2011

Collagen Binding Proteins in Helicobacter Pylori

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By

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MPhil Thesis

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2011

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Abstract

Helicobacter pylori is a human pathogen and the causative agent of chronic gastritis, peptic ulcer and gastric malignancies. A number of *H. pylori* virulence factors have been described including, CagA, VacA, neutrophil activating protein, and expression of receptors for binding to extracellular matrix proteins (adhesins). *H. pylori* interacts with extracellular matrix (ECM) proteins such as collagen IV, laminin, vitronectin and fibronectin, that can be exposed due to loss of integrity of the gastric epithelial cell layer. These interactions may be important in the pathogenesis of the organism.

This study examines the interaction of *H. pylori* with collagen IV in an attempt to identify a *H. pylori* collagen IV adhesin. Collagen IV makes up a large proportion of the basement membranes, and has been implicated in microbial adherence. FITC labelling of bacteria and a bacteria overlay method showed binding of *H. pylori* to ECM proteins collagen IV, laminin and fibronectin. Binding was observed under a variety of culture conditions. Receptor activity-directed tagging known as "Retagging" was employed in an attempt to purify the collagen IV adhesin. *H. pylori* catalase was identified as a possible collagen adhesin, This enzyme was isolated and its interaction with collagen IV *in vitro* was studied. However, *in vitro* binding of collagen IV to catalase could not be demonstrated despite repeated attempts. Immunoblot and receptor overlay techniques were investigated in further attempts to identify the *H. pylori* collagen IV binding protein. The role of collagen IV in *H. pylori* ECM binding is discussed.

Declaration Page

I certify that this thesis which I now submit for examination for the award of _____, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

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Acknowledgements

Firstly I would sincerely like to thank Gary Henehan and Henry Windle for their tireless guidance and supervision during the course of this research. I would like to thank each of you for your time and advice in bringing my thesis to a completion point. I would also like to thank Dermot Kelleher for the use of laboratory and facilities in the IMM, St James's Hospital, Trinity College Dublin.

I would also like to thank my parents for all their huge amount of support over the last few years, and for putting up with me! I couldn't have done any of this without you. Thanks to the rest of my family, my friends and my boyfriend.

A special thanks to Denise Cornally for her help in the early stages of my research. I would have been very lost and lonely without you!

I am extremely grateful to Steffi Gieseler for all her help and also for being my best friend throughout my time in the IMM.

I would also like thank all the post grads in the IMM and wish them luck, in particular Paul, Daniella, Ruth, Fiona, Aoife, Sean, Julia, Anne and Jenny.

Finally, I would like to dedicate this thesis to my cousin Marguerite Kennedy, who sadly passed away in June 2010 from Gastric cancer.

Abbreviations

APS	Ammonium persulfate
BSA	Bovine Serum Albumin
CagA	Cytotoxin associated protein
Cag PAI	Cag pathogenicity island
DTT	Dithiothreitol
ECM	Extracellular Matrix
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
FITC	fluorescein isothiocyanate
H. pylori	*Helicobacter pylori*
kDa	Kilo Dalton
OD	Optical density
PBS	Phosphate buffered saline
PVDT	Polyvinylidene Fluoride
RPM	Rotations per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electerophoresis
SulfoSBED	Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azidobenzamido)-hexanoamido) ethyl-1,3'-dithioproprionate
Vac A	Vacuolating cytotoxin

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Chapter 1

Introduction

(1.0) Introduction

(1.1) Helicobacter pylori

Helicobacter pylori (Figure 1.1) is a gram negative, flagellated, spiral shaped bacterium found in the gastric mucosa of humans. Marshall and Warren first discovered the bacterium when they cultured it from a gastric biopsy (Marshall and Warren, 1984). It is a causative agent in gastritis, both peptic and duodenal ulcer disease (Morris & Nicholson 1987, and Blaser 1992), and is a risk factor for gastric carcinoma and mucosal-associated-lymphoid-tissue (MALT) lymphoma (Parsonnet *et al*., 1991).

Figure.1.1 *Helicobacter pylori*

H. pylori colonises a hostile gastric environment where few other organisms can survive, with a tissue tropism for the gastric mucosa of humans. Tissue tropism is the bacterial preference for certain tissues for growth. The bacteria survives the low pH of the stomach by producing urease. Urease hydrolyzes urea into ammonia and carbon dioxide, thus buffering the pH of the microenvironment surrounding the bacteria. *H. pylori* is sensitive to oxygen and therefore requires a microaerophillic atmosphere of 5%

O_2 and 5-10% CO_2 for growth. Another important colonisation factor for the bacterium is the possession of two to six polar sheathed flagellae, with two types of filaments encoded by the *flaA* and *flab* genes. These flagellae are essential for *H. pylori* motility, allowing the bacterium to propel itself through the mucus layer of the stomach (Suerbaum *et al.,* 1993, and Josenhans *et al.,* 1995).

The proposal that this bacterium could cause gastric disorders was initially greeted with scepticism. In order to convince colleagues, and the public, that this bacterium could cause disease such as gastritis and peptic ulcers, Barry Marshall drank a cultured suspension of *H. pylori*, and showed it caused stomach inflammation, a potential precursor of an ulcer (Marshall *et al*., 1985). Once established in the gastric mucosa, the bacterium usually persists for life unless treated with antibiotics.

(1.2) Epidemiology of H. pylori Infection

Helicobacter pylori is thought to infect about 50% of the worlds population, and is one of the most common bacterial pathogens in humans. Although infection occurs worldwide, the overall prevalence of *H. pylori* infection in developed countries is lower than that in developing countries. In developing countries, 70 – 90% of the population carries *H. pylori*, whereas 25 – 50% of the population in developed countries carry the pathogen. The majority of all *H. pylori* infections are initiated in early childhood. In most developed countries the prevalence of infection is substantially lower at all ages (Karaca *et al.,* 2004). Primary acquisition in adults, or reinfection after eradication does occur, but is less common. Prevalence can vary by ethnicity, place of birth, and socio-economic groupings among people living in the same country. However, infection is decreasing in many areas due to improvements in sanitation and living standards. Many of those carrying *H. pylori* are asymptomatic, but all, without exception, have

inflammation of the stomach lining, a condition termed gastritis. Gastritis is an underlying condition, which eventually gives rise to ulcers and other digestive disorders.

The mode of transmission of *H. pylori* is unclear, but it is thought that the most likely route of transmission is person to person contact, including oral to oral and fecal to oral transmission. Gastroenterologists or endoscopists may acquire infection through patients, and patients may acquire infection when an endoscope used on one patient is used on another. As a result of the association of *H. pylori* with peptic ulcer disease (Figure 1.2), gastric cancer and malt lymphoma, intervention strategies to prevent the spread of this bacterium are urgently needed.

(1.3) Clinical Manifestation of Helicobacter pylori Infection

Once infected the typical course of infection begins with superficial gastritis. If left untreated infection can lead to gastric adenocarcinoma. Hardin & Wright (2002), report that *H. pylori* infection is associated with an increased rate of development of gastric carcinoma by a factor of up to 90-fold. A selection of host factors may also contribute to carcinoma development. The pro-inflammatory cyclooxygenase (COX)2 has been associated with this increased risk of developing gastric cancer. This enzyme catalyses reactions which promote the formation of inflammatory prostaglandins, and overexpression of (COX)-2 has been shown in patients with both *Helicobacter pylori* $CagA^+$ (Cag positive strain) infection and gastric cancer (Menaker *et al.,* 2004).

Host genetics also play an important role in determining the outcome of a *H. pylori* infection. For example polymorphisms of the IL-1 cytokine are known to be associated with a greater risk of cancer (Crowe, 2005).

Potentially, the combination of host and bacterial genotyping may yield clues to the identification of patients with a high risk of developing *H. pylori* associated gastric cancer.

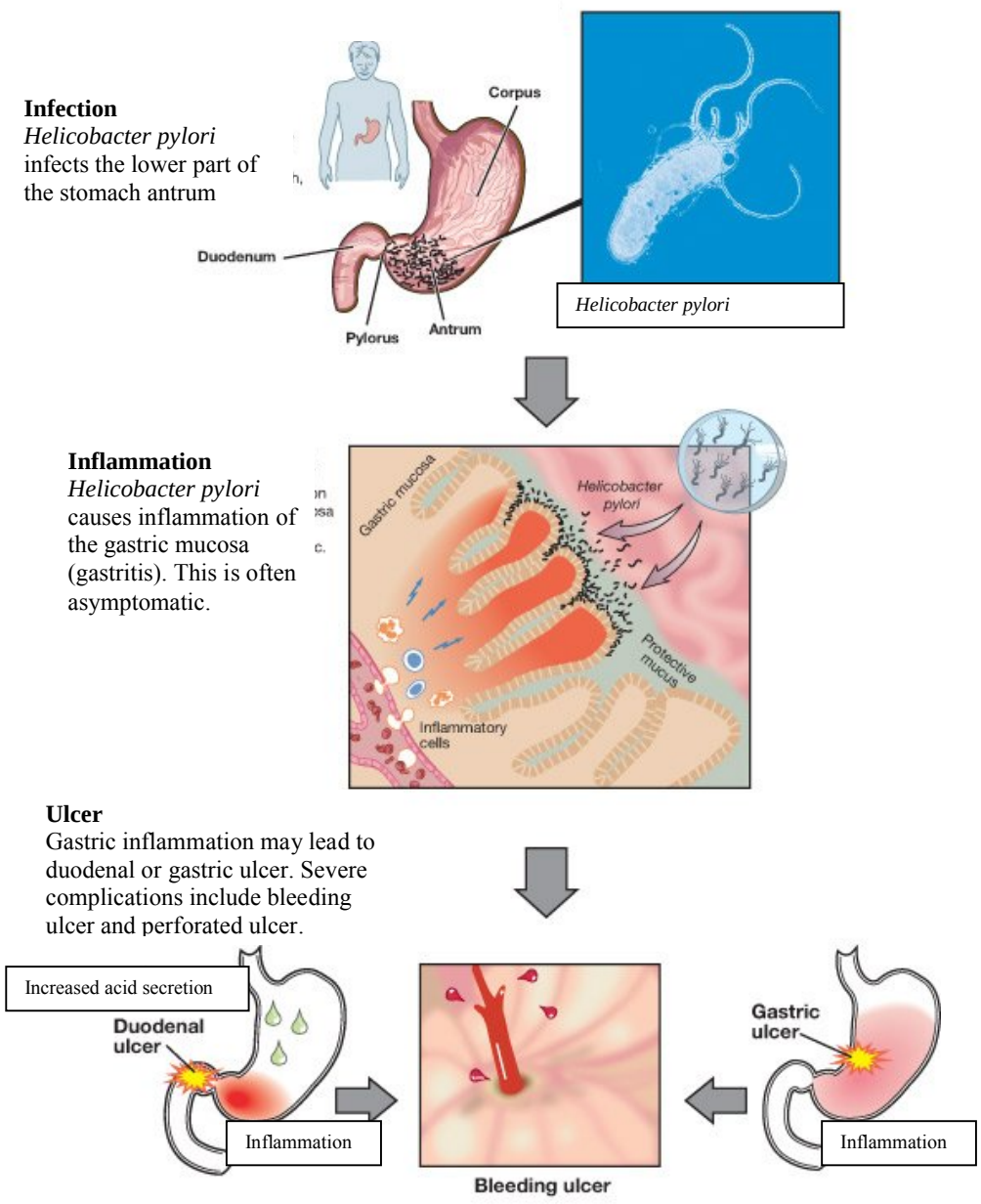


Figure 1.2. Schematic of *Helicobacter pylori* induced peptic ulcer disease.
Taken from http://nobelprize.org/nobel_prizes/medicine/laureates/2005/press.html

(1.4) Virulence Factors

The human stomach is a harsh environment, bathed in gastric juices composed of digestive enzymes and HCl, where food, bacteria, and viruses are all consumed. *Helicobacter pylori* has developed the ability to survive in the ecological niche of the human stomach with the aid of several virulence factors. These are described below and summarised in Table 1.1

(1.4.1) Urease

The gastric mucus protects the stomach from its own gastric juice. *H. pylori* colonises the mucus where it may come into contact with stomach acid. To protect itself against acid damage, *H. pylori* possesses an enzyme called urease. This enzyme hydrolyses urea into carbon dioxide and ammonia, thus neutralizing an area surrounding the bacterium. The reaction is catalysed by urease is:

$$(NH_2)C0 + H_20 \rightarrow C0_2 + 2NH_3$$

Urease may contribute to mucosal damage in addition to conferring acid resistance on *H. pylori*, since it can stimulate upregulation of the inducible NO synthase (iNOS) (Gobert *et al.,* 2002). However, urease may not be the only component involved in acid resistance. It has been reported that urease-negative *H. pylori* were capable of both colonization and inducing gastric ulcer formation in Mongolian gerbils (Mine *et al.,* 2005). *H. pylori* urease can also act as an adhesion factor (Beswick *et al.,* 2006). Although urease is a cytoplasmic protein in log phase bacteria, urease has been found on the cell surface of *H. pylori*, thought to be a result of bacterial lysis or release. Urease present on the bacterial surface binds to class II MHC molecules and CD74.

(1.4.2) VacA

Approximately 50% of *H. pylori* strains produce a vacuolating toxin (VacA), which induces large cytoplasmic vacuoles in cultured eukaryotic cells (Leunk *et al.,* 1988). Studies have shown that it is a major virulence factor that is involved in the pathogenesis of inflammation in *H. pylori* (Papine *et al.,* 2001, Prinz *et al.,* 2003 and Peek 2003). Mature, soluble, 88kDa VacA proteins are secreted into extracellular space (Cover and Blaser, 1992), but have also been found to localise on the surface of *H. pylori* (Ilver *et al.,* 2004). The 88kDa VacA protein can be subdivided into two domains p37 (amino terminal) and p 53(carboxyl terminal). The p37 domain is thought to have vacuolating activity, while the p58 domain is believed to be involved in VacA binding to the cell. Native VacA in *H. pylori* culture medium exists as a large oligomer with a mass of approximately 1000 kDa. The oligomer is composed of two rings consisting of 6-7 VacA monomers, in a flower like shape. Under acidic or alkaline conditions the oligomer dissociates into monomers, increasing the vacoulating activity (Cover et al., 2007, and Yahiro et al., 1999).

VacA has been shown to play a role in colonization of the stomach. Although studies have shown that VacA negative mutants are capable of colonisation of mice and gerbils, Salama *et al.,* (2001), has shown that a VacA producing *H. pylori* strain had a selective advantage over a VacA mutant strain, in colonisation of a mouse stomach.

(1.4.3) Cytotoxin associated geneA – CagA

CagA is an 128kDa immunogenic protein. Antibodies for CagA are detected in up to 80% of *H. pylori* infected persons. It is used as a marker for a large pathogenicity island - a genomic island which is acquired by horizontal gene transfer.

They are incorporated in the genome of pathogenic microorganisms and encode many proteins implicated in pathogenesis. This CagA pathogenicity island is a means of making a distinction among *H. pylori* stains, as the 40kb region is present or absent among strains. CagA was the first gene discovered on the island. CagA positive strainss have been shown to be much more interactive with the host than CagA negative strains. CagA is also associated with VacA (Censini *et al.,* 1996), and strains with both proteins have been implicated in disease at higher frequencies than those without. The CagA Pathogenicity Island (PAI) has a type IV secretion system which allows CagA to be delivered into epithelial cells. CagA is also tyrosine phosphorylated, which allows it to interact with many host signalling molecules. It has recently been shown that CagA injection into host gastric epithelial cells, can induce NF-$_k$B activation and IL-8 production.

(1.4.4) Helicobacter pylori Adhesins

Specific adhesion to host tissue and cells is an essential virulence factor for most bacterial pathogens. The initial step of *H. pylori* colonisation involves the adhesion of the bacterium to human gastric epithelial cells, mediated through interactions between *H. pylori* adhesins and host cell receptors. Adhesion is directly linked to pathogenicity of the organism, and *H. pylori* that does not adhere to gastric mucosa tends to be removed by shedding of surface epithelial cells and the mucous layer.

Adhesins are present on the outer membrane of *H. pylori,* and two of the best known adhesins are BabA and SabA, which bind to fucosylated and sialylated blood group antigens respectively. Bacterial adhesion can result in multiple outcomes depending on the host cell receptor engaged (Testerman *et al.,* 2001). For example, adherence of bacteria to host cell receptors may trigger cellular changes such as signal transduction

cascades, which in turn can lead to inflammation and eventually to persistence of the microorganism (Kronvall & Jonsson, 1999). Adhesins can be surface exposed or released by the bacterium. *H. pylori* also expresses adhesins with affinity for the Extracellular Matrix, which allows the bacterium to gain access to the subepithelial layer through intercellular tight junctions. Identifying adhesins may prove important in the development of strategies against *H. pylori* infection. It is of considerable current interest to analyze the mechanism of adhesion, and the interactions between the adhesins and the receptors. It may be possible to inhibit the adhesin as a mechanism for the prevention of *H. pylori* colonisation.

Table 1.1 Virulence factors in *Helicobacter pylori*

Virulence Factor	**Function and size**	**Reference**
CagA	128 kDa Immunogenic protein	Censini *et al* 1996
VacA	Vacuolating toxin	Telford *et al* 1994
Urease	Enzyme - hydrolyses urea into carbon dioxide and ammonia	Mobley *et al* 1997
Catalase	Enzyme – protects bacterium from oxygen toxicity	Hazell *et al*1991
BabA	Lewis B binding adhesin	Ilver *et al* 1998
Flagellar proteins	Motility	Lingwood *et al* 1993 Suerbaum *et al* 1993 O'Toole *et al* 1994
HP-NAP	Neutrophil activating protein	Evans *et al* 1995

Table adapted from Pantzar 1999.

(1.5) Treatment of H. pylori Infection

Many factors have to be taken into consideration when designing treatments for the successful eradication of *H. pylori* (Figure 1.3). For example, the harsh environment of the human stomach can be a difficult environment for medicines. Development of antibiotic resistance is also proving to be a problem. Many current treatments involve taking a large number of pills per day, thereby causing difficulties for the patient, as well as side effects.

