

**MOLECULAR AND FUNCTIONAL
CHARACTERISATION OF THE IMMUNODOMINANT
ANTIGENS OF THE OBLIGATE INTRACELLULAR
PATHOGEN *LAWSONIA INTRACELLULARIS***

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**A thesis submitted for the degree of Doctor of Philosophy at
the University of Edinburgh**

2005

Declaration

The work reported in this thesis was carried out under the supervision of Professor David G.E. Smith at the Zoonotic and Animal Pathogens Laboratory, University of Edinburgh and latterly at the Moredun Research Institute. All results present, unless otherwise stated, are the sole work of this author, as is the composition of this thesis.

Signed

Date 30/3/06

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Abstract

Lawsonia intracellularis is an obligately intracellular pathogen which is the cause of the disease complex known as proliferative enteropathy (PE) or ileitis. This bacterium is pathogenic in a broad range of animal hosts, disease being most notable in pigs. *L. intracellularis* has a tropism for immature (crypt) epithelial cells and disease is characterised by epithelial hyperplasia in infected crypts. This pathology presumably reflects expression of novel virulence factors during infection. To date few genes have been identified and of these only *lsaA*, a *tlyA* homologue, has any function ascribed. TlyA proteins of bacteria belong to a novel family identified across a phylogenetically diverse range of bacteria. These include several gastrointestinal pathogens such as *Helicobacter pylori*, *Campylobacter jejuni*, *Brachyspira hyodysenteriae* and *Lawsonia intracellularis*. These proteins have been identified mainly through genomic sequencing and their expression and role(s) during infection remain to be fully defined. TlyA deletion mutants in *H. pylori* and *B. hyodysenteriae* are attenuated, suggesting that these proteins perform important roles during infection. LsaA (lawsonia surface antigen) the *L. intracellularis* orthologue, is expressed during infection *in vitro* and *in vivo*, which suggests that this factor is involved during adherence and/or invasion of intestinal epithelial cells. The principal aim was to characterise function(s) of LsaA. Specifically the putative function as an adhesin was investigated further using a combination of biochemical and molecular approaches (including affinity purification and yeast 2-hybrid analysis) to elucidate possible receptor(s). However, no consistent partner was evident therefore mammalian epithelial cell receptors could not be defined using this range of approaches. It is possible that LsaA's role in adherence is adventitious – for example, it has been proposed that the TlyA family of proteins possess a regulatory role in bacterial colonisation as opposed to a direct involvement in bacterial adherence. The existence of two conserved putative functional domains, S4 RNA binding and methyltransferase motifs have been noted in all members of the TlyA family examined to date. These domains are found separately in several protein families known to be involved in gene regulation. Since no system has been developed for mutating genes in *L. intracellularis* the proteome of a TlyA deletion mutant of *H. pylori* was compared to

its parent to further this potentially new and interesting function of TlyA family proteins. Notably, flagellin B and catalase were absent in the *tlyA* mutant. Since deletion of *tlyA* corresponds with changes in expression of several *H. pylori* genes, it can be concluded that reduced colonisation of *H. pylori tlyA* mutant is likely to be as a result of effects on expression of virulence genes rather than a direct role in adherence.

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List of abbreviations

α	alpha
aa.....	Amino acid
Amp.....	ampicillin
ATP.....	adenosine triphosphate
~.....	approximately
β	Beta
β -gal.....	Betagalactosidase
bp.....	base pairs
BHI.....	brain heart infusion broth
BSA.....	bovine serum albumin
CaCl ₂	Calcium chloride
cDNA.....	complementary deoxyribonucleic acid
cfu.....	colony forming units
CNBr.....	Cyanogen bromide
2D-LC-MS/MS.....	Two-dimensional liquid chromatography-tandem mass spectrometry
2D-PAGE.....	Two-dimensional polyacrylamide gel electrophoresis
DAB.....	diaminobenzidine
DAS.....	Dense alignment surface
DMSO.....	dimethyl sulphoxide
DNA.....	deoxyribonucleic acid
dNTP:-	deoxynucleotide triphosphate
dATP.....	Deoxyadenosine triphosphate
dCTP.....	Deoxycytosine triphosphate
dGTP.....	Deoxyguanosine triphosphate
dTTP.....	Deoxythymidine triphosphate
DTT.....	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
γ	gamma
g.....	Gram
HBSS.....	Hanks balanced salt solution
HCl.....	hydrochloric acid
HPLC.....	high performance liquid chromatography
hrs.....	Hours
HRP.....	horseradish peroxidase
IFN- γ	interferon gamma
IPTG.....	isopropyl β -D-thiogalactopyranoside
kb.....	kilobase pairs
kDa.....	Kilodaltons
kg.....	kilogram

L.....	litre
M.....	Molar
<i>M.</i>	<i>Mycobacterium</i>
mA.....	milliampere
MALDI-ToF.....	Matrix-Assisted Laser Desorption Ionization – Time of Flight
mg.....	milligram (10^{-3} gram)
MHC.....	Major histocompatibility complex
MgCl ₂	magnesium chloride
MgSO ₄	magnesium sulphate
min.....	minute(s)
ml.....	millilitre (10^{-3} litre)
mM.....	millimolar (10^{-3} molar)
MRI.....	Moredun Research Institute
MudPIT.....	Multidimensional Protein Identification Technology
NaCl.....	sodium chloride
Na ₂ CO ₃	sodium carbonate
Na ₂ EDTA.....	ethylenediaminetetraacetic acid disodium salt
NaHCO ₃	sodium bicarbonate
Na ₂ HPO ₄	disodium hydrogen orthophosphate
NaH ₂ PO ₄	sodium dihydrogen orthophosphate
NaOH.....	sodium hydroxide
ng.....	nanogram (10^{-9} gram)
(NH ₄) ₂ SO ₄	ammonium sulphate
OD.....	optical density
ORF.....	open reading frame
PAGE.....	polyacrylamide gel electrophoresis
PBS.....	phosphate buffered saline
PCR.....	polymerase chain reaction
PEG.....	polyethylene glycol
Pfu.....	plaque forming unit
pmol.....	picomole (10^{-12} moles)
PMSF.....	phenylmethylsulphonyl fluoride
RBS.....	ribosome binding site
RNA:-.....	ribonucleic acid
mRNA.....	messenger ribonucleic acid
rRNA.....	ribosomal ribonucleic acid
tRNA.....	transfer ribonucleic acid
SAP.....	alkaline phosphatase
SDS.....	sodium dodecyl sulphate
TEMED.....	N,N,N',N'-tetramethylethyldiamine
T _m	melting temperature
µg.....	microgram (10^{-6} gram)
µl.....	microlitre (10^{-6} litre)

μM	micromolar (10^{-6} molar)
V.....	Volt
v/v.....	volume to volume
w/v.....	weight to volume
w/w _t	weight to weight
X-gal.....	5-bromo-4-chloro-3-indoly- β -D-galactopyranoside

Acknowledgements

Firstly, I am extremely grateful to my supervisor David Smith for his ongoing support and encouragement. I would also like to thank Neil Inglis for his insight into the world of protein biochemistry but more especially for his advice and well timed words of wisdom.

There are many people who offered me invaluable help and practical advice during my PhD. Linda Wilson and Arvind Mahajan for their help and expertise with staining and confocal microscopy respectively, Mara Rocchi for her time and help with flow cytometry techniques, the staff of the Functional Genomics unit whose knowledge of all things proteomic was invaluable, Nicola Holden for radioactive labelling of cDNA, Luke Tysal for his help with many things computer related and all the other 'Zappers' who helped me during my time there.

I would like to say how much I have enjoyed my time at Moredun Research Institute. Although stressful at first it was definitely a move in the right direction. Members of the MCI lab who I will miss are Pilar Alberdi, Edith Paxton, Carol Currie, Pamela Cameron, Arvind Mahajan, Ewan Clark, Douglas Fraser-Pitt, Melinda Hough, Agent Orange, and not forgetting other PhD office associates for their banter.

I would also like to thank Vicky Thomas for her timely 'moral' support and also my friend David for putting up with my many annoying habits for so long.

Lastly, I would like to thank my parents and my Auntie Ina. Without whose unwavering help and support (financial and otherwise) I would not be where I am today.

This work was supported by the BBSRC.

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INTRODUCTION

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1.1 General Introduction

Enteric bacterial infections are among the most common and economically significant diseases affecting swine production worldwide. The most common etiological agents include *Escherichia coli*, *Clostridium perfringens*, *Lawsonia intracellularis*, *Salmonella enterica*, and *Brachyspira (Serpulina) spp.* (Moxley & Duhamel 1999 377 /id}. Of these, *Lawsonia intracellularis* and *Brachyspira hyodysenteriae* are ultimately responsible for two of the most important diseases: proliferative enteropathy and swine dysentery respectively (McOrist and Gebhart, 1999, Harris *et al.*, 1999).

