Tp 1 (PVQT) vs. O69174

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

50.0% identity in 4 aa overlap; score: 16 E(10,000):
5.3e+03

Tp      PVQT
       :: : 
O69174  PIIT

50.0% identity in 4 aa overlap; score: 16 E(10,000):
5.3e+03

Tp      PVQT
       :: : 
O69174  PIIT

100.0% identity in 2 aa overlap; score: 15 E(10,000):
6.1e+03

Tp      PV
       :: 
O69174  PV
lalign output for Tp 2 (LWIQ) vs. HP0671

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

100.0% identity in 1 aa overlap; score: 15 E(10,000):
4.3e+03

Tp   W
    :
HP0671 W

100.0% identity in 1 aa overlap; score: 15 E(10,000):
4.3e+03

Tp   W
    :
HP0671 W

100.0% identity in 1 aa overlap; score: 15 E(10,000):
4.3e+03

Tp   W
    :
HP0671 W
lalign output for Tp 2 (LWIQ) vs. HP0725

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

50.0% identity in 2 aa overlap; score: 18 \( E(10,000): 5.3 \times 10^3 \)

Tp LW
HP0725 MW

50.0% identity in 2 aa overlap; score: 17 \( E(10,000): 6 \times 10^3 \)

Tp WI
HP0725 WL

50.0% identity in 2 aa overlap; score: 17 \( E(10,000): 6 \times 10^3 \)

Tp LW
HP0725 IW

264
lalign output for Tp 2 (LWIQ) vs. HP0797

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

100.0% identity in 1 aa overlap; score: 15 $E(10,000)$: 4.1e+03

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100.0% identity in 2 aa overlap; score: 12 $E(10,000)$: 6.3e+03

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100.0% identity in 2 aa overlap; score: 12 $E(10,000)$: 6.3e+03

<table>
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<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>HP0797</td>
<td>IQ</td>
</tr>
</tbody>
</table>

190
lalign output for Tp 2 (LWIQ) vs. 069174

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 19 E(10,000):
3.4e+03

Tp  WIQ
    :
069174  WKQ

100.0% identity in 1 aa overlap; score: 15 E(10,000):
6.1e+03

Tp  W
    :
069174  W

100.0% identity in 1 aa overlap; score: 15 E(10,000):
6.1e+03

Tp  W
    :
069174  W
lalign output for Tp 3 (TAIS) vs. HP0671

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

100.0% identity in 2 aa overlap; score: 10 E(10,000):
7.9e+03

Tp AI
::
HP0671 AI

50.0% identity in 4 aa overlap; score: 10 E(10,000):
7.9e+03

Tp TAIS
:: :
HP0671 TSYS

100.0% identity in 2 aa overlap; score: 10 E(10,000):
7.9e+03

Tp IS
::
HP0671 IS
lalign output for Tp 3 (TAIS) vs. HP0725

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 12 E(10,000): 9.2e+03

Tp AIS
:::
HP0725 ALS
170

66.7% identity in 3 aa overlap; score: 12 E(10,000): 9.2e+03

Tp AIS
:::
HP0725 AIT

66.7% identity in 3 aa overlap; score: 12 E(10,000): 9.2e+03

Tp TAI
:::
HP0725 TAM
lalign output for Tp 3 (TAIS) vs. HP0797

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 12 E(10,000):
6.3e+03

Tp       TAI
  ::.
HP0797   TAL

100.0% identity in 2 aa overlap; score: 10 E(10,000):
7.7e+03

Tp       AI
  ::
HP0797   AI
  220

100.0% identity in 2 aa overlap; score: 10 E(10,000):
7.7e+03

Tp       IS
  ::
HP0797   IS
  100
lalign output for Tp 3 (TAIS) vs. 069174

[ISREC-Server] using matrix file: BL50, gap penalties: -14/-4

66.7% identity in 3 aa overlap; score: 14 E(10,000):
6.8e+03

Tp TAI
:::
069174 TAV

66.7% identity in 3 aa overlap; score: 14 E(10,000):
6.8e+03

Tp TAI
:::
069174 TAV

50.0% identity in 4 aa overlap; score: 11 E(10,000):
8.8e+03

Tp TAIS
:::
069174 TTIA
380