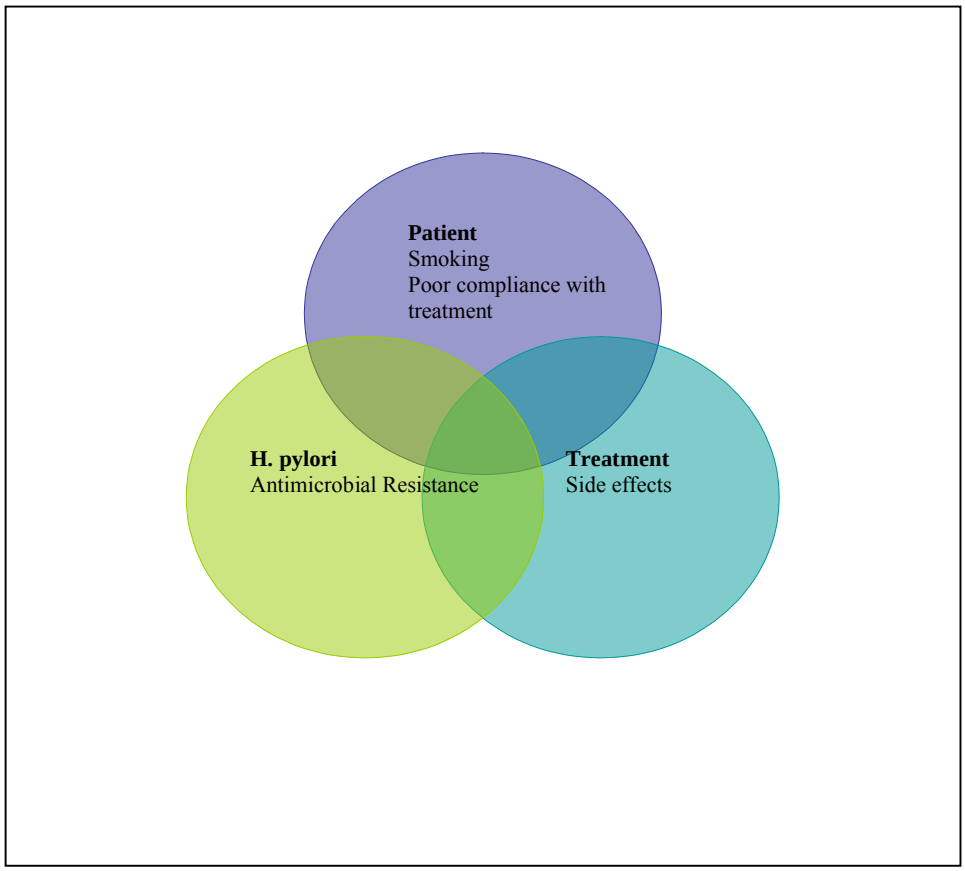


Figure 1.3. Factors to consider in the treatment of *Helicobacter pylori* infection.

Current treatment uses a triple therapy, a combination of two antibiotic agents administered with an adjunctive agent, such as a proton pump inhibitor. Omeprazole is a widely studied proton pump inhibitor, which reduces acid production and raises the pH

in the stomach. A proton pump inhibitor may be used along with a combination of meatronidazole, clarithromycin, amoxicillin, and tetracycline antibiotics. Antimicrobial resistance and incomplete treatment are the major reasons underlying treatment failure. This has made it necessary in some cases to use a quadruple therapy using four antibiotics and a proton pump inhibitor (Malfertheiner *et al,* 2002). Some current treatment regimens for *Helicobacter pylori* infection are outlined in Table 1.2.

Table 1.2. Treatment Regimens for *Helicobacter pylori* Infection

Triple Therapy 1	**Triple Therapy 2**	**Quadruple Therapy 1**	**Quadruple Therapy 2**
Omerprazole (PPI)	Ranitidine bismuth citrate	Bismuth subsalicylate	Bismuth subsalicylate
or	**and**	**and**	**and**
Lansoprazole (PPI)	Clarithromycin	Metronidazole	Metronidazole
and	**or**	**and**	**and**
Metronidazole	Metronidazole	Tetracycline	Tetracycline
or	**and**	**and**	**and**
Amoxicillin	Amoxicillin	H_2RA	PPI
and	**or**		
Clarithromycin	Tetracycline		

Adapted from Malfertheiner *et al,* 2002.

In addition to antibiotic resistance, prohibitive costs of treatment, especially in developing nations where *H. pylori* infection is endemic, may add to the problems surrounding successful treatment and eradication (Ruggiero *et al,* 2003).

Some workers have suggested that immunisation may be a more appropriate treatment of *H. pylori* infection (Hardin & Wright, 2002). The availability of a safe, effective, and cost effective vaccine would be a potential benefit to society.

(1.6) Collagen IV

Collagens are a large family of proteins that are found in all multicellular animals, and are responsible for a wide variety of functions, such as cell migration, cell adhesion, as well as playing a structural role in tissue integrity. At least 28 different collagens occur in vertebrates. Although there is no agreed definition for a collagen, they are regarded in general as triple helicle proteins that function in tissue assembly and maintenance (Kadler *et al*, 2007). Table 1.3 shows a list of some of the known collagens and their properties.

Collagen IV is a large structural protein, found exclusively in basement membranes, and makes up a large component of the mammalian extracellular matrix (ECM) (Kefalides 1973). This collagen is also termed network forming due to its ability to self assemble into organized networks. This property makes type IV collagen different to the fibrillar forming collagens (types I, II, & III). Collagen IV is composed of three domains (Figure.1.4.A). The first is a central triple helical structure, where 3 polypeptide chains called α chains are wound around one another in a rope-like superhelix (Figure.1.4.B). This structure is rich in glycine, and is composed of a series of Gly-X-Y sequences, where X and Y are frequently occupied by proline and hydroxyproline residues. The other two domains are the N terminal (7S) domain and the C terminal Globular (NC1) domain. Disulfide bonds link the NC1 domain of one molecule to the same domain of another, while 7S domains associate to form cross links (Furthmayr, 1993). There are 6 distinct α chain polypeptides in mammalian collagen IV, which have

Table 1.3: Collagens and their Polymerised Form and Tissue Distribution

	Type	Polymerised Form	Tissue Distribution
Fibril Forming	I	fibril	Bone, skin, tendons Ligaments, cornea, internal organs
	II	fibril	Cartilage, invertebral disc, notochord
	III	fibril	Skin, blood vessels internal organs
	V	fibril	As with type I
	XI	fibril	As for type II
Fibril Associated	IX	Lateral association with type II fibrils	Cartilage
	XII	Lateral association with some type I fibrils	Tendons, ligaments, some other tissues
Network Forming	IV	Sheetlike Network	Basal Lamina
	VII	Anchoring fibrils	Beneath stratified squamous epithelia
Transmembrane	XVII	Not known	hemidesmosomes
Other	XVIII	Not known	Basal lamina around blood vessels

Adapted from Kadler *et al.,* 2007.

similar domain structures and have between 50-70% homology at the amino acid level (Hudson *et al,* 1993 and Kalluri, 2003).

(A)

(B)

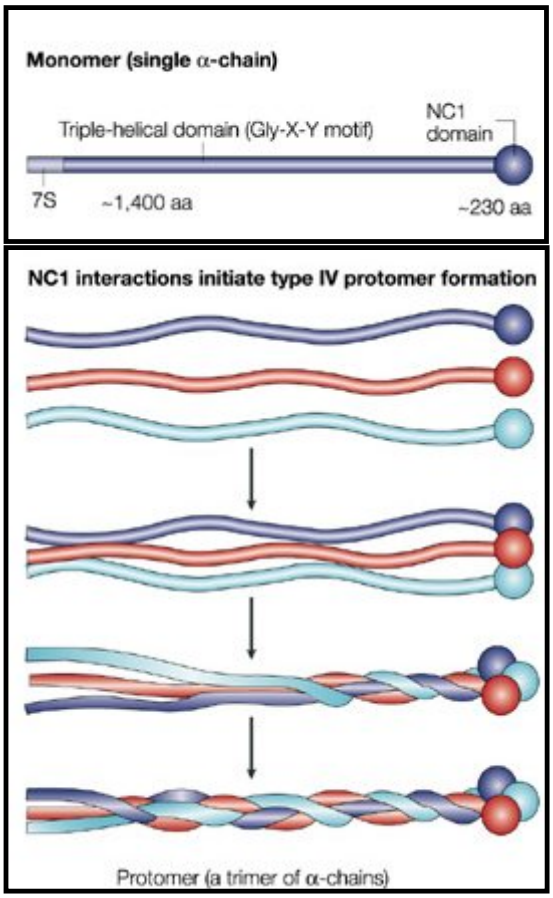


Figure 1.4: Schematic showing **(A)** the three domains of the Collagen IV α chain, and **(B),** the triple helical structure composed of three α chains (Kalluri 2003).

Although collagen IV plays a key role in scaffolding of the ECM, other biological roles, such as binding of collagen IV to cells, proteins or bacterial adhesins, may be just as important to explore. Collagen IV is found exclusively in basement membranes; the extracellular structures which under-lays most epithelial cells and surrounds muscle, smooth muscle cells nerves and fat. The ability of a microorganism to adhere to such an ubiquitous protein would be an obvious advantage for pathogenesis. Several bacterial species have shown binding to collagen IV including *Enterococcus faecalis* (Xiao *et al.,* 1998), *Psuedomonas aeruginosa* (de Bentzmann *et al.,* 1996) and *Staphylococcus aureus* (Switalski *et al.,* 1989).

Trust *et al* (1991) first reported binding of *Heliocbacter pylori* to collagen IV. Testing various *H. pylori* isolates, it was found that binding occurred at high levels (average binding 27%, highest binding 60%) and was of high affinity (K_d = 16nM). Binding was also found to be rapid, insensitive to pH and saturable. Boiling the cells and treating with either trypsin or protein kinase K, significantly reduced binding indicating that binding is most likely mediated by surface proteins present on the bacterial surface. Reduced binding also occurred for bacteria that were treated for 20 minutes at 80ºC. This temperature does not denature the proteins but causes a perturbation of macromolecular assembled proteins. From this data Trust *et al,* suggest that a supermolecular protein structure such as a fimbria (a proteinaceous appendage in many Gram-negative bacteria) might be involved in binding. *H. pylori* binding to collagen IV could be important for colonization and invasion of the basement membranes and the lamina propria in the process of eliciting ulcerative lesions.

(1.7) Laminin

Laminin is a large non collagenous protein which makes up a significant part of basement membranes. The first laminin trimer was isolated from a Engelbreth-Holm-Swarm tumour (Chung *et al*, 1979, and Timpl *et al*, 1979). The structure of a typical laminin molecule (Figure 1.5) is made up of 3 chains held together by disulfide and non-covalent bonds in a cross shaped structure. The first chain is an A chain ~ 400kDa. The remaining two chains are made up of a B1 chain, and a B2 chain each approximately 220kDa, which associate forming a cross linked structure (Chung, 1995). Laminin has a large globular domain at its base as well as smaller globular domains on each arm (Chung, 1993).

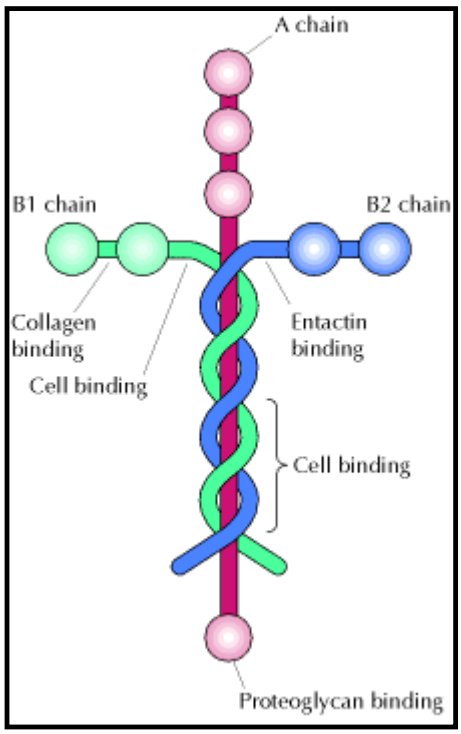


Figure 1.5: Structure of Laminin. Laminin consists of three polypeptide chains designated A, B1 and B2. Binding sites for entactin, collagen IV, proteoglycans, and cell surface receptors are indicated.
Source:http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=cooper&part=A2035&rendertype=figure&id=A2055

(1.8) Fibronectin

Fibronectin is a major component of the ECM. Here it exists in an insoluble form, but it also exists in plasma and other bodily fluids in a soluble form.

It exists as a dimeric glycoprotein, with subunits of 250 kDa, joined by disulfide bonds at one end. Each subunit consists of a series of repeating modules, consisting of 12 type I modules, 2 type II modules, 15 to 17 type III modules, and a variable sequence (Figure 1.6). Fibronectin has roles in adhesion, migration, differentiation and proliferation, and is therefore important in many physiological processes (Hynes, 1990). Fibronectin is also found in the early stages of embryonic development and is critical for normal biological development, especially in vascular structures.

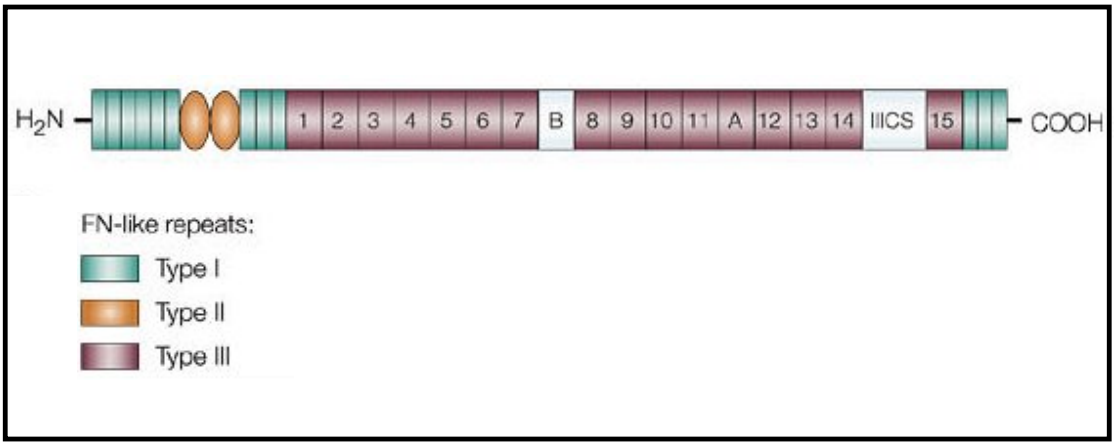


Figure: 1.6.The modular structure of fibronectin. Only one 250 kDa subunit is shown here for clarity (taken from Neri & Bicknell, 2005).

Some of the soluble fibronectin may become incorporated covalently into extracellular tissues, the rest is synthesized directly into multimeric fibres without being released in a

soluble form (Proctor, 1987). This insoluble fibronectin found on cell surfaces and in the extracellular matrix has a more structural role and forms the scaffold upon which collagen, heparin sulfate, proteoglycans and other ECM components are deposited. Here, it functions as an adhesive protein for cell attachment and tissue organization. Fibronectin interacts with several kinds of macromolecules, cells and bacteria. Its ability to bind Staphylococci was first reported in 1978 by Kuusela, and since then, both gram positive and gram negative bacteria have been shown to interact with fibronectin (Proctor, 1988). Fibronectins have specific binding sites for binding of collagen, actin, hyaluronic acid, and heparin. Pankov and Yamada (2002) describe how fibronectin is subject to cell-specific mRNA splicing, which results in polypeptide chains differing in the number of internal repeats. Plasma fibronectin is synthesized predominantly in the liver by hepatocytes, and shows a relatively simple pattern of splicing. On the other hand, cellular fibronectin is much larger and produces a large number of variants, resulting in fibronectins with different cell binding, ligand binding and solubility properties.

The adhesion of bacteria to fibronectin is believed to represent a significant virulence factor for many microorganisms (Joh *et al.,* 1999). Apart from Staphylococci, a number of other bacteria have been shown to bind specifically to fibronectin with some of their adhesins have been identified (Table 1.4).

Table 1.4: Fibronectin binding proteins identified from bacterial species.

Bacterial Species	**Protein**	**Reference**
Staphylococcus aureus	FnbpA & FnbpB	Signas et al (1989), Johnson et al 1991)
Streptococcus pyrogenes	SfbI/ protein F1	Hanski & Caperon (1992), Talay et al (1993)
Escherichia coli	P Fimbriae Type 1 fimbriae	Westerlund et al (1989, 1991) Sokerenko et al (1992)
Campylobacter jejuni	Cad F Flagellin	Konkel et al (1997) Moser et al (1997)

Adapted from Joh *et al.,* 1999.

(1.9) The Extracellular Matrix

Tissues are composed of biological cells that have a specialized function. The extracellular space surrounding cells contains a network of macromolecules known as the extracellular matrix. The extracellular matrix (ECM) is composed of a variety of proteins and polysaccharides that are secreted locally, and assembled into an organized meshwork between clusters of cells. These molecules provide the mechanical strength required for proper structural support for each tissue, but also provide cells with positional and environmental information. The ECM allows information exchange (i.e. signaling) between adjacent cells and molecules of the ECM itself. ECM proteins typically have multiple domains with specific binding sites for other matrix proteins and for receptors on adjacent

cells, therefore contributing to the organization of the matrix and allowing other cells to attach (Alberts, 2007).

While the composition of the ECM can vary in different organs there are many macromolecules that are prevalent throughout. There are two main classes of macromolecules that make up the matrix. Firstly, polysaccharide chains called glycocosyaminoglycans (GAG's), which are usually found covalently linked to a protein component in the form of proteoglycans. Secondly, fibrous proteins including collagen, elastin, fibronectin and laminan, having both structural and adhesive properties. The macromolecules that make up the ECM are mainly produced locally by cells in the matrix. The extracellular matrix is not a static structure, in the sense that it is continually being produced and remodeled, and cell mediated matrix assembly is a highly controlled process (Schwarzbauer 1999).

Many bacteria have receptors with specific affinity for components of the ECM. This was first recognized by Kuusela (1978) with the discovery of interactions between *Staphylococcus aureus* and fibronectin. Since then it has been shown that several pathogens bind components of the ECM, including mucosal pathogens.

Basement membranes are a specialized type of ECM found underlying epithelial cells. The epithelial cell layer consists of cells attached together at several cell junctions, including tight junctions, anchoring junctions, and communicating junctions. Beneath the epithelial cells is a thin layer of basal laminae or basement membranes containing a framework of ECM components. In a normal healthy individual the ECM is not exposed. However, injury due to physical or chemical factors can cause a loss of integrity of the ECM, leaving it

exposed. This allows bacteria with affinity for ECM proteins, to gain access via the cell junctions. They bind to the ECM, facilitating their ability to cause long lasting infection.

Helicobacter pylori penetrates the gastric mucous and binds gastric epithelial cells and mucins which allows colonization of the gastric epithelium. After disruption to the epithelial cell layer, ECM binding may occur which may have a role in subepithelial tissue damage in chronic type B gastritis and gastric duodenal ulcers (Dubreuil *et al*., 2002, Trust *et al*., 1991).