Proliferative enteropathy (PE), also known as ileitis, is characterised by thickening of the intestinal mucosa resulting from adenomatous proliferation of enterocytes in the lower ileum and colon (McOrist *et al.*, 1992, McOrist *et al.*, 1993, Jensen *et al.*, 1997, McOrist and Gebhart, 1999). These pathological effects lead to severe economic losses due to reduced weight gain and mortality (Veenhuisen *et al.*, 1998, McOrist *et al.*, 1997b, Holyoake *et al.*, 1996). For example, PE has recently been estimated to cause losses of £2-4 million per annum in the UK alone in terms of treatment costs and reduced productivity (Lawson and Gebhart, 2000).

In the mid nineties the aetiological agent of this enteric disease was identified as the Gram negative, obligate intracellular bacterium *Lawsonia intracellularis* (Lawson *et al.*, 1993, McOrist *et al.*, 1995a). The obligate intracellular lifestyle of this bacterium has made it a very difficult subject to investigate and hence few virulence factors have been identified to give an insight into this problematic disease complex.

1.2 Proliferative Enteropathy

PE was first recognised as a specific disease in pigs in United States in 1931 (Beister, 1931). Although most significant in pigs, PE has been detected in a wide range of hosts including ostriches (Cooper *et al.*, 1997), deer (Drolet *et al.*, 1996), foxes (Erikson *et al.*, 1990), rabbits (Schoeb and Fox, 1990), primates (Klein *et al.*, 1999)

and horses (Williams *et al.*, 1996). Detection in this broad range of hosts suggests that intra- and/or interspecies transmission appears likely since *L. intracellularis* isolated from different species have little genetic variation based in 16S sequence comparisons (>98% 16S rDNA similarity), (McOrist *et al.*, 2003). Comparison of the GroEL sequence from *L. intracellularis* with those available also supports this phylogenetic placement (Dale *et al.*, 1998).

1.2.1 Clinical Symptoms

PE is a clinical syndrome resulting in dehydration and weight loss in weanling and young adult pigs. However, the disease has several clinicopathological manifestations and normally manifests in pigs in one of two major forms: porcine intestinal adenomatosis (PIA) and porcine haemorrhagic enteropathy (PHE). Young growing animals between 8 and 20 weeks of age are most susceptible to PIA following weaning from their mother (Rowland and Lawson, 1975b). In these animals clinical symptoms include a low-grade diarrhoea causing the affected animals to lose condition. PIA associated mortality is usually low (1-5%) with most affected animals recovering in 6 - 8 weeks accompanied by resolution of the lesions. However, despite apparent recovery of affected pigs, 15% will fail to reach market weight (Lawson *et al.*, 1979) leading to economic losses. Clinical porcine haemorrhagic enteropathy (PHE) is more common in young adult swine of ages 3 to 12 months. In severe acute cases in fattening pigs or young gilts or boars it causes sudden death, known as 'bloody gut', where the pigs are pale and their faeces are black and bloody (Rowland and Lawson, 1975a). The consistent pathological features of intestinal hyperplasia the two forms of disease has suggested that the latter represents a progression of underlying adenomatosis (due to its resemblance of adenomatous neoplasia), but the reason for the haemorrhagic complications remain unresolved (Rowland and Rowntree, 1972, Rowland and Lawson, 1975a) but may be of an immunopathological nature (MacIntyre *et al.*, 2003). Two other forms of PE, necrotic enteritis and regional ileitis, occur in 4- to 5- month-old pigs, but there is a significant component of secondary infection and mucosal destruction in these forms,

making investigation of pathogenesis even more complex (Rowland and Lawson, 1975a).

1.2.2 Diagnosis

For many years the most comprehensive indication of infection was the identification of gross pathology, histological detection of lesions and the presence of intracellular bacteria. However such techniques are laborious and not always conclusive. Post-mortem detection has now improved with immunohistological detection of *L. intracellularis* in intestinal mucosa with specific antibody reagents or by detecting the organism's genome by genomic probe or, most commonly, PCR. Although not ideal, post mortem PCR is the most sensitive method of detection (detecting as little as 10^1 *L. intracellularis* per g of tissue) (Dunser *et al.*, 2003). It is also possible to perform PCR on faeces although this is less sensitive (10^3 - 10^4 *L. intracellularis* per g of faeces), and only feasible if the animal is shedding which does not always coincide with ileal colonisation (Jones *et al.*, 1993, McOrist *et al.*, 1994). Nested PCR has been developed to detect *L. intracellularis* infection in faecal samples, which reportedly increases sensitivity 10-100 fold (Chang *et al.*, 1997). A decreased sensitivity may be due to PCR-inhibitory factors that can be present in complex biological molecules such as faeces and nested PCR on boiled samples has been shown to be the most efficient preparation method, however the time consuming nature of the technique precluded its routine use (Jacobson *et al.*, 2004). A far superior method of detection would appear to be the serum indirect fluorescent antibody test (IFAT). When the two methods were compared PCR was capable of detecting 4 of 23 positive blood and faecal samples compared to the 21 detected by IFAT (Jones *et al.*, 1993, Knittel *et al.*, 1998). Huerta *et al.* (2003) also supported this finding (Huerta *et al.*, 2003). However, IFAT depends on the availability of monoclonal antibody against *L. intracellularis* which, due to difficulties in propagating the bacterium and lack of commercial availability, may not always be readily available in many laboratories (Moore and Shryock, 1996, Guedes *et al.*, 2002). In 1987, McOrist *et al.*, (1987, 1989) developed two mAbs, IG4 (VPM53)

and 4F5, with specificity to a surface protein of approximately 25-27kDa. VPM53 is specific for the 27kDa surface antigen, LsaA (*Lawsonia* surface antigen A).

Recently a monoclonal antibody against a periodate-sensitive 21 kDa antigen has been produced which has shown promise as a diagnostic tool for *Lawsonia* infection (Boesen *et al.*, 2005). Guedes and Gebhart (2003) have also produced a mAb with specificity to an outer membrane antigen of 77 kDa (Guedes and Gebhart, 2003b).

Sero-conversion of pigs infected with *L. intracellularis* is first detected at 12-14 weeks of age (Stege *et al.*, 2004), usually at least 2 weeks after the onset of infection (Jacobson *et al.*, 2004), and although IgA, IgM and IgG antibodies are detected, titres are low or short lived (Lawson *et al.*, 1988, Holyoake *et al.*, 1994b). The presence of serum IgM antibody to *L. intracellularis* can be used to diagnose current disease in growing pigs but will not identify recovered animals (Knittel *et al.*, 1998).

1.2.3 Treatment

Difficulty in diagnosis of the disease in live pigs had led to the widespread use of preventative anti-microbial medications in the feed without a rational basis for their use (McOrist *et al.*, 1995c). When it became possible to cultivate *L. intracellularis* *in vitro* a range of drugs were tested for ability to prevent or control intracellular infection of rat enterocytes. European strains of *L. intracellularis* proved particularly sensitive to chlorotetracycline, penicillin, erythromycin, virginiamycin and difloxacin (minimum inhibitory concentration [MIC] $\leq 1 \mu\text{g/ml}$). Tiamulin and tilmicosin were less active ($< 4 \mu\text{g/ml}$) and other drugs (lincomycin, tylosin and the aminoglycosides) showed little activity ($> 32 \mu\text{g/ml}$) (McOrist *et al.*, 1995c).

Latterly, tylosin phosphate was shown to be effective as a preventative measure, when administered prior to exposure, and also in treatment of established infections when administered 7 days post exposure. However, a subsequent study showed tylosin treated pigs to shed as heavily as untreated controls giving rise to speculation over the efficacy of the drug (McOrist *et al.*, 1997a).

Although antibiotics can prove effective in controlling *L. intracellularis*, widespread use is unfavourable and alternative control methods are necessary. Recently a commercially attenuated vaccine strain of *L. intracellularis* has become available (Enterisol[®] Ileitis, Boehringer Ingelheim Vetmedica GmbH). Clinical trials on large-scale swine production farms in the USA showed a 39.9% reduction in mortality rates in vaccinated individuals with reduced medication programmes compared to fully medicated, non-vaccinated controls. Post mortem antigen detection (via IHC) or genomic detection (PCR) determined that vaccinated individuals were eight times less likely to be infected with *L. intracellularis*. This was translated into a significantly improved daily weight gain, on average 6.1% ($p < 0.05$) (Kolb, 2004). Some animals have been shown to remain seropositive up to 13 weeks after exposure to vaccine strain (Guedes and Gebhart, 2003a). However, optimism regarding the efficacy of such treatment must be guarded, as with all attenuated vaccines there is the ever present possibility of a return to virulence and the search for a permanent solution to the problem of proliferative enteropathy must continue.

1.2.4 Epidemiology

A recent study, using IFAT, detected *L. intracellularis* infection in 90% of the herds tested (Chouet *et al.*, 2003), suggesting infection is highly prevalent. A variable but generally small proportion of animals, around 1%, will be clinically affected with PHE (Lawson and Gebhart, 2000). Morbidity is usually higher with 5% to 20% of animals failing to achieve market weight (McOrist *et al.*, 1995c).

Smith *et al.*, (1998) found slatted or mesh flooring, often found in post weaning accommodation, to be a significant risk factor for PE probably due to insufficient cleaning (Smith *et al.*, 1998). Continuous production and purchased feed have also been highlighted as risk factors for *L. intracellularis* infection (Stege *et al.*, 2001). In the past PE has been associated with high-health herds although this observation is yet to be statistically supported (Lawson and Gebhart, 2000). Herd size was found to be a significant risk factor by Holyoake *et al.*, (1994) and Smith *et al.*, (1998) who showed an increased risk of PE in herds of 80 or 500 sows respectively (Smith *et al.*,

1998, Holyoake *et al.*, 1994a). Spread of disease among animals will be aided by reportedly long shedding periods, which have been measured up to 10 weeks in some cases (Smith and McOrist, 1997). Most *L. intracellularis* infected pigs were shedding at 10-12 weeks of age and shed for 2-6 successive weeks (Stege *et al.*, 2004). Furthermore, suckling animals, which were previously considered to be immune to infection due to the presence of colostral antibodies, are now known to be susceptible to disease and this may have important epidemiological implications (Moller *et al.*, 1998). There is evidence of an association between the occurrence of PHE and nucleus herds (Smith *et al.*, 1998). The epidemiology of infection in such cases of PHE is unclear where there has been no introduction of new animals and may be attributed to introduction via another species, symptomless infection, absence of contact with other pigs or elimination of exposure to infection through medication may all play a role on such occasions (Lawson and Gebhart, 2000). However, prevalence of PE in weaning pigs and growing animals has been suggested to represent an endemic herd infection and the absence of treatment or management procedures that modify the disease process (Lawson and Gebhart, 2000).

1.3.1 Identification and classification of *Lawsonia intracellularis*

Rowland *et al.*, (1973) were the first to describe the presence of bacteria in the proliferative lesions of PE. For several years the bacterium in question was thought to be a member of the *Campylobacter* spp., and was referred to as Campylobacter-like organism (CLO), members of which genus had been cultivated from PE lesions (Gebhart *et al.*, 1983, Lawson and Rowland, 1974, Rowland and Lawson, 1974). However, none of the *Campylobacter* spp. cultivated from the lesions was capable of reproducing the disease. It was not until Lawson *et al.*, (1985) immunized rabbits with a fairly pure preparation of the intracellular bacteria and produced antibody that failed to recognise any of the *Campylobacter* spp. that the intracellular bacteria were recognised as a separate entity (Lawson *et al.*, 1985). Initially this distinct bacterium was referred to as *Ileobacter intracellularis* until finally the name *Lawsonia intracellularis* was formalized (Gebhart *et al.*, 1993). Lawson and colleagues demonstrated that this organism could only be cultured *in vitro* in association with

mammalian epithelial cells and subsequent work has confirmed this organism is obligately intracellular (Lawson *et al.*, 1993, Lawson *et al.*, 1995, Stills, 1991).

Desulfovibrio species were originally believed to be the most closely related to *L. intracellularis* based on 16s rDNA sequence comparisons (91% similarity). However, biological differences led to a more appropriate classification of *L. intracellularis* as a separate genus in the delta division of the *Proteobacteria* (Gebhart *et al.*, 1993), taxonomically distinct from other intracellular bacteria. Subsequent sequence comparisons showed a 92% 16S sequence identity with the free living anaerobic human pathogen, *Bilophila wadsworthia* (Dale *et al.*, 1998, Sapico *et al.*, 1994). The distinction of *L. intracellularis*, both in biological and taxonomical terms, would perhaps point towards the expectation of equally distinct pathogenic mechanisms.

1.3.2 Growth of *L. intracellularis* in vivo and in vitro

1.3.2.1 Colonisation of the intestinal epithelium

L. intracellularis can be cultured *in vitro* as an obligately intracellular pathogen in intestinal epithelial cell cultures under microaerobic conditions. The requirement for reduced oxygen tension *in vivo* is also apparent *in vitro*, with optimal growth observed at a reduced oxygen tension of 8% O₂ (Lawson *et al.*, 1993, Lawson *et al.*, 1995, Stills, 1991). This may explain *L. intracellularis* tropism for the crypts within the porcine intestine, where the dissolved oxygen tension is between 5 and 10% (Hillman *et al.*, 1993). A mouse model of infection has also been described (Smith *et al.*, 2000). However, initial attempts at reproducing the disease in gnotobiotic pigs using a pure bacterial inoculum proved unsuccessful (McOrist *et al.*, 1993). At the time this was attributed to differences in the oxygen tension and crypt architecture of gnotobiotic piglets. It was subsequently confirmed that this was the case and infection of gnotobiotic pigs with *L. intracellularis* was successful in the presence of even a minimal gut flora (McOrist *et al.*, 1994). The presence of gut flora has also been shown to be essential for colonization of *Serpulina hyodysenteriae*, an anaerobic spirochaete pathogen of the porcine large intestine (Harris and Glock,

1981). Presence of gut bacteria increases cell turnover and cellularity of the mucosa and lamina propria which may therefore enhance availability of susceptible cells for infection (McOrist *et al.*, 1994).

Following oral exposure to *L. intracellularis* 5 days elapse before bacteria are detectable in the gut epithelial cells (Figure 1.1) (Smith and Lawson, 2001). Entry of the organism into enterocytes is closely correlated with the onset of epithelial hyperplasia (Lawson *et al.*, 1985, Rowland and Rowntree, 1972). The immature enterocytes apparently fail to mature and continue to undergo mitosis. This leads to the formation of elongated and branched glands (since the cells are not shed) and the absence of secondary goblet cells (Rowland and Lawson, 1974). Data from cell culture has also suggested that *L. intracellularis* is highly adapted for growth in dividing cells (Lawson *et al.*, 1993) Visible hyperplasia of enterocytes is seen from 10 days p.i. and gross lesions from 21 days (McOrist *et al.*, 1994).

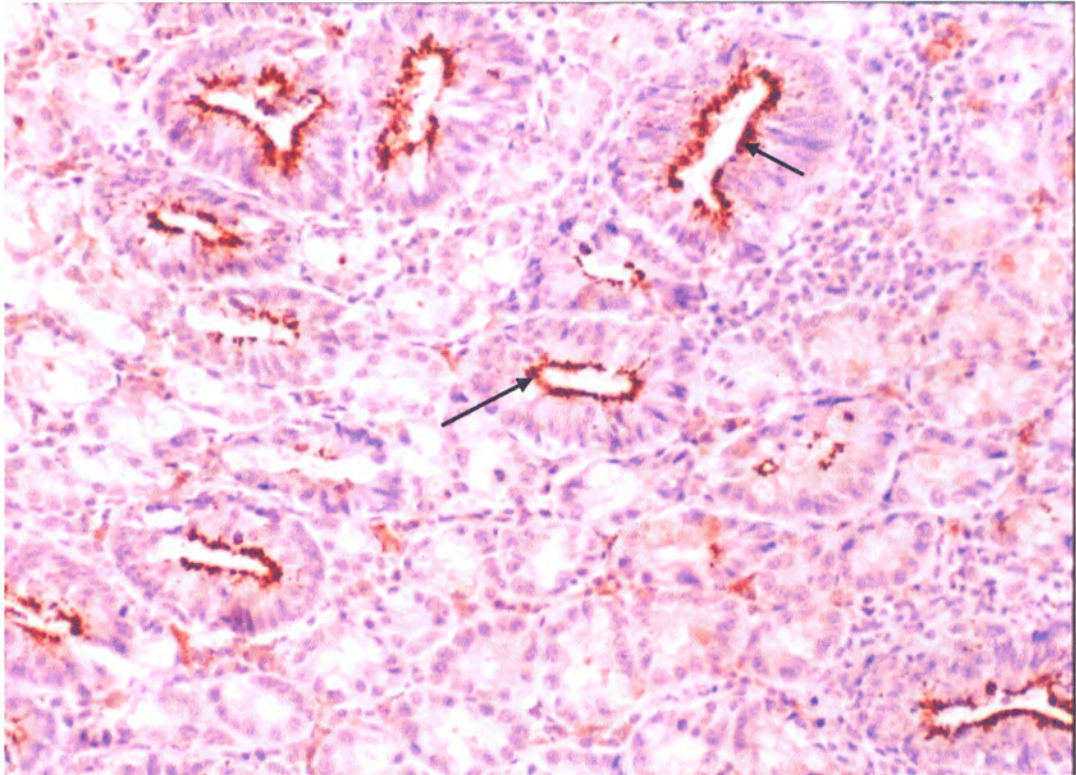


Figure 1.1: Immunohistochemical identification of *L. intracellularis* in crypts from porcine ileal tissue. Arrows indicate the bacteria localising to the sub-apical region of intestinal epithelium.

1.3.2.2 Attachment and Entry

This is a crucial stage for obligate intracellular bacteria and such bacteria targeting the epithelium utilize multiple mechanisms to gain entry to host cells e.g.

Chlamydiae (discussed in Section 1.7.2). *L. intracellularis* is non-motile and, while no fimbriae or pili have been observed, a long, single, uni-polar flagellum has been seen via electron microscopy in several isolates (Lawson and Gebhart, 2000).

Flagella are known to confer motility via chemotaxis through mucus to the crypt lumen and crypt basal/progenitor cells (Macnab, 1996, Bourret and Stock, 2002).