(1.10) Bacterial Adherance

Adherance: Important for bacterial Colonization and Virulence

To be infectious bacteria need to colonize host tissues they encounter. They do this, in the first instance, by means of adhesion using specialized adhesins present on their surface. Each bacterial adhesin binds to specific host cell receptors. For molecules to function as adhesins they must be presented at the bacterial cell surface and the binding domain must be capable of docking with the complementary host cell receptor. Usually, only certain regions of the adhesin and receptor are involved in the binding process. Adhesion is a critical step in the colonization process. It allows the bacterium to resist removal from the host by physical or mechanical forces. Binding to host cell receptors also triggers cellular changes such as signal transduction cascades. This leads an inflammatory response where infiltration of neutrophils and monocytes occurs, and eventually persistence of the bacterium (Testerman *et al,* 2001). These are called secondary effects of binding.

However, bacteria have been shown to interact with other host proteins such as serum and extracellular matrix proteins, where there is no secondary effect of binding. Kronvall and Jonsson (1999) describe how in this case the adhesion has been denoted by a new term receptin. Receptins are microbial binding proteins that interact with mammalian target proteins.

The main type of surface bacteria encounter are epithelial cells. These include the epithelial cells of the skin, upper and lower GI tract and urinary tract which all share the same basic characteristics that make up an epithelium; (1) the cells are joined by junctions, (2) the cell layer has a free apical surface that may be keratinized or has a mucous layer, (3) beneath epithelial cells is a thin layer of basal laminae containing collagens laminans and other molecules which make up an extracellular matrix. When the epithelial cell layer loses integrity due to physical or chemical forces, bacteria can access these sub-epithelial components. Adherence has an especially important role in the colonization of mucosal surfaces which are constantly subjected to clearance mechanisms (Odenbreit 2005). Adherence to host tissue surfaces has been an important factor in the survival of microorganisms. Therefore adhesins must have evolved over time as the bacterium has evolved, to adapt to new hosts or niches. Many bacterial species are capable of producing multiple adhesins, and can alter expression from one adhesive activity to another, and modify the strength of binding by means of antigenic variation. This can help the bacterium overcome host immune recognition, but, of course, makes vaccine development targeting specific adhesins difficult.

(1.11) Helicobacter Pylori binding to ECM

A large number of microorganisms have been shown to adhere to the mammalian Extra Cellular Matrix (ECM) (see Table 1.5) and that adherence has been shown to contribute to their virulence (Patti & Hook, 1994) and (Westerlund & Korhonen, 1993). *H. pylori* has been shown to adhere specifically to several proteins in the extracellular matrix (see Dubreuil *et al.,* 2002 for review) including laminin and collagen IV, vitronectin and heparin sulphate (Trust *et al.,* 1991, Valkonen *et al.,*1993, Moran *et al.,* 1993). Expression of receptins may only appear at certain phases of bacterial growth, for example *H.* pylori only expresses ECM-binding molecules at the stationary phase of growth, and for specific ECM molecules it was shown that both coccoidal and spiral forms of the organism express similar ECM binding patterns (Khin *et al.,* 1996).

Several ECM receptins have been identified for *H. pylori* (see Table 1.6). Laminan has a high carbohydrate content, mainly complex oligosaccharides, therefore binding to microorganisms has been found through lectin interactions (Ljungh *et al.,* 1996). Laminin binds *H. pylori* via surface proteins (Valkonen *et al.,* 1993 and Valkonen *et al.,* 1994). Trust *et al.,* (1991), showed that binding of *H. pylori* to laminin was rapid (15-30 mins), of high affinity K_d = 7.9nM and partially reversible. The binding interaction involves bacterial receptins recognizing certain sialylated oligosaccharides of the glycoprotein. Hemagglutinating strains have shown enhanced binding of laminin compared to non hemagglutinating strains (Valkonen *et al.,* 1994). A *H. pylori* 25 kDa outer membrane protein which acts as a lectin-like molecule was identified as a laminin binding protein (Valkonen *et al.,* 1997), and another interaction between laminin and *H. pylori* has been proposed to be mediated by lipopolysaccahrides (Valkonen *et al.,* 1994).

Table 1.5. Some bacterial receptins with binding sites for mammalian proteins

Collagen receptin	*Staphylococcus aureus* *Staphylococcus lugdunensis* *Streptococcus pyogenes* *Streptococcus pneumoniae* *Escherichia coli* *Helicobacter pylori* *Aeromonas salmonicida* *Treponema pallidum* *Yersinia pestis*
Fibrinogen receptin	*Staphylococcus aureus* *Staphylococcus lugdunensis* *Streptococcus pyogenes* *Streptococcus agalactiae* *Streptococcus canis* *Porphyromomas intermedius* *Porphyromonas gingivalis*
Fibronectin receptin	*Staphylococcus aureus* *Staphylococcus hyicus* Streptococci, grp A, C, G *Streptococcus equi subsp. Zooepidemicus* Streptococcus, a-hemolytic *Porphyromonas gingivalis* *Neisseria meningitides* *Treponema pallidum* *Mycobacterium tuberculosis* *Mycobacterium paratuberculosis*
Lactoferrin receptin	*Staphylococcus aureus* *Escherichia coli* *Neisseria gonorrhoeae* *Aeromonas hydrophila* *Porphyromonas gingivalis* *Prevotella intermedia*
Laminin receptin	*Staphylococcus aureus* *Streptococcus pyogenes* *Streptococcus pneumoniae* *Escherichia coli* *Treponema pallidum* *Actinobacillus actinomycetemcomitans*
Plasminogen/plasmin receptin	*Staphylococcus aureus* *Streptococcus pyogenes* Streptococci, grp C, G

Table 1.5. Continued

	Streptococcus equisimilis *Streptococcus pneumoniae* *Haemophilis influenzae* *Moraxella eatarrhalis* *Escherichia coli* *Proteus mirabilis* *Pseudomonas aeeruginosa* *Neisseria gonorrhoeae* *Neisseria meningitides* *Borrelia burgdorferi* *Yersinia pestis*
Vitronectin receptin	*Staphylococcus aureus* Streptococci, grp A, C Streptococci, grp C, G Streptococcus grp G *Streptococcus pneumoniae* *Escherichia coli* *Neisseria gonorrhoeae* *Helicobacter pylori*

Table adapted from Kronvall and Jonsson 1999.

The ECM glycoprotein plasminogen is a 90 kDa single chain pro-enzyme in its native form. Plasminogen binding to *H. pylori* is a common CagA independent phenomenom among *H. pylori* strains, and generally independent of the morphological state, spiral or coccoid, of the bacteria (Ringner *et al*., 1994). *H. pylori* CCUG 17874 interacts with the fifth kringle of the plasminogen molecule through two surface proteins of 42 and 57 kDa (Pantzar *et al.,* 1998). Cell surface bound plasminogen is easily activated to plasmin – an enzyme that dissolves fibrin in blood clots. This activation of plasmin is a common mechanism used by certain invasive bacteria to facilitate movement through tissue barriers (Lottenberg *et al.,* 1994). It has been suggested that *H. pylori* bound plasmin may be a

mechanism to degrade the extracellular matrix, which could delay the healing of gastric ulcers (Pantzar *et al.,* 1998).

Pantzar *et al.,* (1998), also identified a 57 kDa vitronectin binding protein using a surface protein extract immunolblot assay. The 57 kDa vitronectin binding protein had an amino terminal amino acid sequence identical with the amino-terminal of *H. pylori* catalase. Although catalase is normally a cytoplasmic protein, it is thought that it may become associated with the cell surface when cells undergo autolysis (Phadnis *et al.,* 1996), or through a specific transport mechanism (Vanet and Labigne 1998). Pantzar e*t al.,* suggested both of these hypotheses to explain surface location of proteins that are normally cytoplasmic, and therefore the possibility that catalase might function as an adhesin that binds vitronectin.

Other binding proteins have been identified for heparin and heparan sulfate - 47, 66 and 71 kDa proteins and the 58 kDa subunit of VacA cytotoxin (Ascencio *et al.*, 1993, Ruiz-Bustos *et al*., 2000 and Utt *et al*., 2001). The techniques for ECM receptin identification include radiolabelling, OMP isolation, and 2D gel analysis. Another method that has been used for receptin identification is affinity chromatography, where the sepharose is coupled to the ECM protein of interest and a bacterial cell lysate is applied to this resin in a chromatography column.

Table 1.6: *Helicobacter pylori* ECM Receptins

ECM Protein	**Receptin**	**Reference**
Laminin	LPS, 25 & 67 kDa proteins	Trust *et al.,* 1991. Valkonen *et al.,* 1994 & 1997.

Heparin & Heparan sulfate	47, 66 and 71 kDa proteins 58 kDa subunit of VacA cytotoxin	Ascencio *et al.*, 1993. Ruiz-Bustos *et al*., 2000. Utt *et al*., 2001.
Plasminogen & Plasmin	42, 57 and 58.9 kDa proteins	Pantzar *et al.,* 1998, Ringner *et al.,*
Fibronectin and Fibrinogen	Not Identified	Trust *et al.,* 1991.
Vitronectin	57kDa protein	Pantzar *et al.,* 1998.
Collagen IV	Not Identified	Trust *et al.,* 1991. Khin *et al.,* 1996.

(Table 1.6. Adapted from Dubreuil *et al.,* 2002).

(1.12) Implications for therapy

The ECM binding bacterial adhesins can be used as vaccine candidates in an anti-adhesin vaccine. With the increasing prevalence of antibiotic-resistant strains of bacteria, this new approach could provide a promising alternative treatment. Potential targets for the development of new strategies could include subunit vaccines or receptin blockers. These strategies could however be complicated by the fact that environmental factors can affect expression of these genes or by the fact that not all adhesin genes are present in all strains. Because microorganisms can have several mechanisms of adhesion to colonize and infect host tissues, anti adhesion drugs may have to be composed of a cocktail of inhibitors. Targeting therapy against microbial interactions with ECM components could also prevent deep tissue infections as it is believed that *H. pylori* gain access to the subepithelial layer by means of ECM receptins on their surface.

For *H. pylori*, interactions with certain host ECM proteins are of high affinity and cannot be ignored as possible pathogenicity attributes. When adherent to animal cells or tissues bacteria acquire greater resistance to normal immune responses and antibiotics and are better able to gain nutrients. The possibility that a bacterium can coat itself in ECM proteins to evade immune recognition initiates and permits lifelong infection. It has been proposed that interactions of *H. pylori* and ECM proteins represent a mechanism of adherence to gastric tissue thereby playing a role in development of chronic B gastritis and peptic ulcer disease (Ringner *et al.,* 1994). Moreover, the iron acquisition system of *H. pylori* by using the human lactoferrin receptor system may play a role in the virulence of *H. pylori* infection (Husson *et al.,* 1993).

For certain microorganisms receptins binding to host proteins have been shown to contribute to virulence in animal models. *Staphylococcus aureus* produces a variety of cell wall associated proteins that interact with ECM proteins of the host. *S. aureus* binding ECM binding proteins such as fibronectin, fibrinogen and collagen have been investigated as components of vaccines to protect against *S. aureus* infections in laboratory animals. Mamo *et al* (2000) reported that mice vaccinated with a combination of two *S. aureus* antigens consisting of a recombinant collagen binding protein (CnBP) and alpha-toxoid were significantly protected from intramammary challenge infection with *S. aureus.* The average number of bacteria recovered from the glands of mice vaccinated with a combination of CnBP/alpha-toxoid was significantly lower compared to the average number of bacteria recovered from the glands of mice vaccinated with only CnBP or alpha-toxoid or controls.

Schennings *et al* (1993) prepared a fusion protein by cloning the *lacZ* gene encoding beta-galactosidase(gal) in frame with a region of the *S. aureus* fibronectin binding protein *fnbA*. Rats were immunized with the gal-FnBP in Freund's adjuvant and the resulting antibodies blocked the *in vitro* binding of *S. aureus* to immobilised fibronectin.

Mice immunised with gal-FnBP (with Freund's adjuvant) showed fewer cases of severe mastitis than control mice (immunized with adjuvant alone) and fewer bacteria were recovered from the mammary glands of vaccines than from those of control mice (Mamo *et al.*, 1994).

Another study revealed that heparin as well as polysulphated agents, inhibited binding of fibronectin, vitronectin and lactoferrin by *S. aureus* (Pascu *et al.,* 1995). These could provide alternatives to antibiotic therapy for treatment of *S. aureus* infected wounds and superficial infections.

Another benefit of understanding microbial interactions with the ECM could prove useful in the area of biomaterials where devices are implanted into patients. Infection of these biomaterials can give rise to serious complications especially in tissue penetrating devices. Host proteins absorb onto the biomaterial surface and when bacteria come into contact with the surface they can bind to the adsorbed proteins. Fibronectin and fibrinogen have been proposed to mediate adhesion of bacteria to these devices (Vaudaux *et al.,* 1993 and Herman *et al.,* 1991) as well as vitronectin (Fabrizus-Homan & Cooper, 1991). Treatment of these infections has been reported to be almost impossible (Gristina, 1987). Therefore preventative measures that block adhesion may be a useful tool to overcome these problems.

Although this thesis has mainly focused on pathogenesis of ECM binding, it is worth noting that many probiotic bacteria also bind to the ECM and a lot of research has been carried out on this area also to date. For example Lactobacilli bind to immobilised collagen and fibronectin (Lorca *et al.,* 2002) and Styriak *et al* (2003) report probiotic bacteria binding to ECM molecules. Probiotics have many beneficial effects in various food matrices and milks as well as having a role in the prevention of gastrointestinal infections (Nemcova *et al.,* 1998, Alvarez-Olmos & Oberhelman, 2001). Probiotic bacteria have the potential to prevent attachment of pathogens such as *E. coli* and *H. pylori* to mucosal surfaces by colonising the surfaces and blocking attachment (Lee *et al.,* 2000, Mukai e*t al.,* 2002). As probiotic strains bind the ECM, they should be able to compete with pathogens for the same receptors and to occupy their binding sites (Neeser *et al.,* 2000).This research into the area of probiotic bacteria interacting with ECM molecules could prove useful for probiotic applications but also in the treatment or prevention of infectious gastrointestinal bacteria for example *H. pylori*.

(1.13) Aims and objectives of project.

This project attempts to identify collagen binding adhesins on *Helicobacter pylori.* Such adhesins are possible targets for therapeutic intervention. For example ECM binding proteins may represent potential targets for the development of novel antimicrobial agents, including vaccines and adhesion blockers. Despite indications of specific binding no Collagen IV binding protein has been identified in *H. pylori* to date.

The potential benefits of identifying the Collagen receptin in *H. pylori* are many:

- Interactions of bacteria with the ECM are thought to be of pathogenic significance, therefore possibly adding to the understanding of pathogenesis.
- Knowledge of the molecular basis of adherence.
- Aid in the development of novel antimicrobial therapies, including vaccines.
- Binding to Collagen may equip the bacterium with a mode of immune system evasion, as well as contributing to deep tissue invasion and persistence in the host.

The approach adopted in this work was to use the ReTagging Method in order to isolate *Helicobacter pylori* collagen IV binding proteins. Binding of *H. pylori* to ECM proteins was first confirmed using a bacterial overlay dot blot method. Once this was confirmed a ReTagging Method was employed in an attempt to identify the collagen binding protein. A variety of methods of fractionation of *H. pylori* proteins was examined in an attempt to circumvent difficulties with non specific binding.

Finally an *H. pylori* outer membrane fraction was probed using a receptor overlay immunoblot technique in a further attempt to identify a collagen IV receptin.

Chapter 2

Materials & Methods

(2.0) Materials and Methods

(2.1) Reagents

Bovine serum albumin, acrylamide:bisacrylamide (29:1), ammonium persulphate, β-mercaptoethanol, sodium chloride, sodium hydroxide, magnesium chloride, potassium chloride, Tris(hydroxylmethyl)aminomethane (Tris), glycine, TEMED, Tween-20, Dimethyl sulfoxide and sodium dodecyl sulphate were obtained from Sigma Aldrich. Brain Heart Infusion Medium was obtained from GIBCO BRL (Life Technologies, Scotland). Ethanol, methanol, glacial acetic acid, orthophosphoric acid, hydrochloric acid and glycerol were obtained from BDH Ltd. (Poole, Dorset, UK).
All buffer reagents for SDS PAGE were prepared in deionised water (Elga Prima reverse osmosis).

(2.2) SDS Polyacrylamide Gel Electrophoresis and Western Immunoblotting

(2.2.1) Sample and Molecular weight standards preparation

Protein samples were resuspended in 1x reducing sample buffer for loading onto gels (see Apendix A) unless otherwise stated. Bacterial pellets were resuspended in 2x reducing or non reducing sample buffer unless otherwise stated. For concentration of proteins, a TCA precipitation method was used. TCA was added to the protein sample to 13% of the final volume and stood at 4˚C for at least 30 minutes. The precipitated protein was removed by centrifugation at 15,000 rpm at 4˚C for 5 mins, and the supernatant was removed by aspiration and discarded. The pellet containing the precipitated protein was resuspended in 2x reducing sample buffer. All protein samples,

along with molecular weight standards, were boiled for 5 minutes at 100˚C, prior to loading onto an SDS gel.

(2.2.2) SDS-PAGE

An ATTO system was used for SDS-PAGE gels (ATTO Corporation, Japan). Electrophoresis was carried out using a Consort electrophoresis power supply unit. Proteins were separated on reducing gels, with a discontinuous buffer system using the method of Laemmli (1970) as adopted by Sambrook *et al* (1989). Resolving and stacking acrylamide gels were prepared to the percentage of required acrylamide as indicated in Tables 2.1 and 2.2 below.

Either APS or TEMED were added last for polymerisation of the gel. Electrophoresis was carried out at 25mA per gel for approximately 1.5 hours, or until the tracking dye had reached 5mm above the gel base, at which stage electrophoresis was terminated.

Table 2.1: Solutions for preparing Resolving Gels for SDS-Polyacrylamide Gel Electrophoresis.

	% Acrylamide		
Component	**8%**	**10%**	**12%**
H_2O	9.3ml	7.9ml	6.6ml
Acryl/Bis 30%	5.3ml	6.7ml	8.0ml
1.5M Tris pH 8.8	5.0ml	5.0ml	5.0ml
10% SDS	200µl	200µl	200µl
10% Ammonium Persulfate	200µl	200µl	200µl

TEMED	12µl	8µl	8µl

Table 2.2: Solutions for preparing stacking gels for SDS-Polyacrylamide Gel Electrophoresis.

Component	**Volume**
H_2O	3.4ml
Acryl/Bis 30%	830µl
1M Tris pH 6.8	630µl
10% SDS	50µl
10% Ammonium Persulfate	50µl
TEMED	5µl

(2.2.3) Western Immunoblotting

Western Immunoblotting was carried out using the semi-dry method of transfer of electrophoresed proteins to immobilising membranes as described by Towbin (1979) and was performed using an ATTO semi-dry transfer system (ATTO Medical Supplies , Japan). Prior to transfer Polyvinylidene fluoride (PVDF) transfer membrane of dimensions 6.5 x 9 cm was wetted in methanol for 10-15 seconds and then equilibrated in transfer buffer (See Apendix A for composition). Whatmann 3 MM filter paper was also soaked in transfer buffer prior to semi dry blot sandwich construction, which was assembled in the order of cathode, filter paper, PVDF membrane, acylamide gel, filter paper and finally anode. Electrophoretic transfer was performed at 100 mA per gel for 60 minutes. Unless biotinylated molecular weight markers or prestained markers were included, the lane containing the molecular weight markers was stained with

Coomassie Blue R-250, followed by destaining in a destain solution containing methanol, water and acetic acid (See Apendix A for composition).

(2.2.4) Immunoblot detection and development

Following semi-dry transfer, non-specific sites on the PVDF membrane were blocked by incubation with freshly prepared TBS buffer containing 5% skimmed dried milk (Blocking solution) for one hour with gentle rolling on a roller mixer (Stuart Scientific, UK). Blots were incubated for 2 hours with primary antibody diluted 1:1000 in antibody diluent. Following incubation with primary antibody, membranes were washed 3 times, for 10 minutes each with TBS-Tween (0.1%) washing buffer. Membranes were then incubated with the relevant horseradish peroxidase-conjugated secondary antibody in diluent solution (See Apendix A) for 1 hour at room temperature on a roller mixer (SRT6, Stuart Scientific). For detection of biotinylated proteins, membranes were incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:1000 in Antibody diluent solution. Following incubation with secondary antibody, blots were finally washed 3 times for 20 minutes each, with TBS-Tween (0.1%) pH 7.4 washing buffer.

Development of Immunoblots was carried out using the enhanced chemiluminescence (ECL) method. Membranes were incubated for 1 minute in ECL Western Blotting Detection Reagents (Amersham). The membrane was removed and placed between acetate sheets, which were then exposed to Kodak film for the appropriate time period (range 1 min to 10 mins). Exposed films were developed using an automatic developer (CURIX 60, AGFA, Type 9642/100/140, Agfa-Gevaert AG, Munich, Germany).

(2.3) Binding of Helicobacter pylori to ECM proteins using a Dot Blot Method

(2.3.1) Bacterial strains and growth conditions

Helicobacter pylori SS1 were grown for 48 hours on Columbia agar plates supplemented with 10% horse blood serum at 37°C in a microaerobic (Anoxomat) atmosphere generated using a MART Microbiology Anoxomat system (Lichtenvoorde, the Netherlands). For liquid culture, strains were grown in Brucella broth or Brain Heart Infusion medium supplemented with 7% fetal bovine serum, in a microaerobic atmosphere, at 37˚C and 120rpm in an orbital incubator (S1 50, Stuart Scientific). For iron reduced conditions 100μm desferroxamine was added to the medium.

(2.3.2) Labelling H. Pylori with FITC (fluorescein isothiocyanate)

2 days after inoculation, bacteria were removed from the agar plate with a sterile loop and resuspended in 1ml 0.15M NaCl, 0.1M sodium carbonate, pH 9.0, by gentle pipetting. 10μl of a 10mg/ml FITC solution in dimethyl sulfoxide (freshly prepared) was added into the cell culture suspension and incubated for 30 minutes at room temperature in the dark. Bacteria were centrifuged at 3000g (5700rpm) for 5 minutes and resuspended in 1ml PBS by gentle pipetting. The wash cycle was repeated 3 times. Aliquots were used immediately or stored at -20°C for further use.

(2.3.3) Extracellular Matrix (ECM) Proteins

ECM proteins used were Laminin (Mouse 1, Cultrex, Trevigene), Fibronectin (from Human Plasma, Sigma), Collagen IV (from Engelbreth Holm Swarm Sarcoma, Sigma),

Lactoferrin (Sigma), HSA (Sigma), Hemin, HSA:Hemin complex. BSA (Sigma) was included as a negative control.

(2.3.4) Preparation of HSA:Hemin Complex

Hemin was dissolved at 10mM in DMSO immediately before use in a foil wrapped microcentrifuge tube. Hemin and HSA solutions were mixed to give a molar ratio of 1.1 : 1.0. The mixture was incubated with constant rotation, at room temperature overnight for at least 12 hours. The tube was protected from light.

(2.3.5) Bacterial Overlay Method (Dot Blot)

This overlay method was performed essentially as described by Ruhl *et al.,* (2000) and adapted by Walz *et al.,* (2005) where fluorescence labelled bacteria were used as probes. PVDF membranes were first wet in methanol followed by TBS buffer. Membranes were then transferred to a moist chamber to prevent drying. PVDF membranes were spotted with 5µl volumes of ECM proteins at 1mg/ml. Membranes were blocked for one hour at room temperature in Blocking buffer (50mM Tris pH7.5; 150mM NaCl, containing 5% BSA, 1mM $MgCl_2$, 1mM $CaCl_2$). 1ml of fluorescein labelled bacterial suspension was added to the membrane and sealed in a bag. The overlays were incubated with labelled bacteria for 30 minutes at 4°C in the dark without mixing, and washed on a rotary shaker (Stuart Scientific, UK) for 3 x 5 minutes in TBS-T containing 1mM $MgCl_2$, 1mM $CaCl_2$. The membranes were protected from light throughout. The fluorescence of adherent bacteria was visualised using a UV light source (10 W).

(2.4) Identification of Collagen IV binding proteins using Sulfo-SBED Biotin Label Transfer Kit

In this work we attempt to identify the collagen IV receptor by use of direct binding measurements and Tagging Technology. The tagging method was adapted from Ilver *et al* 1998, using a Receptor Activity- Directed Affinity Tagging method called "Retagging" (Figure 2.1). This technique employs an ECM protein covalently attached to a multifunctional crosslinker, which, following UV irradiation and reduction, transfers a biotin tag to the cognate adhesin (or receptin) on *H. pylori*. The adhesin is then identified by Western Blot analysis using streptavidin tagged Horseradish peroxidase (HRP). After detergent solubilisation, the adhesin may be isolated from cell suspensions by use of streptavidin coated magnetic beads. The beads provide a method to affinity purify adhesion proteins labelled by the tagging technique. A kit for retagging of proteins is commercially available from Pierce Chemicals.

Thus, in step 1 collagen is covalently attached to the tag by modification of a surface amino acid on collagen with the tag's N-hydroxysuccinimide (NHS) moiety. NHS esters react with primary amines on protein side chains at pH 7-9 to form covalent amide bonds. In step 2, collagen with its attached tag is incubated with *H. pylori* cells where the collagen binds to the *H. pylori* adhesin (putative). In step 3 exposure to UV light causes activation of the aryl azide group causing it to react with amino acid residues on proteins adjacent to the bound collagen. In step 4 Collagen is removed by reduction of a disulfide bond that links the NHS moiety of the tag to the modified collagen amino acid. The result is that the protein adjacent to bound collagen becomes labelled with a biotin tag. This protein is assumed to be the receptin/adhesin for collagen. The biotin labelled protein may subsequently be identified following separation by electrophoresis (or a similar method) and probing with sterptavidin HRP.

The numerous steps in this tagging method and the requirement to carry out a series of tagging reactions in sequence can potentially give rise to problems and great care is required in preparation and handling of reagents.

(2.4.1) Labelling of Bait Protein usingProfound Sulfo-SBED Biotin Label Transfer Kit (Pierce)

The manufacturer's instructions for use of this labelling kit were followed. A brief description of the process is given below.

Collagen IV (from Engelbreth Holm Swarm Sarcoma, Sigma) was diluted to give 200µg/ml in PBS (Pierce Kit) adjusted to pH 8.0. The collagen was labelled using manufacturers guidelines for the kit. Sulfo-N-hydroxysuccinimidyl-2-(6-[biotinamido]-2-(p-azido benzamido)-hexanoamido) ethyl-1,3'-dithioproprionate (Sulfo –SBED) labelled protein was divided into single-use aliquots and stored protected from light at -80°C. Label incorporation was assessed by performing a dot blot using Streptavidin-HRP as a probe. Briefly, a few microlitres of labelled collagen were spotted onto one side of a PVDF membrane along with unlabelled collagen for use as a control. The membrane was blocked by washing with a skimmed milk powder solution (PBS containing Marvel, 5%, w/v) and probed with streptavidin HRP which reacts specifically with biotin.

(2.4.2) Identification of Collagen Binding Proteins from H. pylori using ProFound Sulfo SBED Biotin Label Transfer Kit

H. pylori SS1Rf were grown for 48 hrs on Columbia agar plates. Bacteria were harvested from the agar plate and resuspended in 1 ml PBS (supplied with Pierce Kit), washed twice in PBS and adjusted to an OD_{600} of 1.0. SBED-labelled protein was added

to the bacterial suspension and incubated under microaerophillic conditions for 1hr at room temperature and protected from light. This exposure initiated the reaction between the aryl azide moiety of the tag and the putative collagen receptor(s).

The suspension of bacteria with the collagen-crosslinker conjugate was mixed every 15 minutes. Samples were transferred to a 96 well plate and the SBED conjugate was photoactivated using a UV light source for 10 minutes at a distance of 5 cm. Samples were removed to minifuge tubes and centrifuged for 3 minutes at top speed. Pellets were resuspended in sample buffer and

DTT at a concentration of 100mM was added to samples that required complete disulfide bond reduction. Samples were boiled for 5 minutes at 100°C. Proteins were separated by electrophoresis and probed with streptavidin-HRP. Each sample was loaded on the electrophoresis gel under reducing conditions. A control for each sample was loaded under non reducing conditions. Reducing conditions (the final concentration of DTT must be 100mM for complete disulfide bond reduction) resulted in the cleavage of the disulfide bond on the crossslinker.

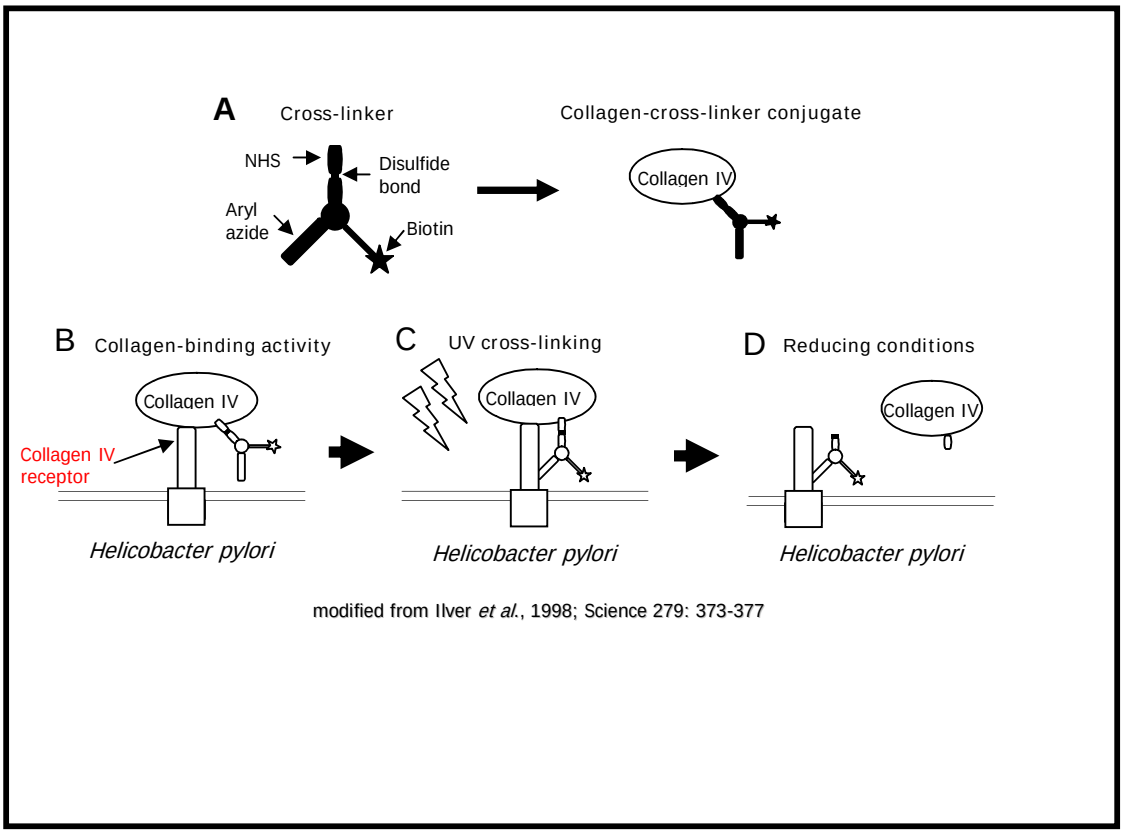


Figure 2.1: Overview of Retagging Method for purification of collagen IV binding protein. (A) The Sulfo-SBED crosslinker has an NHS group for conjugation to collagen IV, a central disulfide bond, a photoreactive arylazide group and a biotin side group. (B) The Collagen labelled with crosslinker is incubated with *Helicobacter pylori*, bringing the crosslinker close to the adhesin protein. (C) The cells are exposed to UV light, causing the photoreactive sidegroup to form a covalent bond to the adhesin protein. (D) Reducing conditions result in the cleavage of the disulfide bond in the cross linker. Collagen IV is subsequently released and washed away. The end result is that the adhesin is labelled with biotin.

(2.4.3) SDS - Polyacrylamide gel electerophoresis and Western Blot Analysis

Samples were applied to a 10% polyacrylamide gel and transferred to a PVDF membrane. Membranes were blocked in 5% Skimmed Milk Powder in TBS and incubated with Streptavidin HRP (1:2000 dilution) for at least 1 hour.

(2.4.4) Purification of biotinylated proteins using streptavidin coated magnetic beads

MyOne Streptavidin T1 Dynabeads (Invitrogen), were prepared as described by the manufacturer. Beads were incubated with biotinylated proteins in Lane Marker Reducing Sample Buffer (Thermo Scientific) for 30 minutes at room temperature using gentle rotation. Protein coated beads were separated using a magnetic rack for 2-3 minutes and beads were washed 5 times in PBS and resuspended in sample buffer. The biotin-streptavidin bond was broken by boiling the samples for 5 minutes. Samples were loaded on a 12% polyacrylamide gel and transferred to a PVDF membrane.

(2.4.5) Coomassie Blue G-250 staining (for Mass Spectrometric analysis)

The SDS gel was rocked overnight in fixation solvent (50 % methanol, 2 % phosphoric acid, 85 %), washed 3 times in 500 ml water followed by shaking for one hour in incubation solvent (34% methanol, 2% phosphoric acid, 85%, 17% ammonium sulphate). Coomassie G-250 (Bio-rad) solution was added to the incubation solvent (0.025% Coomassie G-250 in 20ml methanol) to give 0.25g/L incubation solvent. Staining was for 2-3days, followed by washing in water then washing twice in 25% methanol. The Gel was washed in water and sealed at stored at 4 ºC. The gel was visualised and stained protein bands were excised for mass spectrometric analysis.

(2.4.6) Purification of H. pylori Catalase

Helicobacter pylori SS1 were grown for 48 hours on Columbia agar plates supplemented with 10% horse blood serum in a microaerobic (Anoxomat) atmosphere at 37°C. Bacteria were harvested by suspension in 25mM sodium phosphate buffer pH 7.5. After centrifugation the pellets were resuspended in buffer and sonicated for 8 x 30 second intervals, keeping samples on ice throughout. Cellular material was removed by centrifugation at 10,000g for 5 minutes at 4°C. The supernatant was collected and filtered through a 0.22µm-pore-size filter. Extracts were kept on ice throughout.

The filtrate was loaded onto an ion exchange column (Resource S, 1ml, GE Healthcare) equilibrated in 25mM sodium phosphate buffer, pH 7.5.

Proteins were eluted by gradient of 0-1M NaCl in 25mM sodium phosphate buffer pH 7.5. Catalase positive fractions were selected by checking oxygen reducing activity in the presence of 3% H_20_2. The purity of catalase was confirmed by Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS PAGE,12% polyacrylamide).

The fractions collected were stored at 4ºC and were stable at this temperature for several days.

(2.4.7) Gel Filtration analysis of Collagen and H. pylori Catalase to check for binding activity

Gel filtration was performed using a Superdex 200 gel filtration column equilibrated in PBS buffer pH 8.0 using an AKTA FPLC system (Amersham Pharmacia, UK). A standard curve was constructed using molecular weight standards. Collagen and *H. pylori* catalase were applied separately to the column and the volume at which each protein was eluted was noted. A collagen and *H. pylori* catalase mixture, incubated for

half an hour on ice, was applied to the column and elution point noted. The flow rate in all cases was 0.4ml/min.

(2.4.8) Binding assay to test for Collagen IV binding to H. pylori Catalase

A 96 well plate was coated with collagen IV at concentrations of 100, 50 and 10 µg/ml and left overnight at 4°C. PBS was used as a control. Excess liquid was removed from the plates and they were blocked for one hour in 5% BSA at room temperature. Excess liquid was removed and 100µl *H. pylori* catalase was added and incubated for one hour at room temperature. The plate was washed with PBS buffer three times. 100 µl PBS was added and 3% hydrogen peroxide was used to test for catalase activity.

(2.5) Identification of Collagen IV binding proteins using Immunoblot Methods.

(2.5.1) Preparation of H. pylori Outer Membrane Protein Fraction

Helicobacter pylori SS1 were grown for 48 hours on Columbia agar plates supplemented with 10% horse blood serum in a microaerobic (Anoxomat) atmosphere at 37°C. Cells were harvested and washed three times (4,000rpm, 10 minutes) with 20mM Tris-HCl, pH 8.0. Cells were sonicated on ice (8 times for 30 seconds, intervals of 1 minute). Unbroken cells and debris were removed by centrifugation (4,000rpm, 10 minutes, 4°C). The supernatants were ultracentrifuged at 45,000g for 20 minutes at 4°C, and pellets were washed three times with 20mM Tris-HCl, pH 8.0. A bicinchoninic acid (BCA) protein assay (Thermo Scientific Pierce) was carried out to determine the protein concentration. The pellets were resuspended in 0.6% N-lauroyl sarcosine-20mM Tris-HCl, pH 8.0 to a final concentration of 1mg of protein/ml and incubated at room

temperature for 20 minutes. Outer membrane proteins were collected by ultracentrifugation at 45,000g for 20 minutes at 4°C.

The sarcosine insoluble pellets were dissolved in 20mM Tris-HCl, pH 8.0 for protein determination. Supernatants containing sarcosine soluble fraction were retained.