These structures have also been shown to be involved of adhesion of *C. difficile*, *P. aeruginosa* and some enteropathogenic *E. coli* to epithelial cells and for invasion of such by several bacteria including *Yersinia enterocolitica* (Ramos *et al.*, 2004).

Association of *L. intracellularis* with the cell membrane of enterocytes has been observed *in vitro* up until three hours following initial challenge of the cell monolayer and *in vivo* studies have demonstrated entry of *L. intracellularis* from the crypt lumen following attachment to the microvillus brush border (Jasni *et al.*, 1994).

This evidence would indicate an adhesin is involved in attachment to host cells.

However, PCR with degenerate primers has found no homologues from the intimin/invasin family, involved in adhesion/invasion of several bacteria, in *L. intracellularis* (Lawson *et al.*, 1995, McOrist *et al.*, 1997c). Electron microscopy studies also found no evidence of an S-layer (McOrist *et al.*, 1997c), another structure commonly involved in bacterial adherence to the host epithelium (Sleytr and Beveridge, 1999).

Recently-entered *L. intracellularis* have been observed to show some morphological association with small pits or vesicles of the cell membrane (McOrist *et al.*, 1995c), similar to the association between bacterial entry and clathrin-coated pits described in both *Chlamydia trachomatis* and *Chlamydophila psittaci* (Reynolds and Pearce, 1991). Thus despite importance of adherence to *L. intracellularis* the only possible adhesion characterized to date is *Lawsonia* surface antigen A (LsaA). *In vitro* attachment studies have shown that the monoclonal antibody VPM53, specific for LsaA, is capable of reducing attachment/entry of all tested strains (McOrist *et al.*, 1997c).

1.3.2.3 Escape from the phagosome

There are many pathogens that will spend their entire intracellular life enclosed within the endocytic vesicle. However, this depends on the ability of the pathogen to control the development of the endocytic vesicle so that it fails to mature into a hydrolase-rich phagolysosome. *Mycobacteria*, *Salmonella*, *Brucella*, *Chlamydia*, *Coxiella*, *Leishmania* and *Toxoplasma* have all developed unique strategies for controlling the biogenesis of their endocytotic vesicles (Amer and Swanson, 2002). For example, *Toxoplasma gondii* rapidly removes from its vacuole the proteins that were derived from the host plasma membrane (Suss-Toby *et al.*, 1996), thereby depriving the vacuole of recognition signals required for fusion with endocytic compartments and rendering the membrane truly non-fusogenic (Vogel and Isberg, 1999). Other strategies such as inhibiting phagosome acidification or by adapting to survive in the acidic conditions have also been adopted by other pathogens (Meresse *et al.*, 1999).

When *L. intracellularis* enters the cell individual bacteria are initially enclosed in a vacuole where they may divide and/or fuse with other vacuoles containing bacteria (McOrist *et al.*, 1995b). However, 24 hours later all bacteria will have escaped from the endocytic vacuole in which they were internalised. Following escape from the endocytic vacuole, *L. intracellularis* exists freely within the apical cytoplasm of crypt epithelial cells (McOrist *et al.*, 1994), although reasons for this remain unknown. As such *L. intracellularis* joins the ranks of intracellular pathogens of the genera *Listeria*, *Shigella*, *Rickettsia* and *Trypanosoma*, who, following uptake into the host cell, are known to destroy the endocytic vacuole and multiply in the nutrient-rich environment of the cytoplasm (Almeida-Campos *et al.*, 2002). Escape from the phagosome typically involves membranolytic proteins, the most studied and well-established example being listeriolysin O (LLO), a pore forming cytolysin produced by *Listeria monocytogenes*. LLO-negative mutants are non-virulent, failing to escape from primary vacuoles (Portnoy *et al.*, 1992b, Gedde *et al.*, 2000). The clear role of LLO in vacuolar rupture was established when an attenuated *Salmonella dublin aroA* strain expressing an active hybrid cytolysin consisting of LLO and the C-terminal secretion signal of *E. coli* haemolysin. The haemolytic *S. dublin* strain

was shown to be partially released into the cytoplasm of the host cell following uptake by J774 macrophage cells, whereas the nonhaemolytic *S. dublin AroA* strain remained in the cytoplasm (Gentshev *et al.*, 1995). Recently it was shown that LLO is also required for the escape from the secondary double-membrane vacuole formed upon cell-to-cell spread (Gedde *et al.*, 2000). Likewise, *Rickettsia* invade the cytoplasm, and eventually the nucleus by involvement of a phospholipase (Heinzen *et al.*, 1999). Bacterial phospholipases comprise a diverse group of proteins that have a range of effects *in vivo* and *in vitro* ranging from minor alterations in cell membrane composition to cell lethality at low concentrations (Songer, 1997). *L. monocytogenes* also uses phospholipases to escape the vacuole (Cossart and Lecuit, 1998). It encodes two distinct phospholipases, a phosphatidylinositol-specific phospholipase C (PI-PLC; encoded by *plcA*) (Camilli *et al.*, 1991) and a 'broad-activity' phosphatidylcholine-specific phospholipase C (PC-PLC; encoded by *plcB*) (Vazquez-Boland *et al.*, 1992). LLO and broad-range phospholipase (*plcB*) double mutants are unable to escape from the Henle 407 human epithelial cell line (Marquis *et al.*, 1995). However, *L. monocytogenes plcA* mutants are partially defective in disrupting bone marrow derived macrophage vacuoles, suggesting that PI-PLC may play an accessory role in lysing the primary phagosome (Goldfine *et al.*, 1995). A model has been suggested whereby the pore-forming toxin acts first on the membrane resulting in exposure of phospholipids which are then degraded by the phospholipases (Wadsworth and Goldfine, 1999).

It was initially proposed that LsaA from *L. intracellularis*, as a member of the TlyA haemolysin family, in addition to being involved in adherence may have a similar function. However, it is now thought to be devoid of haemolytic activity (McCluskey *et al.*, 2002). Such a dual function is evident in *Shigella* where the macropinocytotic vacuole containing the bacterium is rapidly lysed by the IpaB invasin, which acts as a membranolytic toxin in the phagosome membrane, releasing *Shigella* into the host cell cytoplasm (High *et al.*, 1992). The lysis of the phagosome may also involve IpaC, which is able to disrupt phospholipid membranes upon insertion of its hydrophobic regions (De Geyter *et al.*, 1997, Kuelzto *et al.*, 2003).

1.3.2.4 Replication and Spread

One essential aspect in the life cycle of any intracellular pathogen is its ability to enter and leave host cells to facilitate its continued spread. The microbial pathogens *Listeria*, *Shigella* and *Rickettsia*, following escape from the endocytic vacuole, have evolved mechanisms to utilise pre-existing components of the actin cytoskeleton to generate their own motility within and between cells. Once in the cytoplasm each of these microbes recruits to its surface host actin and other cytoskeletal proteins and activates the assembly of an actin tail. The continuous assembly of which provides sufficient force to propel the organisms through the cytoplasm of the infected cell and into adjacent cells. Each organism has evolved protein(s) which bind and activate one or more components of a mammalian actin assembly pathway thereby inducing the downstream events which bring about actin polymerisation (Goldberg, 2001). For example, *L. monocytogenes* surface protein ActA is responsible and sufficient for actin-based motility and similarly the *Shigella* bacterial surface protein IcsA. Both proteins subvert the normal regulatory systems that control actin polymerisation, turning them to their own advantage (Frischknecht and Way, 2001). The ability to spread directly between cells allows pathogens to avoid many components of the host immune response (Goldberg, 2001).

In contrast, there is no evidence for actin based motility in *L. intracellularis*, rather it is partitioned alongside cellular components as the cell divides (Collins *et al.*, 1996, Lawson *et al.*, 1993). Infected cells therefore populate the epithelium in the manner of normal epithelial replacement (Gebhart *et al.*, 1993). In cell culture, most heavily infected cells will be present in such foci indicating that transfer of infection between cells, other than by cell division, is a limited event in the initial stages of infection (Smith and Lawson, 2001).

Eventual bacterial release is thought to occur via balloon-like protrusions of epithelial cells, enabling spread of the bacterium to new cells and other animals via the faeces. The presence of bacteria-filled protrusions has been observed in both tissue culture (Lawson *et al.*, 1993, McOrist *et al.*, 1996) and in the ileum of experimentally infected pigs (McOrist *et al.*, 1996). In the latter, aggregates of

Lawsonia have been observed congregating at the basal cytoplasm of epithelial cells in pigs exhibiting the haemorrhagic form of the disease (MacIntyre *et al.*, 2003).

1.4.1 Immunopathology of *L. intracellularis* infection.

The lesions produced during PE are distinct from other bacterial induced pathology.

In pigs PE is characterized by a thickening of the mucosa in the lower ileum and colon, the extent of which varies according to the intensity of the disease.

Interestingly, however, there is a marked absence of inflammation or immune response to the infected tissue (McOrist *et al.*, 1992) neither is there evidence for any dissemination of bacteria beyond the mucosa (Smith *et al.*, 2000).