For SDS-PAGE, samples were TCA precipitated to yield a concentration of 100µg of protein per lane. The OMP fraction is insoluble in sarcosine, whereas the cytoplasmic fraction is totally soluble. 100µg of each protein fraction, as well as a whole cell extract were TCA precipitated and resuspended in 2X reducing sample buffer.

(2.5.2) Outer Membrane Protein immunoblot

100µg of Outer membrane proteins were TCA precipitated and resuspended in 2X reducing sample buffer. Samples were boiled for 5 minutes and loaded onto a 12% polyacrylamide gel and run for 1 hour and 20 minutes at 25mA. Proteins were transferred to PVDF membrane for 1 hour at 100mA. Membranes were blocked for one hour in 5% skimmed milk powder in TBS-T. Membranes were then incubated with Collagen IV, X-linked Collagen IV or X-linked Fibronectin, at concentrations of 20µg/ml for 2 hours. Collagen IV and X-linked Collagen IV were diluted in PBS pH 8.0, and Fibronectin was diluted in PBS, pH 7.2. Membranes were washed for 3 x 5 minutes in TBS-T. Membranes treated with X-linked Collagen and X-linked Fibronectin were probed with Streptavidin-HRP (Pierce) (1:1000 dilution), for one hour, and washed for 3 x 20 minutes with TBS-T. Membranes treated with Collagen IV were probed with Rabbit Anti Collagen IV (Acris Antibodies) for 2 hours, washed 3 x 20 minutes with TBS-T, and probed for one hour with Goat anti Rabbit HRP (Santa Cruz Antibodies). It was expected that collagen would bind directly to its *H. pylori* adhesin on the PVDF membrane and that this interaction could be observed by probing

with antibodies. We hoped that by using this method we could overcome some of the possible problems encountered with the crosslinker such as non-specific binding, and the potential of the crosslinker to attach to proteins other than the collagen adhesin protein.

Chapter 3
Results

(3.0) Results

(3.1) Introduction

Various investigations have revealed that many microorganisms interact with the mammalian extracellular matrix, and have shown the importance of these interactions for pathogenicity. *H. pylori* is known to interact with a number of Extracellular Matrix (ECM) components such as laminin and plasminogen. Some reports have suggested that collagen IV interacts with *H. pylori* but an adhesin for this ECM protein has not been identified. Identification of a collagen IV receptor could yield benefits for the treatment of *H. pylori* infection. For example, such receptors may represent potential targets for the development of novel antimicrobial agents, including vaccines and adhesion blockers.

(3.2) Binding of Collagen and ECM proteins to H. pylori cells.

(3.2.1) Labelling H. pylori with FITC (fluorescein isothiocyanate)

H. pylori was labelled using fluorescein isothiocyanate (FITC) as described in Materials and Methods (Section 2.3.2). Labelling was verified by visualisation of green fluorescence of cells by microscopy (data not shown).

(3.2.2) Bacterial Overlay Method (Dot Blot)

H. pylori labelled with FITC were applied to PVDF membranes spotted with ECM proteins. The ECM proteins used were laminin, fibronectin, collagen IV, lactoferrin, HSA:hemin complex, HSA and hemin. It is important to note that hemin is dark in colour and could therefore be seen as a dark spot after applying onto the membrane.

BSA was used as a control. In all cases binding was observed to laminin, fibronectin and collagen IV(Figures 3.1, 3.2, & 3.3).

(3.2.3) Assessment of H. pylori Binding to ECM Proteins under Different Growth Conditions.

Changes in growth conditions are known to affect expression of adhesins. We examined collagen binding under different growth conditions.

Binding of *H. pylori* was assessed under the following growth conditions - directly from agar plates (Figure 3.1), from liquid culture (Figure 3.2.) and from liquid culture under iron reduced conditions using the iron chelator desferroxamine (See Fig. 3.3).

Binding of *H. pylori* to ECM proteins was observed under the three growth conditions examined. This experiment clearly showed binding of *H. pylori* to the ECM proteins collagen IV, laminin and fibronectin. Under the same conditions *H. pylori* did not bind to lactoferrin, HSA, HSA:hemin complex hemin, or the control BSA.

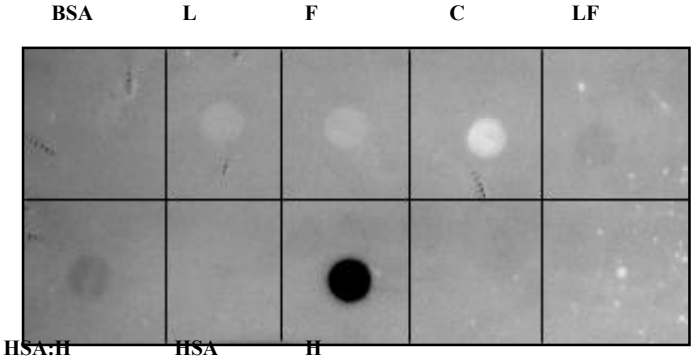


Figure 3.1. Bacterial Overlay: The adhesion of fluorescein-5-isothiocyanate (FITC) – labelled *H. pylori* to ECM proteins. ECM proteins were spotted onto PVDF membranes and overlaid with FITC labelled bacteria. Adherent bacteria were visualised under UV light. This shows *H. pylori* labelled from an agar plate. **L** = Laminin, **F** = Fibronectin, **C** = Collagen IV, **LF** = Lactoferrin, **HSA:H** = HSA Hemin complex, **HSA** = Human Serum Albumin, **H** = Hemin, and **BSA** was used as the control. The green fluorescence is observed as white spots on the membranes shown above.

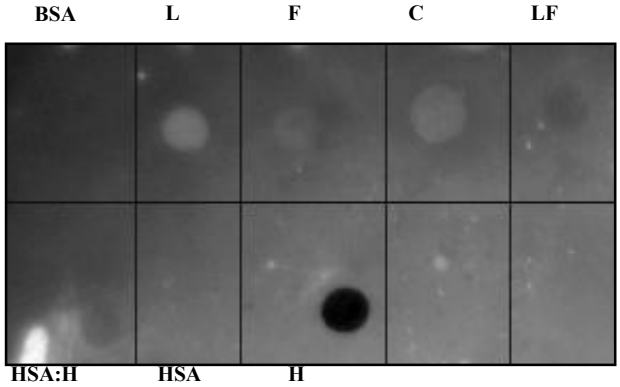


Figure 3.2. Bacterial Overlay: The adhesion of fluorescein-5-isothiocyanate (FITC) – labelled *H. pylori* to ECM proteins. ECM proteins were spotted onto PVDF membranes and overlaid with FITC labelled bacteria. Adherent bacteria were visualised under UV light. This shows labelled *H. pylori* from liquid culture.Where **L** = Laminin, **F** = Fibronectin, **C** = Collagen IV, **LF** = Lactoferrin, **HSA:H** = HSA Hemin complex, **HSA** = Human Serum Albumin, **H** = Hemin, and **BSA** was used as the control. The green fluorescence is observed as white spots on the membranes shown above.

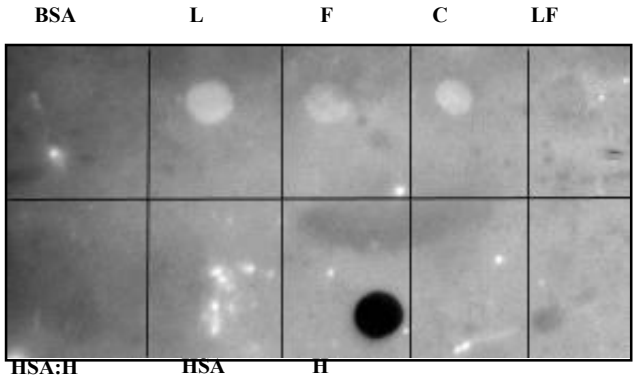


Figure 3.3. Bacterial Overlay: The adhesion of fluorescein-5-isothiocyanate (FITC) – labelled *H. pylori* to ECM proteins. ECM proteins were spotted onto PVDF membranes and overlaid with FITC labelled bacteria. Adherent bacteria were visualised under UV light. This shows labelled *H. pylori* from liquid culture under iron reduced conditions. Where **L** = Laminin, **F** = Fibronectin, **C** = Collagen IV, **LF** = Lactoferrin, **HSA:H** = HSA Hemin complex, **HSA** = Human Serum Albumin, **H** = Hemin, and **BSA** was used as the control. The green fluorescence is observed as white spots on the membranes shown above.

(3.3) Isolation of Collagen IV Adhesin by Retagging

Having established collagen binding to *H. pylori* the retagging technology was used in attempt to isolate the cell surface adhesion responsible for collagen binding.

(3.3.1) Collagen Labelling using Profound Sulfo-SBED Biotin Label

The labelling of Collagen IV with crosslinker was assessed by performing a dot blot using Streptavidin-HRP as a probe (Figure 3.4.). Figure 3.4(B)clearly shows the biotin was detected in the labelled collagen-crosslinker conjugate. Figure 3.4(A) (control) shows no reaction as expected. Thus, it was clear that the crosslinker had been successfully incorporated onto collagen IV.

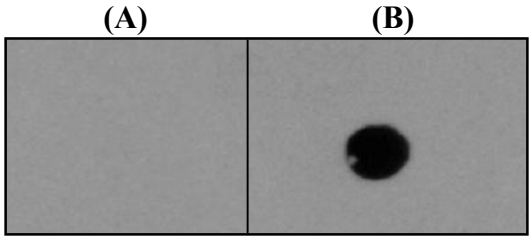


Figure 3.4. Construction of crosslinker-collagen conjugate probed with streptavidin HRP. **(A)** Unlabelled Collagen **(B)** Sulfo-SBED tagged Collagen. The collagen with its covalently attached tag binds to streptavidin-HRP causing luminescence to be observed as a dark spot.

(3.3.2) Identification of Collagen Binding Proteins from H. pylori using ProFound Sulfo SBED Biotin Label Transfer Kit

Having verified that covalent modification of collagen was successful, direct labelling of the collagen receptor was attempted.

Initial attempts showed labelling of a number of biotin-tagged proteins detectable by Western Blot (Figure 3.5, lane 4). Non specific binding of Strepatavidin was observed in control experiments under non-reducing and reducing conditions (Figure 3.5, lanes 1 and 2). Streptavidin binding was observed in the presence of bound, tagged Collagen (Figure 3.5, lane 3). Removal of collagen under reducing conditions showed labelling of a large number of proteins (See Figure 3.5, lane 4). It was unlikely that all of these were collagen receptins and non-specific binding was suspected. Separation of these proteins was poor and did not allow ready identification of tagged proteins.

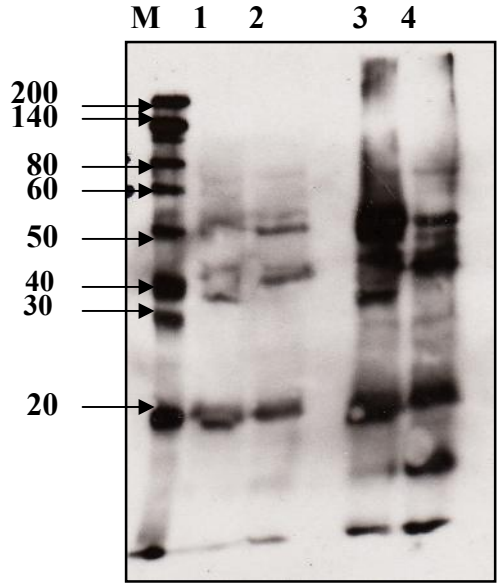


Figure 3.5. ReTagging Method showing Western blot analysis of biotinylated binding proteins. After Retagging, biotin tagged proteins were identified by SDS-PAGE and Streptavidin probed Wetern Blot. Lane **(M)** is the biotinylated protein molecular weight standards ladder. Lanes **(1)** and **(2)** are controls containing *H. pylori* alone where **(1)** is without reducing agent and **(2)** is in the presence of reducing agent. Lane **(3)** shows collagen crosslinking non-reducing, and **(4)** shows collagen crosslinking under reducing conditions and shows detection of putative biotin tagged collagen binding proteins.

To enhance the specificity of the procedure the amount of tagged collagen probe was increased and, in addition, a larger resolving gel was used to improve protein separation. Using this approach, labelling of a large number of *H. pylori* proteins was still observed (Figure 3.6 Lane 2). This experiment was repeated and gave the same pattern. The large number of proteins labelled seemed to indicate extensive non specific binding of streptavidin-HRP to *H. pylori* proteins.

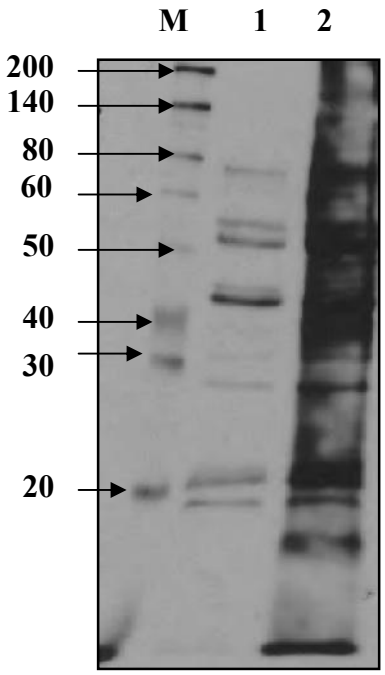


Figure 3.6. ReTagging Method showing Western blot analysis of biotinylated binding proteins. After Retagging, biotin tagged proteins were identified by SDS-PAGE and Streptavidin probed Wetern Blot. Lane **(1)** is a control containing *H. pylori* alone. Lane **(2)** shows detection of putative biotin tagged collagen binding proteins. Lane **(M)** is the biotinylated protein molecular weight standards ladder.

In order to further purify these potential proteins of interest from proteins that might be responsible for non-specific binding, magnetic beads were used to enrich or purify a biotin labelled protein fraction.

(3.3.3) Purification of Biotinylated Proteins using Streptavidin Coated Magnetic Beads.

Since the Western blot analysis showed up a large number of labelled proteins (section 3.3.2) it was necessary to attempt to further purify these proteins in order to identify collagen binding protein(s). It was possible, for example, that the crosslinker had bound non-specifically to other proteins in the immediate vicinity of the collagen IV binding protein or that some non specific binding of collagen to cell surface proteins was occurring. Streptavidin coated magnetic beads function as a method of direct isolation of biotinylated molecules. The beads exhibit low non-specific binding in the presence of complex biological samples such as cell lysates. These beads were used in order to enrich and affinity purify a biotinylated collagen adhesion fraction (see Section 2.4.4). Figure 3.7 shows SDS-PAGE analysis of the magnetic bead purification and the Western blot analysis is shown in Figure 3.8.

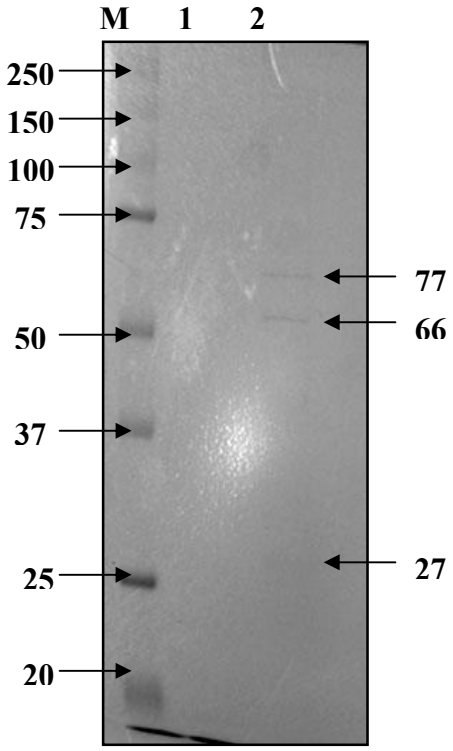


Figure 3.7. Retagging of the *H. pylori* Collagen IV binding protein – Magnetic bead purification (SDS-PAGE Analysis). After Retagging with crosslinker-collagen conjugate, biotin tagged proteins were visualised by SDS-PAGE after magnetic-bead-purification (Section 3.3.3). The figure shows a colloidal Coomassie stained gel where Lane 1 is the control and Lane 2 the protein fraction eluted from magnetic beads. Significant protein bands are shown at 77, 66 and 27kDa.

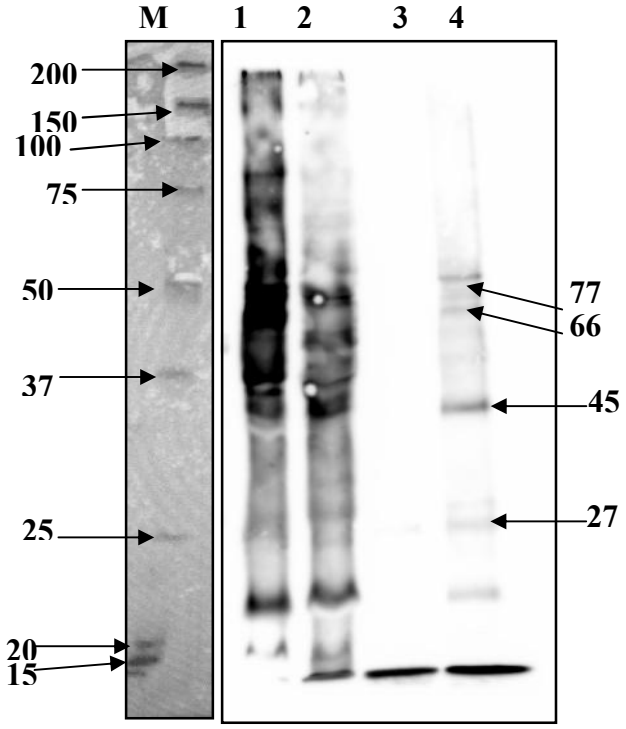


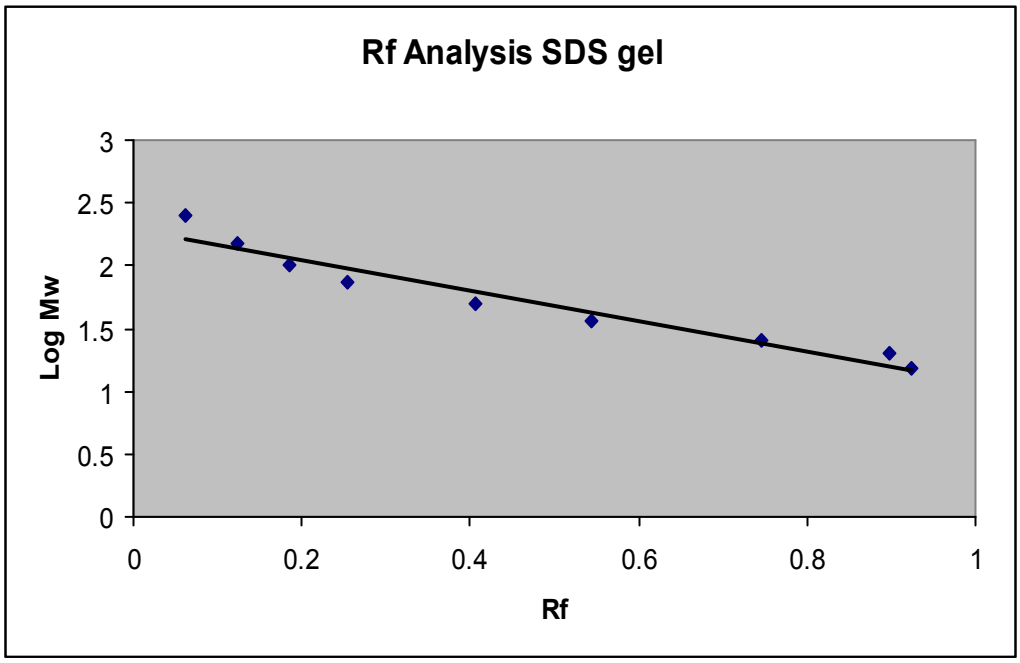
Figure 3.8. Retagging of the *H. pylori* Collagen IV binding protein-Magnetic bead purification (Western blot analysis). After Retagging with crosslinker-collagen conjugate, biotin tagged proteins were identified by western blotting as described earlier. Lanes **(1)** and **(2)** show blot analysis of biotinylated binding proteins non-reduced and reduced respectively. Lanes **(3)** and **(4)** show blot analysis of proteins eluted from Streptavidin coated beads where Lane 3 is the control and Lane 4 are the eluted proteins. Several bands were observed including three that corresponded with those observed by Coomassie staining at 77, 66 and 27 kDa. A prominent band at 45 kDa was present in the Western blot that was not seen by Coomassie staining. This is due to the greater sensitivity of Western blot staining.

The SDS gel showed labelling of a smaller number of proteins than was observed with Streptavidin interaction with less purified cell suspensions (Figure 3.5 & Figure 3.6). Major proteins at 77, 67 and 27 kDa were observed in the colloidal Coomassie stained gel (Figure 3.7). Proteins with the same molecular weight were observed in the Western Blot (Figure 3.8).

The approximate size of the proteins was calculated using Relative mobility - R_f analysis for both the SDS gel and Western Blot (Figure 3.9).

These results were reproduced in subsequent experiments. The protein bands at bands at sizes 77, 67 and 27 kDa were excised and analysed by Mass Spectrometry (MS) at The University of St Andrews, Fife, Scotland. The band at 27 kDa was contaminated with keratin and could not be identified. The pattern obtained in MS analysis identified the 77 kDa protein as *H. pylori* urease β subunit. This was interesting as urease β subunit is known to bind CD74 on MHC II and has been shown to bind mucins and some glycoproteins. The 67 kDa protein was identified by mass spectrometry as *H. pylori* catalase.

A



B

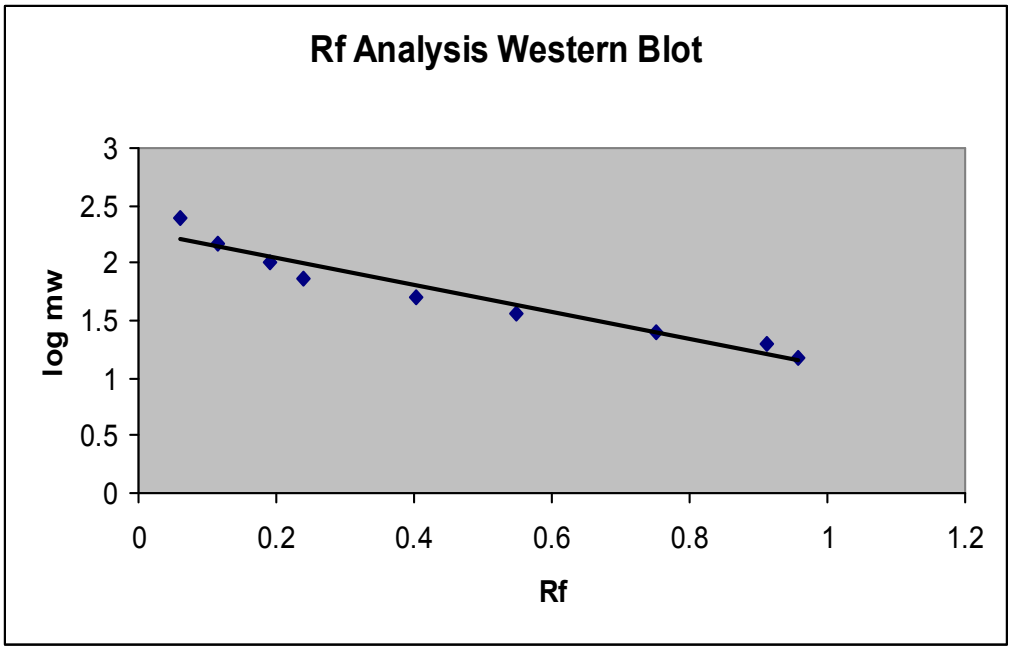


Figure 3.9. Graph of Relative Mobility (R_f) Analysis of *H. pylori* purified biontinylated proteins. Standard protein mobility data was used to prepare a graph of the relationship between the molecular weight of standard proteins and their R_f. Graph **A** shows the R_f analysis on the SDS Gel (Figure 3.7) and graph **B** shows the graph prepared by R_f analysis on the Western blot (from Figure 3.8). The molecular weights of unknown proteins were calculated using the equation: $\log MW = a + b * R_f$.

(3.4) Urease

Urease is the most abundant *H. pylori* protein representing 5-8% of total cell protein and is also associated with the cell surface. It was thought more likely that binding to this protein was non specific. Therefore, initial investigation instead focussed on catalase involvement in ECM binding. This finding seemed encouraging and was consistent with evidence in the literature that catalase was implicated in collagen binding.

(3.5) Purification of H. pylori Catalase

In order to examine collagen binding to catalase it was decided to purify the *H. pylori* catalase protein in order to carry out direct studies of collagen binding *in vitro*. Indication that catalase was functioning as an adhesin prompted us to attempt purification of this protein. There were several literature reports to support this finding and we therefore decided to further explore a collagen catalase interaction. A simple, one step, protocol for purification of catalase from *H. pylori* was previously described by Hazell *et al.,*(1991). Using this protocol *Helicobacter pylori* catalase was purified using FPLC Cation exchange chromatography. The eluate fractions showed a single peak of protein (Figure 3.10, peak B) and activity. Catalase activity was confirmed by checking for oxygen reducing activity using 3% H_20_2. The purity of the eluted catalase fractions was assessed by 12% SDS-PAGE and the proteins were visualised by Coomassie Brilliant Blue staining (Figure 3.11).

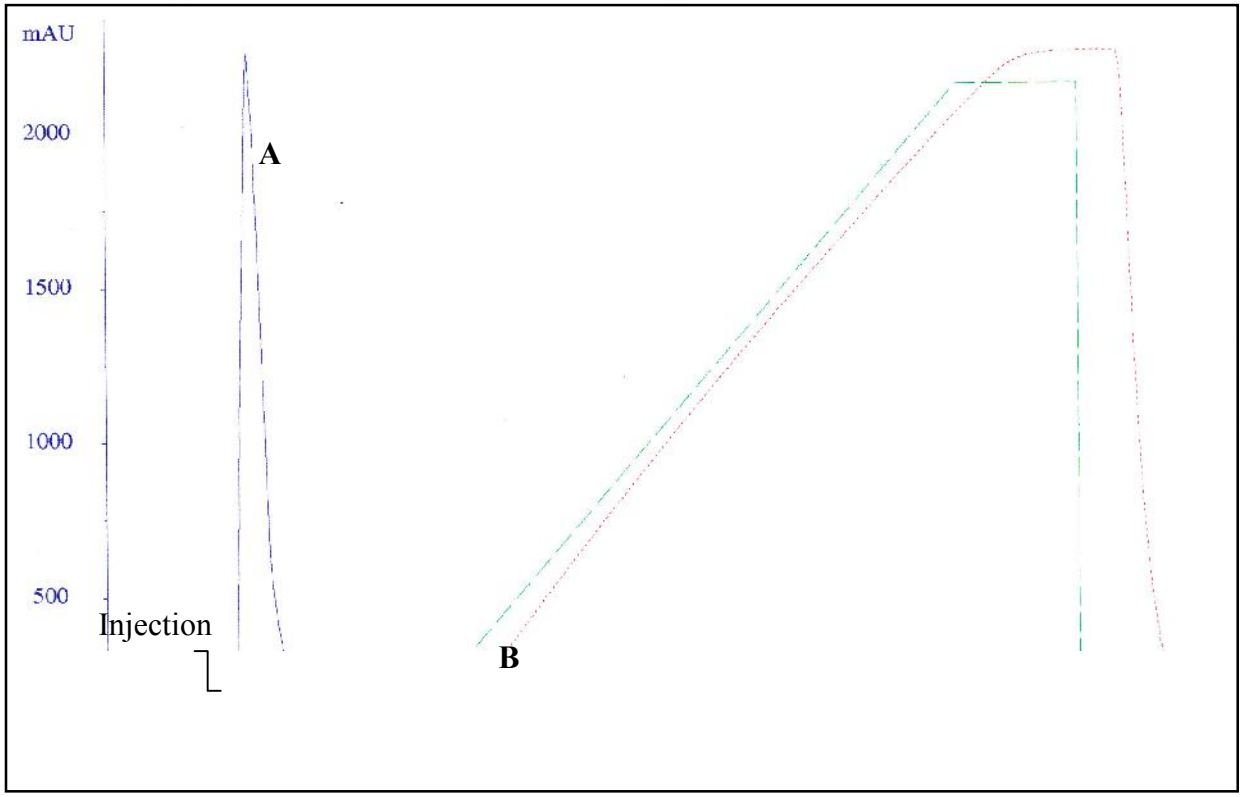


Figure 3.10. Elution profile for Purification of *H. pylori* Catalase by Ion Exchange Chromatography. A *H. pylori* cell lysate was applied to the cation exchange column and eluted by using a salt gradient of 0.1-1M NaCl. Two peaks were apparent on the chromatogram. Peak **A** represents the *H. pylori* flowthrough and **B** represents *H. pylori* catalase protein - fractions A8 –A10. The blue line represents the UV absorbance, the arrow represents the point of injection of the *H. pylori* cell lysate, the red dashed lines represent 1ml fractions collected after injection. The green line represents the salt concentration, and the red dotted line represents conductivity.

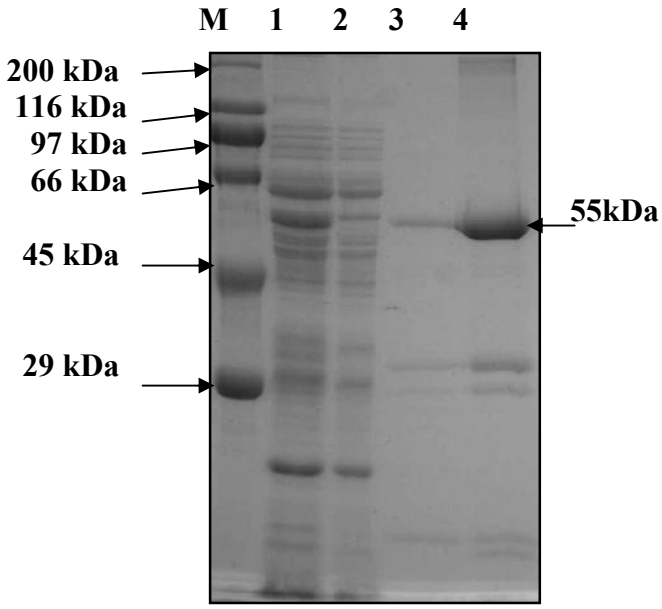


Figure 3.11. 12% SDS-page gel showing the purification of *H. pylori* catalase by cation exchange. Lane M is the molecular weight markers. Lane 1 shows a cell lysate, Lane 2 shows the flow through the cation exchange column, and Lanes 3 and 4 show purified catalase fractions A8 and A9 (Figure 3.10) Catalase was observed as a prominent band at ~ 55 kDa. The SDS-page electrophoresis showed that catalase eluted from the size exclusion column was substantially pure. Minor proteins at 30-40 kDa and faint low molecular weight proteins were the only contaminants.

(3.6) Investigation of In vitro Collagen Binding to Catalase

Collagen-catalase binding interaction analysis was carried out using gel filtration chromatography on a superdex 200 gel. Molecular weight standards were applied to the gel filtration column as described in Section 2.4.7. Collagen and catalase were applied separately to the gel filtration column and their elution volumes noted. A collagen catalase mixture in PBS was incubated for 30 minutes to allow them to bind to one another before being applied to the column. If binding was successful we would expect them to elute together at a combined molecular weight of around 250kDa. However, the elution profile (Figure 3.12) showed that protein eluted from this column across a wide range of molecular weights and was too varied to usefully demonstrate catalase binding to collagen IV. The elution over this range of molecular weights may be the result of self association between collagen monomers and or their association with catalase.

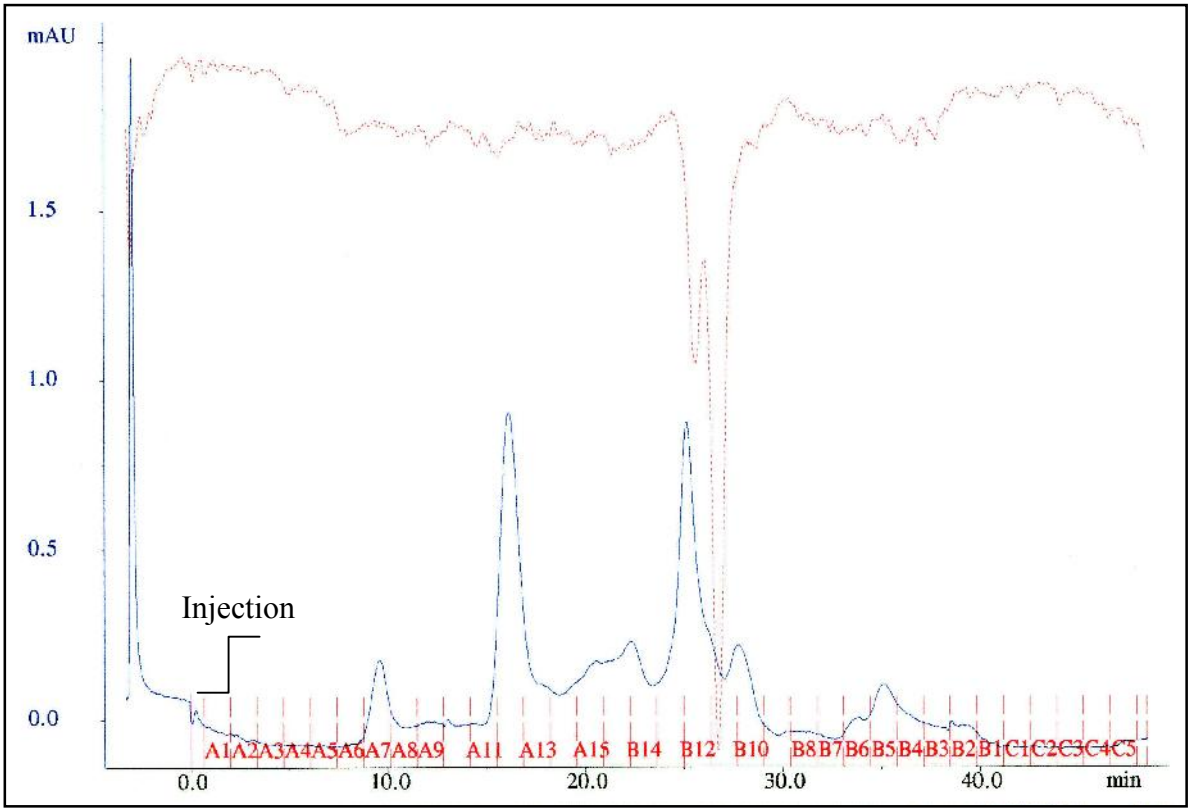


Figure 3.12. Elution profile of Gel Filtration Chromatography of a mixture of *H. pylori* catalase and collagen IV. A Catalase and collagen IV mixture incubated for 30 minutes was applied to the superdex 200 gel filtration column and eluted in PBS pH 8.0. The blue line represents the UV absorbance, the arrow represents the point of injection of the protein mixture, the red dashed lines represent 1ml fractions, and the red dotted line represents conductivity.

(3.7) Binding Assay to test for Collagen IV binding to purified H. pylori Catalase

It was possible that column chromatography conditions were not suitable as a means to measure collagen-catalase association. The tendency of collagen monomers to self associate may have complicated this picture. It was decided to attempt binding assay measurements to further assess collagen-catalase association. 3% hydrogen peroxide was used to test for catalase activity. No catalase activity could be observed using this assay. Thus, it was not possible to show collagen binding to catalase *in vitro*. We therefore decided to examine collagen binding to Urease – the other potential collagen adhesin identified above (Section 3.3.3).

(3.8) Binding of H. pylori Urease β subunit binding to Collagen IV

In order to verify whether the urease β subunit had a role in collagen adhesion we made use of a Urease β subunit-negative isogenic mutant available in the laboratory. *H. pylori* urease β subunit negative mutant was cultured and FITC labelled as described previously (Section 3.2). The wildtype *H. pylori* was used as a control. A dot blot binding assay was used to assess any differences between mutant and wild-type in binding by *H. pylori* to collagen or any of the other ECM proteins. Perhaps surprisingly, the dotblot showed binding of the *H. pylori* urease β subunit negative mutant to collagen, laminin and fibronectin as had been observed before (Figures 3.1, 3.2 and 3.3) with the wildtype strain (see Figure 3.13A &B). Therefore, we conclude that *H. pylori* urease β subunit does not play a role in collagen IV binding. Urease is the most abundant protein in *H. pylori*, and therefore it is most likely to be binding to collagen non-specifically.