Accumulation of IgA and IgM around the site of bacterial infection in enterocytes is observed in proliferative enteropathy and is a feature of other gastrointestinal infections. IgA has also been observed to accumulate within enterocytes adjacent to intracellular bacteria (Lawson *et al.*, 1979), a novel phenomenon. The reason for the accumulation is not apparent, however, it may be due to a deleterious effect of *L. intracellularis* on the ability to process the immunoglobulin via the secretory pathway to the luminal surface. Alternatively the build-up may be specific to the site of infection (McOrist *et al.*, 1992). *L. intracellularis* infection also causes an apparent down regulation of villus epithelial-associated CD3^{+ve} T-cell numbers in heavily infected animals which is most noticeable at day 14 post infection. This loss is expected to be most detrimental to the CD8⁺ T cell population, since 84% of the villus associated T cells are CD8⁺. This is likely to affect the presentation of antigens via the MHC class I pathway which will also affect the ability of the host to defend against intracellular pathogens (MacIntyre *et al.*, 2003). Furthermore, proliferating enterocytes in PE show poor major histocompatibility complex type II expression possibly leading to a depressed immune response. It has been suggested that the loss of this antigen presenting function in infected enterocytes may provide a

protected niche for the growth and division of *L. intracellularis* (McOrist *et al.*, 1992).

An increased macrophage presence in crypts showing hyperplastic lesions, compared to those infected but non-hyperplastic, has also been observed. The greatest increase occurred in the most severely infected animals and was associated with the haemorrhagic form of the disease, PHE. This localisation could be due to the release of chemo-attractants from hyperplastic lesions, or conversely, the release of cytokines by large numbers of macrophages could be the key factor in the development of hyperplasia. Large numbers of *L. intracellularis* have been observed inside macrophages. An accumulation which may be the result of natural phagocytosis or it may indicate that they, like other enteropathogenic bacteria (e.g. *Salmonella*, *Listeria*), may be able to proliferate inside these cells possibly inducing the release of cytokines responsible for increased vascular permeability and haemorrhage (MacIntyre *et al.*, 2003). This theory is supported by the lack of marked tissue destruction or mucosal ulceration associated with haemorrhagic lesions which suggests that some alteration to blood vessel structure and viability may be responsible (McOrist *et al.*, 1992).

1.4.2 Bacterial-induced dysregulation of epithelial proliferation

The group of enterovirulent bacteria that cause cellular proliferation is small but of these *L. intracellularis* is somewhat unique. The pathology of *L. intracellularis* infection bears some resemblance to that observed in mice infected with *Citrobacter rodentium*, the aetiologic agent of transmissible murine colonic hyperplasia (TMCH) (Luperchio *et al.*, 2000). Infection with *C. rodentium* is characterised by colonic hyperplasia primarily at the distal end of the colon and spreading up towards the caecum with increasing severity of infection (Schauer *et al.*, 1995). Experiments with *C. rodentium* have shown that the severe mucosal thickening is mediated by an immune response involving a CD4⁺ T cell infiltrate and a distinct Th1 mucosal immune response and characterised by increased levels of IL-12, interferon- γ , and TNF- α mRNA (Higgins *et al.*, 1999b). The crypt distortion and branching is identical to that seen in IBD (Irritable Bowel Disease), where it can also be attributed

to T cell responses, and it appears this is a typical response of the colonic epithelium to this stimulation (Higgins *et al.*, 1999a). Mice lacking a TNF- α receptor show higher bacterial burdens, increased IL-12 production and greater hyperplasia, suggesting that TNF- α may modulate the Th1 mucosal response and serve to limit pathology in wild type animals. However, infection of IFN- γ R⁻ mice with *C. rodentium* failed to produce hyperplasia (Higgins *et al.*, 1999) indicating the potent role of this cytokine in induction of the pathology. A Th1 immune-induced gastric mucosal hyperplasia also occurs as a consequence of *H. pylori* infection and is thought to be an important precursor to intestinal metaplasia and gastric cancer (Moss, 2001, Peek *et al.*, 1997). It was consequently proposed that IFN- γ may play a similar role in controlling *L. intracellularis* infection. However, its role in the infections described above appears to be the converse of the protective role it was found to play in *L. intracellularis* infection. When challenged with *L. intracellularis*, IFN γ R⁻ mice appear to be more easily and severely infected, a greater proportion of crypts are infected, and the hyperplasia is much more marked compared to that of wild-type mice. The means by which IFN- γ exerts this control however is unclear (Smith *et al.*, 2000).

Evidently, the role of the Th1 immune response in *L. intracellularis* infection is complex since this may contribute to both pathology (hyperplasia) and cell-mediated immunity against *L. intracellularis*. These pathogens obviously employ different mechanisms to elicit similar effects although they may all benefit from the resulting hyperplasia. It has been suggested that thickening of the mucosa following the initial infection would benefit the pathogen by increasing the surface area of crypts. This would result in an increased surface area for colonisation and an escalating rate of cell renewal ensures a fresh supply of cells for infection. Furthermore, the increased shedding of colonised epithelial cells would augment the transmission of organisms (Higgins *et al.*, 1999a). This certainly holds in the case of *L. intracellularis* in which a clear link between infection, cellular and bacterial proliferation has been established.

Alterations in epithelial cell surface markers, markers of proliferation/apoptosis, and in host cell protein phosphorylation have been noted in pigs naturally and

experimentally infected with *L. intracellularis* (Smith and Lawson, 2001). It is plausible that *L. intracellularis* may downregulate or interfere with apoptotic pathways in some way thereby contributing to the proliferation of epithelial cells (McOrist *et al.*, 1994, MacIntyre, 2001). Although the details are still a subject of much study, a decrease in apoptotic activity during *H. pylori* infection is also expected to contribute to carcinogenesis (Moss, 2001). It was recently shown that both c-Myc and Bcl-2 are upregulated in hyperplastic crypts of *L. intracellularis* infected gut epithelium (MacIntyre, 2001). The c-Myc proto-oncogene has been assigned roles in cellular proliferation, growth differentiation and apoptosis (Facchini and Penn, 1998), where it is known to be capable of increasing the rate of cellular proliferation and also apoptosis (Askew *et al.*, 1991). Conversely, Bcl-2 and its relatives are key players in the downregulation of apoptosis (Bonnetoy-Berrard *et al.*, 2004). Therefore upregulation of both these factors will result in a net increase in cellular proliferation and may be the basis of the hyperlasia observed in *L. intracellularis* infection (MacIntyre, 2001).

Crypt distortion and branching have also been associated with infections with the enteroinvasive pathogens *Shigella* and *Salmonella* (Sachdev *et al.*, 1993). *Shigella* infection in the colon is also associated with a decrease in goblet cells numbers and proliferation of crypt epithelial cells at sites distal from the primary ulcers, implicating a role for a secreted factor in the development of these secondary lesions (Islam *et al.*, 1994). Although goblet cell depletion, due to a loss of cellular differentiation, is also a recognized consequence of *L. intracellularis* infection (Rowland and Lawson, 1974) no secreted factors are thought to be involved in *L. intracellularis* induced proliferation where the contrast between regions of diseased tissue and adjacent healthy areas can sometimes be quite distinct (Lawson *et al.*, 1995).

The work summarized above provides some indication of the factors that may be involved in bringing about mucosal hyperplasia, however, much remains undefined for *L. intracellularis*. To date, there are only a handful of *L. intracellularis* antigens

which have been characterised and only one main potential virulence determinant has been identified.

1.5 *L. intracellularis* antigens and virulence determinants

The genome of *L. intracellularis* is currently being sequenced. Once it is published it will be possible to deduce factors involved in pathogenesis. Currently few virulence determinants have been described in *L. intracellularis*. Dale *et al.*, (1998) used sera from *L. intracellularis*-immunised rabbits to screen a partial genomic library. Two sero-reactive clones were identified one of which expressed proteins of 10 and 60 kDa. The sequence of the insert from this clone revealed ORF's with similarity to the *groES/EL* operon of *E. coli*, the 50S ribosomal proteins L21 and L27 of *E. coli*, a GTP-binding protein of *Bacillus subtilis* and a possible protoporphyrinogen oxidase, HemK of *E. coli* (Dale *et al.*, 1998). Our own work has used degenerate oligonucleotide PCR to identify genes of a type three secretion system (Alberdi *et al.*, manuscript in preparation). Polyacrylamide gel electrophoresis of whole cell *L. intracellularis* proteins has shown major bands of molecular weight 53, 42, 37, 30KDa and minor bands of 47, 25 and 27KDa. Monoclonal antibodies against the latter two have been produced and these react in immunoblotting and in immunofluorescence experiments (McOrist and Lawson, 1987). The use of immunoelectron microscopy has shown the 25 - 27 kDa antigens to lie in the bacterial outer membrane (McOrist *et al.*, 1989b, McOrist *et al.*, 1989a). These are also the main proteins identified in immunoblots analysed with hyperimmune rabbit polyclonal antibody raised against bacteria in adjuvant (Lawson *et al.*, 1979, McOrist and Lawson, 1989). Semirandom PCR chromosome walking has led to the sequencing of the ORF which incorporates the larger of the two immunodominant surface antigens, now referred to as LsaA. Besides the *groE* operon (Dale *et al.*, 1998), LsaA was one of the first *L. intracellularis* genes to be sequenced and subsequent comparison with protein and nucleotide databases have shown that LsaA has up to 53.3% identity sequence homology to known members of the TlyA family of bacterial haemolysins (McCluskey *et al.*, 2002).