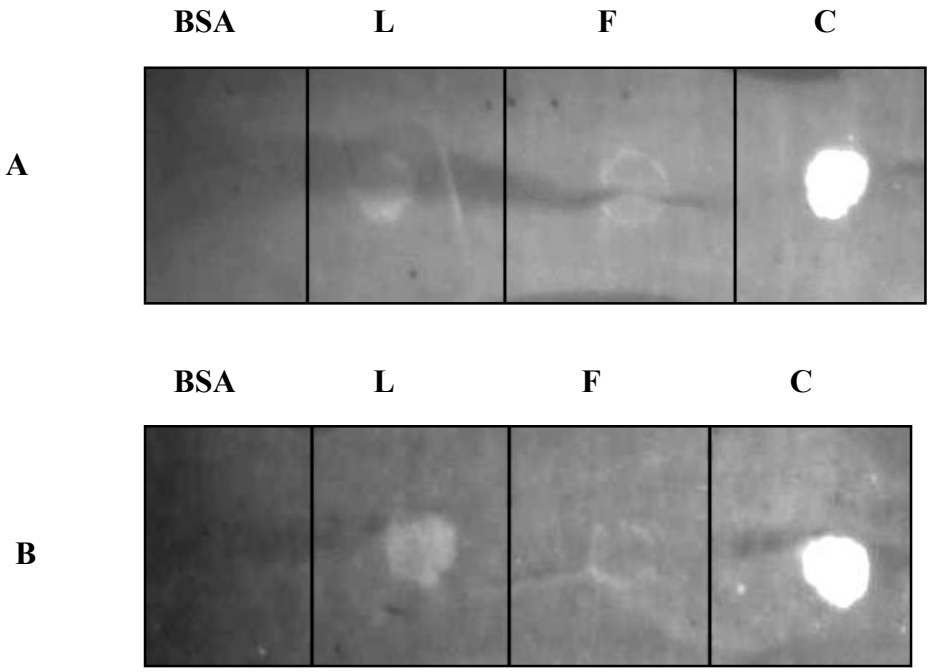


Figure 3.13: Bacteria overlay method using *H. pylori* Urease β subunit deficient strain. A shows the wildtype strain overlay and **B** shows the Urease β subunit deficient strain overlay. Where **BSA** is Bovine Serum Albumin (Control), **L** is Laminin, **F** is Fibronectin, and **C** is Collagen IV. Binding to collagen is seen as an intense white spot in both dot blots while binding to Laminin and Fibronectin is less intense.

(3.9) Collagen binding to agar-cultured H. Pylori

In view of the difficulty in showing collagen binding to *H. pylori* catalase or urease we decided to examine binding to agar cultured cells. It was possible that the adhesin was not expressed abundantly in liquid culture. It is well documented that gene/protein expression profiles are modulated by the growth environment. Figure 3.14 shows a western blot analysis of biotinylated magnetic bead purified *H. pylori* proteins from agar plates. The pattern obtained using agar cultured cells was markedly dissimilar to that obtained using liquid culture. A corresponding SDS gel was also prepared (data not

shown).The main features were bands at ca. 74 and 61 kDa. Although these did not correspond in molecular weight to the proteins observed by Western blot, these bands were excised and submitted for Mass spectrometry analysis. This analysis identified these proteins as urease and elongation factor (EF-tu), both abundant proteins in *H. pylori*.

At this point, for reasons that were not clear, a marked variability in western blot analysis of biotinylated magnetic bead purified proteins was observed. When the experiment shown in Figure 3.14 was repeated, a different pattern of labelling was obtained (see Figure 3.15). Despite repeated attempts no consistency in the pattern of proteins labelled was found.

In an attempt to reduce the complexity and variability of the potential non-specific interactions it was decided to isolate an outer membrane fraction from *H. pylori*. Such a fraction would consist of a smaller number of proteins than whole cell extracts and would therefore decrease the possibility of nonspecific interaction. It would also have the effect of producing a fraction enriched with the adhesin which is assumed to be among the Outer Membrane proteins.

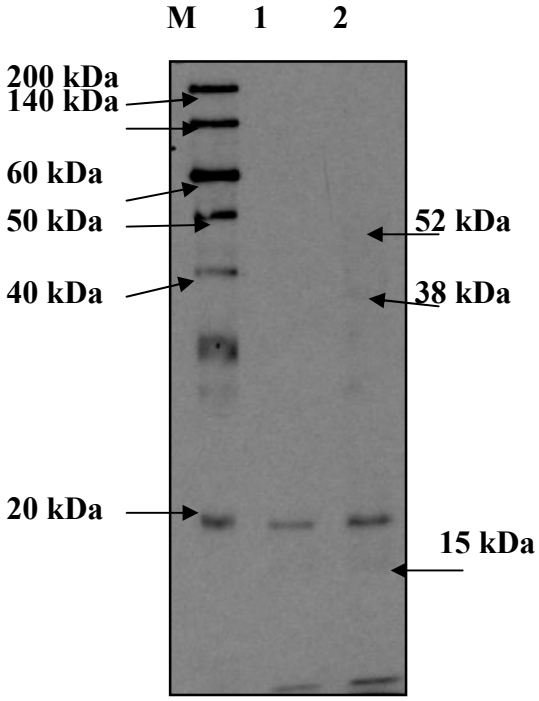


Figure 3.14. Retagging of the *H. pylori* Collagen IV binding protein. After Retagging with crosslinker-collagen conjugate, biotin tagged proteins were identified by SDS-PAGE/streptavidin blot, magnetic-bead-purified and analysed by Mass Spectrometric analysis. This figure shows streptavidin blot analysis of samples eluted from streptavidin coated magnetic beads where Lane **1** is the control and Lane **2** the eluted protein. Lane **M** is the molecular weight marker.

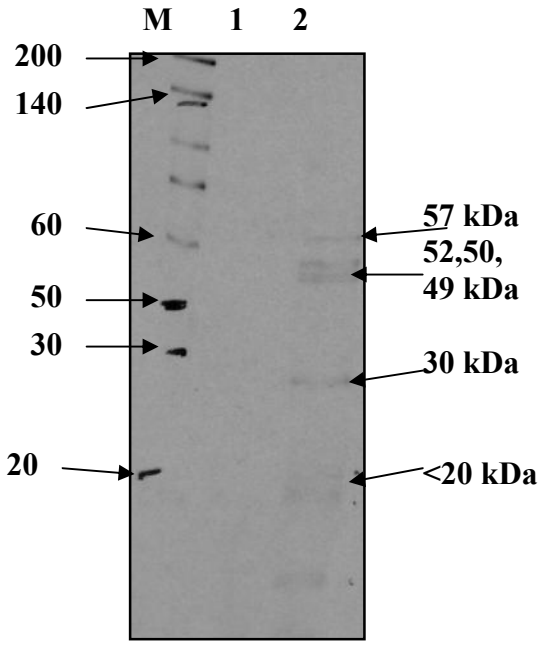


Figure 3.15. Retagging of the *H. pylori* Collagen IV binding protein. After Retagging with crosslinker-collagen conjugate, biotin tagged proteins were identified by SDS-PAGE/streptavidin blot, magnetic-bead-purified and analysed by Mass Spectrometric analysis. This shows streptavidin blot analysis of samples eluted from streptavidin coated magnetic beads where Lane **1** is the control and Lane **2** the eluted protein. Lane **M** is the molecular weight marker.

(3.10) Collagen binding to H. Pylori Outer Membrane Proteins

The Retagging Method gave highly variable and unreliable results, therefore it was decided to use a another approach to attempt to identify a Collagen IV binding protein by extracting the outer membrane proteins, separating them by SDS- PAGE and transferring them to a PVDF membrane. A similar method had previously been successful for Pantzar *et al.,*(1998) in the identification of *H. pylori* vitronectin and plasminogen binding proteins.Outer membrane proteins (OMPs) were isolated from *H. pylori* as discussed in Materials and Methods (Section 2.5.2).

Figure 3.16 shows an OMP immunoblot incubated with collagen IV. Unbound proteins were washed away and bound collagen was visualised using an immunoassay. The assay used anti-collagen antibody, followed by visualisation, using peroxidise labelled goat anti-rabbit antibodies (Figure 3.16B). A control OMP immunoblot treated with antibodies alone (Figure 3.16A). No proteins were detected in B that were not present in the control, indicating that collagen IV was not binding differentially to any proteins on the outer membrane fraction.

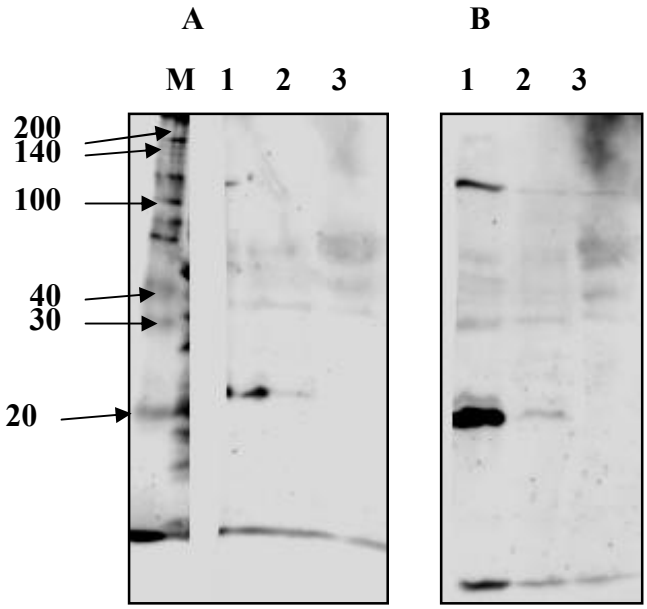


Figure 3.16. *H. pylori* Outer Membrane protein Immunoblot. Outer membrane proteins from *H. pylori* were purified and electrophoretically transferred to a PVDF membrane. The membrane was halved and **B** was incubated with collagen IV and probed with anti-collagen and peroxidase conjugated antibodies, and **A** acted as a control, probed with antibodies alone, without collagen. Lanes: **(1)** represents the sarcosine insoluble outer membrane fraction, **(2)** represents the sarcosine soluble outer membrane fraction, and **(3)** represents the whole cell extract.

We attempted to show binding of tagged collagen to electrophoretically separated outer membrane proteins. Figure 3.17 shows an OMP immunoblot incubated with crosslinker-collagen conjugate and streptavidin HRP antibody (Figure 3.17B), along with a control treated with antibodies alone (Figure 3.17A). There were no proteins detected in B that were not present in the control, thus indicating that the crosslinker-collagen conjugate was not binding to any proteins on the outer membrane fraction. This receptor overlay method had been successfully used by Ilver *et al.,* (1998) to identify the BabA adhesin from *H. pylori.* That group had used this method to

characterise the molecular mass of the BabA adhesin by separating protein extracts using SDS-PAGE, transferring to a PVDF membrane and incubating with a biotinylated fucosylated blood group antigen Lewis b (Le^b) glycoconjugate followed by peroxidase streptavidin. This method allowed them to identify a 75kDa adhesin, and subsequent Retagging analysis confirmed this finding.

However, our attempts using the receptor overlay method yielded no definite result in localising and characterising the collagen IV adhesin. We did not detect unique binding of the collagen-crosslinker conjugate to any of the purified OMP proteins, that were not found in the control.

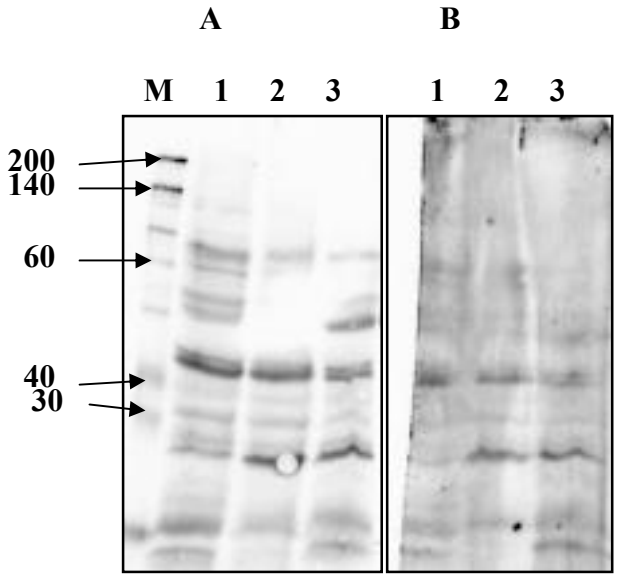


Figure 3.17. *H. pylori* Outer Membrane Immunoblot. Outer membrane proteins of *H. pylori* were purified and electrophoretically transferred to a PVDF membrane. The membrane was halved and **B** was incubated with crosslinker-collagen conjugate and detected with streptavidin HRP. **A** acted as a control, treating only with streptavidin HRP. **(1)** represents the sarcosine insoluble outer membrane fraction, **(2)** represents the sarcosine soluble outer membrane fraction, and **(3)** represents the whole cell extract.

Thus, despite repeated attempts and application of a wide variety of techniques we were unable to demonstrate the presence of a specific collagen IV adhesin in *H. pylori.* It was felt that further studies were not warranted.

Chapter 4
Discussion

(4.1) General Discussion

This thesis describes our attempts to identify a *Helicobacter pylori* collagen IV binding protein or ahdesin/receptin. A bacterial overlay method was initially employed in order to demonstrate binding of FITC-labelled bacteria to extracellular matrix proteins. We successfully showed binding of *H. pylori* to ECM proteins: Laminin, Fibronectin and collagen IV.

Identification of adhesions was attempted using Retagging Technology which employs a multifunctional crosslinker with a biotin side group. The Retagging technique was employed for crosslinker-specific biotin tagging of the putative collagen IV adhesin. Further described herein are receptor overlay immunoblots used in an attempt to identify a specific collagen IV binding protein.

(4.2) Collagen adhesins of H. Pylori

Numerous adhesive properties of *H. pylori* have been described including epithelial cell attachment, hemagluttination, and binding to distinct receptors such as proteins in body fluids and proteins of basement membranes (Trust *et al,* 1991, Gerhard *et al.,* 2001). Adherence to the ECM is an important mechanism of colonisation for many bacterial species. A key feature of many pathogenic bacteria after their initial binding to surfaces is the ability to invade deep tissues. Collagen IV is an abundant molecule in the ECM, and could represent a major target for binding of microorganisms. Trust *et al,* 1991, first reported *H. pylori* binding to collagen IV. Since then, no collagen binding protein (adhesin) has been identified although much work has been carried out on other *H. pylori* ECM adhesins (see Chapter 1, section 1.11). Initial studies focussed on

establishing if Collagen IV and other ECM proteins were capable of binding to *H. pylori*. This binding has been reported but in some cases was observed only when collagen was in an immobilised form. Thus, some workers had described collagen binding to *H. pylori* using a radiolabelling technique (Trust *et al,* 1991). However, other workers were only able to show binding to immobilised collagen and suggested that the soluble collagen did *not* bind to *H. pylori* (Moran *et al.,* 1998).

(4.2.1) Approaches to adhesin identification

(i) Bacterial overlay method

To confirm *H. pylori* binding to collagen IV and other ECM proteins we used a bacteria overlay method. The high specificity and reliability of the overlay method provides us with a useful tool for identification of the binding properties of *H. pylori* to extracellular matrix components. The bacteria overlay method has been helpful in searching for glycoprotein receptors on eukaryotic cell surfaces (Ruhl *et al.,* 2000) and for identifying ligands for bacterial adhesins in complex body fluids (Murray *et al.,* 1992, Ruhl *et al.,* 2004). Our results confirmed binding of fluorescence labelled bacteria to immobilised ECM proteins laminin, fibronectin and collagen IV as described in previous reports (Trust *et al.,* 1991). However, we did not observe binding to Lactoferrin, HSA, HSA:Hemin complex, or Hemin, despite reports that these proteins bind *H. pylori.* Dhaenens *et al.,* 1997, reported identification of a 70 kDa *H. pylori* lactoferrin binding protein which is only present when *H. pylori* are grown in an iron-starved medium. Our results showed no binding of FITC-labelled bacteria binding to lactoferrin when grown under iron reduced conditions. However, it is interesting that Walz *et al.,* 2005, have shown the *H. pylori* J99 strain binding to lactoferrin using a bacterial overlay method, without growing the *H. pylori* in an iron reduced medium.

Binding to laminin and collagen IV was consistent with previous reports (Trust *et al.,* 1991) where high affinity binding of *H. pylori* to both proteins was revealed. These workers reported that the *H. pylori* adhesins appear to be surface proteins, since treatment with trypsin or proteinase K and boiling the cells significantly reduced binding. They proposed that binding to these two major basement proteins is highly conserved among *H. pylori* strains.

It has been suggested that *H. pylori* only expresses ECM receptins during the stationary phase of growth, and other factors including growth medium, pH, iron availability and redox potential can have effects on binding (See Dubreuil *et al.,* 2002 for a review). Moran *et al.,* (1993) proposed in their concluding remarks that because microaerophillic conditions are required to demonstrate reproducible binding to ECM proteins, it suggests that certain metabolic activity may be involved in binding. They support this proposal by adding that decreased binding to ECM has been observed by aberrant forms of *H .pylori* that may be non viable, which has been observed among other microaerophillic bacteria (Moran and Upton, 1987). They further proposed that the receptin or adhesin may be absent from aberrant forms. From our data, binding of FITC-labelled *H. pylori* to laminin, fibronectin and collagen IV was not affected by the growth conditions of the bacteria, as bacteria when harvested from Columbia agar plates, liquid culture, and iron reduced liquid culture, were shown to bind all three ECM proteins. It is important to note however, that as binding was observed by visualising the fluorescence of bound bacteria under a UV light source, there was no precise method used to measure binding affinities. These findings demonstrate that the bacterial overlay method is a useful tool for exploration and characterisation of unknown adhesion specificities of *Helicobacter pylori* and other bacteria.

(ii) Receptor Activity-directed Affinity Tagging (Retagging) technique for Adhesin Protein Identification

The Retagging method involves a technique based on a receptor covalently attached to a multi functional crosslinker, which following UV irradiation, transfers a biotin tag to the bound adhesin. After detergent solubilization the adhesin can be extracted by use of streptavidin coated magnetic beads. Ilver *et al.,* (1998) successfully identified the BabA adhesin in *H. pylori* using this technique and later the *H. pylori* SabA adhesin was identified by retagging (Mahdavi *et al.,* 2002). Possible drawbacks of this method include protein precipitation, poor labelling of bait protein, poor photoactivation, non specific binding of probe, and steric hindrance.

Retagging with the crosslinker-collagen conjugate first isolated 3 proteins at ~ 77, 66 and 27kDa. Mass spectrometric analysis identified that the 77kDa protein was identical to *H. pylori* Urease β subunit. The 66kDa protein was identical to *H. pylori* catalase. Although we assumed these results to be aberrant or artefactual at first, investigation of the literature provided us with a body of evidence to support this finding.