The recent research devoted to the development of new monoclonal antibodies has also led to the identification of a lipopolysaccharide 21 kDa immunodominant surface antigen (Boesen *et al.*, 2005) and five OMP's of 77, 69, 54, 42, and 36 kDa (Guedes and Gebhart, 2003b).

1.6 Bacterial Haemolysins/Cytolysins

In 1981 α -toxin from *Staphylococcus aureus* was the first bacterial toxin recognised to form pores in the membranes of mammalian cells (Fussle *et al.*, 1981). Today pore-forming proteins are known to be produced by the majority of bacterial pathogens (Bhakdi *et al.*, 1998) and are recognised as important virulence factors of many pathogenic Gram-negative and Gram-positive bacteria. Generally they are referred to as haemolysins due to their ability to lyse red blood cells, however, this is somewhat of a misnomer as many have no clearly designated role and may target a variety of cell types *in vivo* hence the term cytolysin. Several families of cytolysins have now been established based on their mechanism of cell membrane disruption whether it be: pore forming, enzymatic (which includes phospholipases), or solubilization of membranes by detergent-like actions (Ludwig, 1996). The TlyA family are thought to use the former mode of action and penetrate the host cell wall via a pore formed by oligomerisation of several toxin subunits (Stabler, R.A., PhD. Thesis, LSHTM). The two main families of pore-forming bacterial haemolysins are the thiol-activated cytolysin (TACY) family, found in over 20 Gram-positive bacteria, (Alouf, 1980) and the RTX (Repeats in ToXin) family, produced by several unrelated Gram-negative bacteria (Strathdee and Lo, 1989, Welch, 1991).

1.6.1 Properties of the TACY family of pore forming cytolysins

The group, which includes *Bacillus*, *Clostridium*, *Listeria* and *Streptococcus*, is named for the sensitivity of their cytolytic activity to oxygen and their activation by reducing compounds (thiol activation). However, the physiological importance of

this phenomenon has been contested. Members of the TACY family have between 30% and 60% identity in amino acid sequence as well as an invariant undecapeptide sequence (ECTGLAWEWWR) close to the C-terminus. Despite the host specificity of a number of TACYS, cholesterol is the major target receptor (Johnson *et al.*, 1980) and free cholesterol can inhibit toxin function by interfering with the oligomerization process after binding to target cells (Jacobs *et al.*, 1998). Binding of membrane cholesterol is probably the initial interaction of the toxin with the membrane (Ohno-Iwashita *et al.*, 1988). This interaction between toxin and cholesterol induces a conformational change in the toxin monomer, presumably converting it from a soluble molecule to a hydrophobic molecule ready for membrane insertion (Jacobs *et al.*, 1998). Toxin molecules will thus collect at cholesterol-containing sections of the membrane thereby promoting oligomerisation and pore formation (Ohno-Iwashita *et al.*, 1988). As for other pore-forming cytolysins, oligomerisation of toxin molecules may take place in the proximity of the membrane prior to insertion (Rossjohn *et al.*, 1997), however, it has been suggested that this would be energetically unfeasible due to the large size of pores formed by TACYs (Palmer *et al.*, 1998). It is more likely that toxin monomers insert partially into the membrane where new monomers are added to the growing oligomers as they insert (Billington and Jost, 2004). This mechanism of pore formation would account for the ability of small oligomers to cause cytoplasmic leakage (Bayley, 1997) and the presence of arc-shaped oligomer ‘intermediates’ by electron microscopy (Palmer *et al.*, 1998). The pore can consist of 40 – 80 monomers, and with a pore reaching up to 30nm in diameter it is particularly large and can allow passage of cell macromolecules (Billington and Jost, 2004).

The only intracellular bacterium to produce a TACY is *Listeria monocytogenes*, which produces listeriolysin O (LLO). LLO enables escape from the phagolysosome preventing destruction of the bacteria in lysosomal degradation pathway (Portnoy *et al.*, 1992a). Lysis of host cells is also used for defence against the immune system, for example perfringolysin O (PFO) from *C. perfringens* is cytotoxic for murine macrophages and bovine and ovine polymorphonuclearleukocytes (PMNs) (Jost *et al.*, 1999). However, the major effects of TACYs in pathogenicity may be subtler

than direct destruction of host cells by lysis. At sublytic concentrations several members of the family have been shown to be capable of interference with host defense and immune cell function and modulation of the inflammatory response (Billington and Jost, 2004). *Streptococcus pneumoniae* pneumolysin (PLY) for example can directly activate the classical complement pathway by binding directly to the Fc region of human IgG (Mitchell and Andrew, 1997) possibly leading to direct complement-mediated attack on host cells. This activity is associated with increased persistence of pneumococci during the later stages of infection of the mouse lung (Mitchell *et al.*, 1991). Many others TACYs are capable of cytokine induction, Streptolysin O induction of IL-1 β , IL6 and IL8 from human keratinocytes, and IL-1 β and TNF α from monocytes (Hackett and Stevens, 1992, Ruiz *et al.*, 1998). LLO has been shown to induce the production of IL-1, IL-12, and TNF- α by macrophages (Nishibori *et al.*, 1996, Yoshikawa *et al.*, 1993), as well as to express an immunodominant H-2d-restricted epitope that is able to induce a specific MHC class I-restricted CD8⁺-mediated protection against *L. monocytogenes* (Sirard *et al.*, 1997). In addition LLO can direct other antigen molecules to the intracellular compartment that leads to MHC class-I restricted presentation (Brunt *et al.*, 1990). Such manipulation of the immune system may prevent clearance of the bacteria during the early stages of infection (Billington and Jost, 2004) and may be equally important for intracellular bacteria.

1.6.2 Properties of the RTX family of pore-forming cytolytins

The large RTX-toxin family is comprised of multi-domain Gram-negative bacterial pore-forming exotoxins, products of a conserved gene cluster found in the *Actinobacillus*, *Bordetella pertussis*, *Escherichia coli*, *Mycoplasma hyopneumoniae*, *Neisseria meningitidis* and *Pasteurella haemolytica*. The operon consists of five genes: the toxin structural gene (*rtxA*) and three transport related proteins (*rtxB*, *rtxD* and *rtxE*). The family name is derived from a repeat domain in the toxin protein, RtxA. The nonapeptide glycine- and aspartate- rich motif has the consensus

sequence GGXGXDX[L/I/V/W/Y/F]X and is repeated 6 to 40 times. The genes are usually transcribed in the order CABD, *rtxE* is often separate in the genome and also referred to as *tolC*. Although no signal sequences have been identified the, toxins are transported to the cell surface by transport proteins encoded by the *rtxB* and *rtxD* genes (Lally *et al.*, 1999). The genes have also been located on transmissible plasmids and have non-characteristic DNA content, e.g. *E. coli* haemolysin RTX operon has a G+C content of 39%, 10% lower than the *E. coli* genome average, and utilises *E. coli* preferred codons. This suggests that there has been horizontal transmission of the RTX operon (Welch, 1991). The toxin is activated by RtxC through fatty acylation and is essential for biological function (Stanley and et al, 1994). Monoclonal antibodies targeted against an epitope located near a putative acylation site have been shown to abolish cytolytic/haemolytic ability suggesting the acyl group could be the initial site of interaction between RTX toxins and their targets cells (Lally *et al.*, 1999). In order to mediate their toxic effects RTX toxins recognize and bind to a $\beta 2$ integrin on the surface of target cells (Lally *et al.*, 1997). Following binding, pore formation is thought to occur via the energetically favorable spontaneous insertion of the hydrophobic amino acid groups into the membrane. The insertion process is independent of signal sequences, molecular chaperones, or ATP/GTP but usually requires oligomerisation of the toxin molecules to provide the energy to penetrate the lipid bilayer (Bhakdi *et al.*, 1998). As with the TACY family, RTX toxins are water soluble despite their ability to insert into the hydrophobic lipid bilayer of the cell membrane. This is thought to be achieved by what has been described as a “molten globule” state in which the hydrophobic residues normally concealed in the proteins interior are exposed, due to conformational changes following binding, to allow for insertion into the host cell membrane (Lally *et al.*, 1999).

Although RTX family members are homologous there is considerable difference in cellular tropism between family members and the family is subdivided into two groups based on specificity. The first group are able to bind and lyse a wide variety of cell types from different species, e.g. *E. coli* α haemolysin and *Actinobacillus pleuropneumoniae* ApxIA which are toxic to erythrocytes from a wide variety of species, chicken embryo fibroblasts, rabbit granulocytes and mouse fibroblasts. The

second group has a restricted range of target cells, e.g. *Pasteurella haemolytica* leukotoxin which is specific to ruminant leukocytes and *Actinobacillus actinomycetemcomitans* leukotoxin that is specific to lymphocytes and granulocytes from humans, great apes and new world monkeys (Taichman *et al.*, 1984, Taichman, 1987). These variations may be accounted for by the varying interaction of RTX haemolysins and cytolytins with their target cells (Lally *et al.*, 1999). The repeat region of RTX cytolytins is involved in toxin-target cell interactions (Cruz, 1990, Lally *et al.*, 1994, Mc Whinney, 1992) and antibodies targeted against this region have been shown to inhibit killing of nucleated cells (Lally *et al.*, 1999). In general, however, RTX haemolysins appear to rely on electrostatic interactions to anchor the toxin onto the cell membrane and the repeat region does not seem necessary for cell lysis (Otero, 1995). Dependence on electrostatic interactions negates the necessity of a specific cellular receptor and as a result these toxins are more promiscuous in their ability to kill cells than RTX cytolytins. Interestingly, RTX cytolytin ability to lyse sheep red blood cells was not inhibited along with their cytolytic action suggesting that the conformational changes which accompany target cell binding differ to those involved in haemolysis (Lally *et al.*, 1999).

1.6.3 The TlyA family of pore forming haemolysins

The TlyA proteins form a novel family identified across a phylogenetically diverse range of bacteria and have been identified mainly through genomic sequencing. Several members have been found as part of an operon (Stabler, R.A., PhD. Thesis, LSHTM), members of the TlyA family are highly heterogeneous, even those with known haemolytic activity showing only around 40% identity in amino acid residues. However an S4 RNA binding domain at the N-terminus and homology to a methyltransferase, FtsJ, at the C-terminus has been observed in the majority of TlyA family members (Table 1.1).

Species	S4 Domain	FtsJ	Identity to LsaA (%)	kDa	pI
<i>Agrobacterium tumefaciens</i>	+	+	34	26.6	5.90
<i>Anabaena variabilis</i>	+	+	33	29.9	5.63
<i>Arabidopsis thaliana</i>	+	+	33	33.2	9.23
<i>Archaeoglobus fulgidus</i>	+	+	28	23.8	5.88
<i>Aquifex aeolicus</i>	+	+	36	29	9.09
<i>Bacillus anthracis</i>	+	+	37	31.1	5.96
<i>Bacillus cereus</i>	+	+	37	31.2	5.97
<i>Bacillus clausii</i>	+	+	32	29.7	6.61
<i>Bacillus halodurans</i>	+	+	35	30.5	8.79
<i>Bacillus licheniformis</i>	+	+	39	31	7.61
<i>Bacillus subtilis</i>	+	+	38	29.7	9.73
<i>Bacillus thuringiensis</i>	+	+	37	31.1	6.04
<i>Bartonella henslae</i>	+	+	39	27.1	6.86
<i>Bartonella quintana</i>	-	+	39	18.7	8.84
<i>Bdellovibrio bacteriovorus</i>	+	+	31	28	7.02
<i>Bifidobacterium longum</i> DJO10A	+	+	34	27.3	4.99
<i>Bifidobacterium longum</i> NCC2705	+	+	34	29.8	5.28
<i>Borrelia burgdorferi</i>	+	+	37	30	9.59
<i>Borrelia garinii</i>	-	+	43	27	9.25
<i>Brachyspira hyodysenteriae</i>	+	+	41	26.9	6.54
<i>Bradyrhizobium japonicum</i>	+	+	37	26	5.72
<i>Brevibacterium linens</i>	+	+	35	29	5.42
<i>Brucella abortus</i>	+	+	30	27.3	8.45
<i>Brucella melitensis</i>	+	+	30	27.3	8.45
<i>Brucella suis</i>	+	+	30	27.3	8.45
<i>Campylobacter coli</i>	+	+	36	28.8	8.12
<i>Campylobacter jejuni</i>	+	+	38	29.2	8.52
<i>Campylobacter jejuni</i> subsp. <i>jejuni</i> NCTC 11168	+	+	38	29.2	8.72
<i>Campylobacter lari</i>	+	+	37	27.96	7.56
<i>Campylobacter upsaliensis</i>	+	+	39	26.9	9.10
<i>Caulobacter crescentus</i>	+	+	34	25.7	5.99
<i>Chloroflexus aurantiacus</i>	+	+	38	27.7	9.68
<i>Chromobacterium violaceum</i>	+	+	33	26.6	6.84
<i>Clostridium acetobutylicum</i>	+	+	35	29.9	7.55
<i>Clostridium perfringens</i>	+	+	35	30	7.60
<i>Clostridium tetani</i>	+	+	41	30.9	7.61
<i>Clostridium thermocellum</i>	+	+	36	31.3	8.74
<i>Corynebacterium diphtheriae</i>	+	+	32	29.6	8.48
<i>Corynebacterium efficiens</i>	+	+	30	29.9	5.30
<i>Corynebacterium glutamicum</i>	+	+	30	29.7	6.03
<i>Crocospaera watsonii</i>	+	+	32	30	8.85
<i>Dechloromonas aromatica</i>	+	+	34	28.9	7.74
<i>Desulfitobacterium hafniense</i>	+	+	33	29.7	6.05

<i>Desulfotalea psychrophila</i>	+	+	32	26.4	8.22
<i>Desulfovibrio desulfuricans</i>	+	+	40	30.4	8.49
<i>Desulfovibrio vulgaris</i>	+	+	34	27.8	6.66
<i>Dictyostelium discoideum</i>	-	-	30	46.7	4.87
<i>Enterococcus faecalis</i>	+	+	36	30	5.95
<i>Enterococcus faecium</i>	+	+	40	29.5	6.88
<i>ErYthrobacter litoralis</i>	+	+	32	26.8	5.75
<i>Exiguobacterium sp. 255n15</i>	+	+	38	29.6	5.51
<i>Fusobacterium nucleatum</i>	+	+	42	30.7	9.09
<i>Geobacillus kaustophilus</i>	+	+	42	30.9	7.75
<i>Geobacillus stearothermophilus</i>	+	+	41	30.9	7.73
<i>Geobacter metallireducens</i>	+	+	36	28.1	6.45
<i>Geobacter sulfurreducens</i>	-	+	34	24.3	5.88
<i>Gloeobacter violaceus</i>	+	+	34	29.7	8.98
<i>Gluconbacter oxydans</i>	+	+	35	27.1	5.92
<i>Helicobacter hepaticus</i>	+	+	34	27.3	6.03
<i>Helicobacter pylori 26695</i>	+	+	39	26.6	9.19
<i>Helicobacter pylori J99</i>	+	+	36	26.6	8.95
<i>Lactobacillus acidophilus</i>	+	+	40	30.2	6.15
<i>Lactobacillus casei</i>	+	+	38	30.3	7.83
<i>Lactobacillus delbrueckii subsp. bulgaricus</i>	+	+	40	30	5.99
<i>Lactobacillus gasseri</i>	+	+	42	30	6.36
<i>Lactobacillus johnsonii</i>	+	+	40	30	7.12
<i>Lactococcus lactis subsp. cremoris</i>	+	+	38	29.7	6.79
<i>Lactococcus lactis subsp. Lactis</i>	+	+	38	26.7	6.03
<i>Lactobacillus plantarum</i>	+	+	34	29.7	5.84
<i>Lawsonia intracellularis</i>	+	+	100	26.6	8.85
<i>Leifsonia xyli subsp. xyli</i>	+	+	35	26.8	10.53
<i>Leptospira interrogans</i>	+	+	38	31.7	9.56
<i>Leuconstoc mesenteroides subsp. Mesenteroides</i>	+	+	36	31	6.03
<i>Listeria innocua</i>	+	+	36	30.4	6.62
<i>Listeria monocytogenes</i>	+	+	36	30.2	6.41
<i>Magnetococcus sp. MCn1</i>	+	+	35	16.9	9.69
<i>Magnetospirillum magnetotacticum</i>	+	+	38	41.3	7.67
<i>Mesoplasma florum</i>	+	+	38	30.6	8.99
<i>Mesorhizobium loti</i>	+	+	32	27.2	6.52
<i>Mesorhizobium sp. BNC1</i>	+	+	33	27.2	6.94
<i>Methanococcoides burtonii</i>	+	+	30	24.8	4.74
<i>Methansarcina barkeri str. Fusaro</i>	+	+	23	26.1	5.34
<i>Microbulbifer degradans</i>	+	+	33	27.9	5.44
<i>Micrococcus luteus</i>	-	+	36	23.9	7.50
<i>Moorella thermoacetica</i>	+	+	38	28.4	7.78
<i>Mycobacterium avium</i>	+	+	32	28	8.43
<i>Mycobacterium bovis</i>	+	+	34	28.1	9.74