Catalase is present in a variety of bacteria and protects the bacterial cell against the toxic effects of oxygen radicals, for example, those produced during the oxidative burst of phagocytosis. The *H. pylori* catalase protein has been previously characterised and cloned showing a gene *katA* and a 58.6 kDa protein (Hazell *et al.,* 1991, Odenbreit *et al*., 1996). The protein purified from *H. pylori* by Lingwood et al., (1993) and the *katA* gene product produced in *E. coli* both run with an apparent molecular massof 62 or 63 kDa in the SDS gel, which is higher than the calculated molecular mass of the KatA protein described byHazell and coworkers. Although catalase is normally thought of as

a cytoplasmic protein in *H. pylori*, examination of the literature revealed that this was not the first time catalase was implicated in adhesion. Lingwood *et al*., (1993) identified a 63 kDa protein suggested to be a lipid binding adhesin of *H. pylori* which corresponded to the N-terminal of *katA*. In addition to this, Pantzer *et al.,* (1998) identified a vitronectin binding protein from *H. pylori* which had an amino terminal sequence identical to that of *H. pylori* catalase. The isolated vitronectin protein showed 92% identity with the *H. pylori* catalase amino terminal amino acid sequence. The observation of catalase binding to vitronectin was encouraging as it demonstrated binding of catalase to an extracellular matrix protein, as well as providing evidence that catalase might function as an adhesin in *H. pylori.*

Several previous studies had noted the binding capabilities of catalase. For instance, catalase adheres to smooth muscle cells (Sundaresan *et al.,* 1995) and it also binds β-amyloid, a major component of the plaques observed in Alzheimer's disease (Milton 1999). Yano *et al* (2004) investigated catalase binding to mitogenic signalling molecules. Serum stimulation of HeLa, Caco-2 and LiSa-2 cells resulted in catalase binding to the signalling molecule Gbr2. In addition, stimulation with the ECM proteins fibrinogen, fibronectin, and laminin, but not collagen types I-V, resulted in binding of the cells in a manner similar to that of serum. It is interesting to note that collagen stimulation did not result in binding of catalase to Gbr2. If catalase does in fact, bind collagen, then collagen might bind to catalase before Gbr2.

A study by Visai *et al* (1990) used ^{125}I labelled collagen to study the binding of collagens to an enterotoxigenic strain of *E. coli*. The specificity of ^{125}I labelled collagen binding to *E. coli* was examined by analysing the inhibitory activity of different collagen types and catalase, a non collagenous protein. All the collagens tested were potent inhibitors and showed essentially the same inhibitory potential when tested at

different concentrations. These workers state that binding of ^{125}I labelled collagen to bacteria in the presence of catalase was not significantly reduced. Our findings do not support this observation. In their data, when the concentration of catalase was increased the inhibitory effect of catalase increased, with a maximum inhibition of binding ^{125}I labelled collagen of approximately 20-25%. From our viewpoint when looking at the potential of catalase binding to collagen this is significant inhibition, and suggests that catalase might compete with the bacteria for binding to collagen.

Furthermore, Peterson *et al* (2004) found that Extracellular Superoxide Dismutase (EC-SOD) binds to type I collagen. Like catalase, EC-SOD has a role in detoxification of reactive oxygen species. The EC-SOD has a C-terminal heparin-binding region which mediates its interaction with collagen. The bound EC-SOD is thought to protect collagen from oxidative fragmentation during oxidative stress. These findings were of interest, as they showed an enzyme, similar to catalase, interacting with collagen.

From our findings and evidence presented in the literature it seemed plausible that catalase could be the binding protein on *H. pylori* for collagen IV. A rapid convenient protocol for *H. pylori* catalase purification had been described by Hazell *et al.*,(1991). We purified *H. pylori* catalase using cation exchange chromatography and examined *in-vitro* Collagen binding to *H. pylori* catalase (see section 4.3). Catalase was identified a second time as a potential collagen receptor, when *H. pylori* were grown from liquid culture and retagging was performed. This time the Western blot detected corresponding biotinylated proteins of the same size as catalase when R_f analysis was carried out. These results were reproducible. The fact that catalase was detected a second time lead us to believe that it may in fact bind collagen IV. There was certainly strong evidence in the literature to support this observation. However, our attempts to investigate collagen-

catalase association (Section 4.3) had been unsuccessful and no further work was carried out to investigate this.

Repeating the crosslinking experiment using bacteria harvested from agar culture did not yield the same results as had been observed with liquid culture. Catalase was not identified in subsequent labelling experiements and therefore this raised doubts whether catalase was truly implicated in *H. pylori* binding to collagen IV. Magnetic bead purification yielded proteins on SDS gels that were not observed on corresponding streptavidin blots, indicating that non-specific binding may have been occurring.

The second protein identified by Mass spectrometric analysis was *H. pylori* Urease enzyme β subunit. Like catalase much of urease is found in the cytoplasm of the bacteria (Scott *et al.,* 2002). However, it has also been shown to be present in association with the outer membrane (Bode *et al.,* 1993, and Rokita *et al.,* 2000). Literature reports pointed to the possibility of urease acting as an adhesin. *H. pylori* urease has been shown to bind gastric mucin, heparin, and related heparinoids (Icatlo *et al.,* 1998) and it has been suggested that urease binds to lipopolysaccharide on the bacterial surface (Icatlo *et al.,* 2000). Fan *et al.,* (2000) have shown that urease binds to Class II MHC on gastric epithelial cells and *H. pylori* urease β subunit binds to CD74 on gastric epithelial cells (Beswick *et al.,* 2005).

With this evidence it was decided to examine urease binding further. A *H. pylori* urease β subunit negative mutant strain was available in the laboratory. The dotblot method is a fast, effective way of measuring binding interactions of bacteria to proteins and was employed for this purpose. The urease β subunit negative mutant strain was labelled with FITC and used in binding analyses of the mutant strain to ECM proteins spotted on a PVDF membrane. If urease was involved in collagen binding we would expect to see

little or no binding to collagen by the urease negative strain when compared to the wildtype strain. However, binding occurred to all three ECM proteins, collagen, fibronectin and laminin, in the mutant strain as well as the wildtype. This indicated that the urease β subunit in *H. pylori* was unlikely to be implicated in collagen binding. One possibility not evaluated in this study is that there might be more than a single collagen adhesin in *H. pylori*. If this were the case and there was an element of redundancy in the system then deletion of a single 'adhesin' would not necessarily impact on or abrogate collagen binding significantly. Alternative methods to evaluate the affinity of the interaction would be required.

One interesting result worth noting was the identification of *H. pylori* EF-Tu elongation factor as a potential adhesin. Initially this finding was considered a false positive, as it was thought that this protein did not normally function as an adhesin. However, Dallo *et al.,* (2002) described a role for EF-Tu in mediating adhesion of bacteria to the extracellular matrix. They reported that the *Mycoplasma pneumonia* EF-Tu elongation factor mediates binding to the ECM component fibronectin through the carboxyl region of the EF-Tu. Balasurbramanian *et al.,* (2009) further explored this interaction, comparing *Mycoplasma pneumonia* to closely related *Mycoplasma genitalium*. Here they found that *M. genitalium* EF-Tu, despite having 96% identity to *M. pneumonia* EF-Tu, does not bind to fibronectin. This showed that there were critical amino acids involved in *M. pneumonia* binding to fibronectin. Further surface-associated EF-Tu proteins from other microorganisms including *Lactobacillus johnsonii*, *Listeria monocytogenes* & *Pseudomonas aeruginosa* were reported to bind mucin (Granato *et al.,* 2004), fibrinogen (Schaumburg *et al.,* 2004), plasminogen and factor H (Kunert *et al.,* 2007). From this data it is interesting that *H. pylori* EF-Tu was identified in this current study as a potential collagen receptor. This was not reproduced.

(iii) Immunoblot and Receptor overlay Technique

As the retagging method was proving unsuccessful in our attempt to identify the *H. pylori* collagen IV binding protein, we decided to explore alternative methods. Several groups have used radiolabelling of ECM proteins as a method for identification of ECM receptins on microorganisms. This method was not an option for us as radioactivity handling was not possible in the laboratory. We decided to examine immunoblotting techniques. Pantzar *et al.,* (1998) described a method they used for the identification of *H. pylori* vitronectin and plasminogen binding proteins. This method involved extracting the outer membrane proteins (OMP's) from *H. pylori*. In the case of gram negative bacteria, many of the surface proteins are constituents of the outer membrane. Pantzar *et al.,* separated OMP proteins on a SDS gel and transferred them to a PVDF membrane. The membranes were firstly incubated with vitronectin or plasmonogen, followed by anti vitroncetin or anti plasmonogen antibodies, and finally developed using peroxidase conjugated antibodies. In this way, they could identify the proteins to which the ECM proteins were binding to on the immunoblot. These workers found a 57kDa vitronectin binding protein (identical to *H. pylori* catalase) and a 42 and 57 kDa plasminogen binding protein.

Outer membrane proteins were isolated from *H. pylori* using ultracentrifugation in the presence of sarcosine (See Results section 3.9). Doig *et al.,* (1994) used sarcosine as a method for extracting OMP's and this method was shown to extract a higher number of protein species than other preparation methods. This method yields two protein preparations; a sarcosine soluble fraction and a sarcsoine insoluble fraction. The outer membrane fraction is insoluble in sarcosine, whereas the cytoplasmic membrane is fully soluble (Filip *et al.,* 1973). Both fractions were analyzed by immunoblotting, as well as the whole cell extract. The protein fractions transferred onto the PVDF membranes were

incubated with collagen IV and an immunoblot procedure was carried out. If collagen IV had interacted with any of the proteins then we would expect to detect a unique species that was not present in the control immunoblot - no unique proteins were observed in the test immunoblot. Although these attempts were unsuccessful, this method was robust and reliable and had been successful in other studies.

In attempts to further identify collagen IV binding proteins, it was decided to probe a collagen-crosslinker conjugate using a immunoblot procedure. *H. pylori* OMP's were transferred to a PVDF membrane and then incubated with a collagen-crosslinker conjugate. Streptavidin HRP was used to detect binding interactions between the collagen conjugate and any of the *H. Pylori* OMPs proteins. This method resulted in blots with high backgrounds and a number of electrophoresis bands indicating that non-specific binding remained a problem. However, we did not observe any unique protein band in the immunoblot that was not present in the control. This method had worked successfully for Ilver *et al*., (1998) in their initial attempts at localizing the *H. pylori* BabA adhesin and they describe it as a "receptor overlay technique".

Either of these immunoblot methods, if successful, would have provided us with an indication of the size of the *H. pylori* collagen binding protein. There are no literature reports to suggest the molecular weight of the protein, although it would be useful to know the approximate size of the protein we were trying to identify. When Pantzar *et al.,* (1998) used the vitroncetin immunoblot procedure, it allowed them to identify a 57 kDa protein, and 2D electrophoresis was used to identify and purify this protein from a crude guanidine-HCl cell extract.

(4.3) In Vitro Collagen binding to H. pylori Catalase

Gel filtration chromatography was used in an attempt to observe collagen IV binding to purified *H. pylori* catalase *in vitro*. These attempts however, were inconclusive as the gel filtration chromatography gave rise to several peaks and it was difficult to determine what these peaks represented. Collagen IV is a large, hydrophobic protein with low water solubility and it is possible it may have precipitated in the column. Thus there was the possibility that collagen might self associate or associate with one or more catalase molecules to produce equilibrium mixtures containing a large number of protein species.

In further attempts to examine collagen IV binding to catalase we coated 96 well plates with collagen IV, blocked with BSA and incubated with catalase. After washing we added hydrogen peroxide to check for a bubbling effect indicating the presence of catalase. We did not observe a catalase activity in the wells. In a few instances a minimal bubbling effect was thought to be observed but was unconvincing. Since we used BSA as a blocking agent, it was difficult to know for certain whether this minimal bubbling effect was a result of residual BSA, as BSA dissolved in solution can cause foam or bubbles.

(4.4) Difficulties with Identification of the Collagen IV Adhesin.

The identification of the *H. pylori* collagen binding protein has proven difficult. This may be the reason why the collagen binding protein has not been identified to date, despite the fact that collagen IV is the major protein present in basement membranes. Considering there has been much research carried out on *H. pylori* interactions with other proteins of the ECM, it is somewhat surprising that no collagen binding proteins have been identified so far.

Although Trust *et al.,* (1991) described high affinity binding of *H. pylori* to collagen IV, Moran *et al.,* (1993) reported different observations. While the latter group found strong binding of *H. pylori* to laminin, they could not demonstrate binding to collagen IV in any of the strains tested. Moran *et al*., found that none of the strains were capable of binding soluble proteins, but a proportion bound immobilised laminin, fibronectin and types I and V collagens. They suggest that in the immobilised form the protein is open and extended, whilst in the soluble form the protein might be folded, thereby not presenting the appropriate binding site for bacterial adhesion. This folded state in the soluble form was observed for fibronectin in a previous report (Rocco *et al.,* 1987). Moran *et al.,* (1993) also propose that it is the insoluble form that is tissue associated *in vivo*, and due to this, the immobilised form may better reflect the ECM as bacteria would encounter it *in vivo*. Another observation to note is that despite Trust *et al,* (1991) reporting that binding to laminin and collagen IV is conserved among *H. pylori* strains, Moran *et al.,* (1993) found binding of some but not all strains of *H. pylori* to laminin, but none to collagen IV, emphasising inter-strain differences in specificity. This binding to immobilized and but soluble forms of proteins has been observed in other studies of interactions of bacteria with ECM proteins. *Lactobacillus acidophilus* binds to immobilised collagen I and fibronectin but not soluble forms (Lorca *et al.,* 2002). Reports that Yad A of *Yersinia enterocolitica* and P fimbriae of *Escherichia coli* bind immobilised but not soluble fibronectin may reflect the fact that these adhesins recognise a domain which is exposed only when fibronectin is immobilised in tissues (Schulze-Koops *et al.,* 1993 and Westerlund *et al.,* 1991)

Therefore, these findings suggest that from our results, even although *H. pylori* binds collagen IV when immobilised on PVDF membranes, it may not bind collagen in the soluble form. Collagen is in its soluble form in the retagging method, and it might be

possible that its binding sites are not accessible at this time. This could explain our variable results when the beads are used to purify biotinylated proteins after the retagging method. The collagen was also in a soluble form during our collagen/ collagen-crosslinker conjugate immunoblot procedure, and this could explain the fact that we did not detect any binding interaction.

(4.5) Conclusion and Future work

Arising from the work presented in this thesis it is evident that further exploration is required in order to identify the collagen IV receptor on *H. pylori*.

It is clear to see that investigations into *H. pylori* and other microorganisms binding host ECM proteins has much potential in the role of treating infection.

Future work that we would suggest would be to construct a *H. pylori* catalase negative strain as catalase was identified by mass spectrometry on two occasions as a potential collagen binding protein. There is also a good amount of evidence to support this result. However, our own attempts to characterise this interaction were unsuccessful. After construction of a catalase mutant we would suggest using the Biocore to characterise this interaction. This instrument has been successfully used by other members in this laboratory to examine protein-protein interactions. This experiment should finally rule out the possibility of catalase as a collagen IV binding protein on *H. pylori.*

Other methods of binding protein identification might include affinity purification, using a sepharose resin coupled to collagen, and applying a *H. pylori* cell lysate.

There are also the options of radiolabelling and ELISA to further characterise binding activities.

It is evident from previous reports and our own bacterial overlay method that *H. pylori* binds collagen IV. Therefore an adhesin most likely is present on the surface of the pathogen. However, identification of this adhesin protein has proven challenging.

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Appendix A

Reagents and Buffers

SDS-PAGE/protein purification reagents

10X Phosphate Buffered Saline

Reagent	**Volume/Quantity**
$Na_2HPO_4.2H_2O$ (8mM)	14.24 g
KH_2PO_4 (1.5mM)	2.04 g
NaCl (137mM)	80.0 g
KCl (2.7mM)	2.0 g
Adjust pH to 7.4 and make up to 1Litre Volume	

Resolving Gel Buffer

Reagent	**Volume/Quantity**
Tris Base	18.165g
Distilled Water	100 mls
Adjust to pH 8.8 with concentrated HCL	

Stacking Gel Buffer

Reagent	**Volume/Quantity**
Tris Base	12.11 g
Distilled Water	100 mls
Adjust to pH 6.8 with concentrated HCL	

10% Ammonium Persulphate

Reagent	**Volume/Quantity**
APS	50 mg
Distilled Water	1 ml
Prepare fresh prior to use	

10X Running Buffer

Reagent	Volume/Quantity
Tris base	30 g
Glycine	114 g
SDS	5.0 g
Distilled Water	1000 ml
Dilute 1:10 prior to use	

5X Reducing Sample Buffer

Reagent	Volume/Quantity
Glycerol	5 ml
β-mercaptoethanol	6.25 ml
20% SDS	5 ml
1.0M Tris pH 6.8	1.25 ml
0.2% Bromophenol Blue	0.3 ml
Make up to 25 ml with distilled water	

Coomassie Blue R-250 Gel Stain

Reagent	Volume/Quantity
Coomassie Brilliant Blue R250	0.5g
Methanol	200 ml
Glacial Acetic Acid	35 ml
Distilled Water	265 ml

Destain Solution

Reagent	Volume/Quantity
Methanol	400 ml
Glacial Acetic Acid	70 ml
Distilled Water	265 ml

Coomassie Blue G-250 stain (for Mass spectrometry)

Fixation Solvent

Reagent	**Volume/Quantity**
50% Methanol	500 ml
2% Phosphoric acid 85%	23.5 ml
Distilled Water	480 ml

Incubation Solvent

Reagent	**Volume/Quantity**
34% Methanol	500 ml
2% Phosphoric acid (85%)	23.5 ml
17% Ammonium sulphate	170 g
Distilled Water	640 ml

Destain Solution

Reagent	**Volume/Quantity**
Methanol	400 ml
Glacial Acetic Acid	70 ml
Distilled Water	530 ml

Immunoblotting

Blocking Solution 5%

Reagent	**Volume/Quantity**
Skimmed dried milk powder	5 g
PBS	100 ml

0.1% Tween Washing Solution

Reagent	**Volume/Quantity**
Tween 20	1 ml
PBS	1000 ml

Primary and Secondary Antibody Diluent Solution 5%

Reagent	Volume/Quantity
Skimmed dried milk powder	5 g
PBS	100ml

Transfer Buffer

Reagent	Volume/Quantity
Tris base	2.9 g
Glycine	1.45 g
SDS	0.185 g
Methanol	100ml
Make up to 500ml with distilled water	

Membrane Stripping Buffer

Reagent	Volume/Quantity
Tris base pH 6.8 (25.5mM)	6.25 ml
β-mercapthoethanol (100mM)	0.68 ml
SDS (2%)	20 ml
Make up to 100ml final volume with distilled water	

Developing Solution

Reagent	Volume/Quantity
Iodophenol	4 mg
Luminol	12 mg
DMSO	0.5 ml
0.1M Tris pH 8.8	50 ml
H_2O_2	18 µl