<i>Mycobacterium leprae</i>	+	+	31	28.2	5.79
<i>Mycobacterium paratuberculosis</i>					
<i>Mycobacterium tuberculosis H37Rv</i>	+	+	34	28.1	9.74
<i>Mycobacterium tuberculosis CDCn1551</i>	+	+	34	28.1	9.74
<i>Mycobacterium ulcerans</i>	+	+	32	29.1	6.53
<i>Mycoplasma hyopneumoniae</i>	+	+	52	26.6	9.48
<i>Mycoplasma mobile</i>	+	+	50	27.4	9.35
<i>Mycoplasma mycoides</i>	-	-	36	11.1	9.55
<i>Mycoplasma mycoides subsp. mycoides</i>	+	+	41	17.2	9.20
<i>Mycoplasma penetrans</i>	+	+	41	28.3	9.23
<i>Mycoplasma pulmonis</i>	+	+	54	27.2	8.26
<i>Nocardia farcinica</i>	+	+	32	29.1	6.86
<i>Nostoc punctiforme</i>	-	+	34	26.8	5.97
<i>Nostoc sp. PCC 7120</i>	-	+	33	26.8	5.97
<i>Novosphingobium aromaticivorans</i>	+	+	38	32.7	8.98
<i>Oceanobacillus iheYensis</i>	+	+	35	30.6	5.95
<i>Oenococcus oeni</i>	+	+	37	22.6	9.21
<i>Pediococcus pentosaceus</i>	+	+	39	30	5.93
<i>Polaromonas sp. JS666</i>	+	+	30	35.4	5.15
<i>Prochlorococcus marinus subsp. marinus str. CCMP1375</i>	+	+	34	29.7	9.67
<i>Prochlorococcus marinus subsp. pastoris</i>	+	+	35	30.1	9.24
<i>Propionibacterium acnes</i>	+	+	33	27.7	6.08
<i>Rhodopseudomonas palustris</i>	+	+	34	26	6.60
<i>Rhodospirillum rubrum</i>	+	+	35	28.1	6.92
<i>Rickettsia akari str. Hartford</i>	+	+	40	28	7.08
<i>Rickettsia conrri</i>	+	+	39	28.4	8.82
<i>Rickettsia conrri str. Malish 7</i>	+	+	39	28.4	8.82
<i>Rickettsia prowazekii</i>	+	+	36	28.3	9.16
<i>Rickettsia prowazekii Madrid E</i>	+	+	36	28.3	9.16
<i>Rickettsia rickettsii</i>	+	+	39	28.3	8.95
<i>Rickettsia sibirica</i>	+	+	39		
<i>Rickettsia typhi</i>	+	+	35	28.4	8.83
<i>Rubrivivax gelatinus</i>	-	+	33	24.8	5.56
<i>Rubrobacter xylanophilus</i>	+	+	33	28.3	9.85
<i>Sinrhizobium meliloti</i>	+	+	31	25.9	5.84
<i>Streptococcus agalactiae</i>	+	+	36	31	6.35
<i>Streptococcus mutans</i>	+	+	37	30.6	6.47
<i>Streptococcus pneumoniae</i>	+	+	37	29.9	5.73
<i>Streptococcus pyogenes</i>	+	+	38	30.4	7.86
<i>Streptococcus suis</i>	+	+	37	30.1	5.86
<i>Streptococcus thermophilus</i>	+	+	38	31.8	5.74
<i>Streptomyces avermitilis</i>	+	+	31	28.3	6.35

<i>Streptomyces coelicolor</i>	+	+	38	31.7	9.56
<i>Symbiobacterium thermophilum</i>	+	+	36	27.3	7.73
<i>Synechococcus elongatus</i>	+	+	33	29.4	6.85
<i>Synechococcus Sp. WH 8102</i>	+	+	35	29	6.13
<i>Synechocystis sp. PCC 6803</i>	+	+	35	29	6.13
<i>Thermoanaerobacter tegcongensis</i>	+	+	33	29.6	8.85
<i>Thermobifida fusca</i>	+	+	36	28.2	6.92
<i>Thermoplasma acidophilum</i>	-	+	32	23.2	8.63
<i>Thermosynechococcus elongates</i>	+	+	32	28.6	8.21
<i>Thermotoga maritima</i>	+	+	38	30	8.96
<i>Thermus thermophilus</i>	+	+	36	26.4	9.57
<i>Trichodesmium erythraeum</i>	+	+	33	31.9	8.67
<i>Ureaplasma parvum serovar 3</i>	+	+	36	21.4	9.36
<i>Ureaplasma urealyticum</i>	+	+	36	28.5	9.24
<i>Wolinella succingenes</i>	+	+	34	26.3	6.37
<i>Zymomonas mobilis subsp. mobilis</i>	+	+	35	27.6	7.02

Table 1.1: Table of organisms containing a homologue of the TlyA family. Presence (+) or absence (-) of the S4 RNA binding domain and FtsJ methyltransferase in each homologue is indicated, as is respective MW and pI. % homology to LsaA from *L. intracellularis* is also displayed.

1.6.3.1 Role of TlyA in *Brachyspira hyodysenteriae*

The index member of the TlyA family is the TlyA protein from *Brachyspira* (formerly *Serpulina*) *hyodysenteriae*. It displays characteristically strong β -haemolysis on blood agar plates and it is this characteristic which distinguishes it from its close relative the non-pathogenic weakly β haemolytic *Serpulina innocens* (Jones *et al.*, 1979). *B. hyodysenteriae* and *B. innocens* are both similar in morphology, growth characteristics and surface antigens (Muir *et al.*, 1992). However, *B. hyodysenteriae* is the agent of swine dysentery (SD) whereas *B. innocens* is non-pathogenic. Accordingly *B. hyodysenteriae* haemolytic activity has been correlated to its virulence, with low haemolytic activity equating to avirulent strains (Hughes *et al.*, 1975). The *tlyA* gene from *B. hyodysenteriae* has been cloned and characterised and is encoded by a 720bp, AT rich (74%) ORF that defines a 240 amino acid, 26.9 KDa polypeptide. It showed no homology to RTX or TACY toxins. Southern blot analysis has shown that, excluding *B. innocens*, this gene is present in all *B. hyodysenteriae* serotypes including a passage-attenuated strain in a single copy (TerHuurne *et al.*, 1992). The *B. hyodysenteriae tlyA* gene has also been transformed into *E. coli* K-12 strain DH5 α producing a haemolytic phenotype. A haemolysin has been purified from *B. hyodysenteriae* culture medium. However, no such haemolytic activity was found in supernatant from *E. coli* expressing the recombinant TlyA (Muir *et al.*, 1992), which was proposed as being due to variation in the method of secretion between the bacteria. Isolated haemolysin has also been shown to cause lesions in swine ileal and colonic loops similar to those found in SD (Lysons *et al.* 1991). It has been suggested that destruction of cell membranes may fulfill the requirements of *B. hyodysenteriae* for cholesterol and phospholipids whilst enabling invasion of the host cell (Muir *et al.*, 1992). The function of TlyA from *B. hyodysenteriae* was further investigated when ter Huurne *et al.* (1992) constructed an insertional mutation in the TlyA gene using a kanamycin resistance cassette (TerHuurne *et al.*, 1992). The mutant initially showed reduced haemolytic activity although this phenotype was lost after several passages possibly due to complementation by other *B. hyodysenteriae* genes (TerHuurne *et al.*, 1994). Although it was still able to colonise mice caeca the mutant produced fewer lesions than the wild type, indicating a reduction in virulence (TerHuurne *et al.*, 1992).

Experiments with piglets produced similar results where, although colonisation was present, it was accompanied by a reduction in clinical signs and lesions. Subsequent wild type challenge produced less severe lesions and reduced the duration of bacterial shedding (Hyatt *et al.*, 1994). Such a reduction in virulence would suggest that these factors may show potential as candidates for vaccine studies.

1.6.3.2 Role of TlyA in *Helicobacter pylori*

Helicobacter pylori is a Gram-negative human pathogen that survives largely within the gastric mucous layer in the stomach (Blaser, 1997). It has been shown to be haemolytic when grown on unlysed blood agar plates and is known to contain several haemolytic factors including a TlyA homologue (Segal and Tompkins, 1994). Martino *et al.*, (2001) have reported that disruption of the *tlyA* gene in the SS1 strain of *H. pylori* with a kanamycin resistance cassette has severe effects on haemolytic activity and virulence. When grown *in vitro* the *tlyA* mutant, RS7, showed reduced haemolytic activity. Addition of dextran 5000 to the medium reduced haemolytic activity of the wild-type to levels similar to those of the knockout, which was unaffected by the addition of dextran 5000, indicating a pore forming mode of action for the TlyA haemolysin. As for *S. hyodysenteriae* reduction in haemolytic activity also seems to correlate with a reduction in adherence. Adherence of RS7 to AGS cells was measured at 71.4% +/- 7.5% compared to 92.8% +/- 2.6% observed with SS1, a highly significant difference ($p=0.0017$). Furthermore, RS7 colonisation of the mouse model was also impaired. At the 10 day time point (1 week after final challenge) only 1 of 34 (2.9%) mice inoculated with the TlyA mutant were colonised with *H.pylori* in contrast to the 21 of 29 mice inoculated with the wild-type SS1 strain at the same time point. At the 31 day time point (four weeks after final challenge) all the mice inoculated with SS1 were colonised whereas none of the mice challenged with the TlyA knockout were colonised (Martino *et al.*, 2001).

1.6.3.3 Role of TlyA in *Campylobacter jejuni*

An insertional mutant of the *TlyA* gene from *C. jejuni* NCTC 11168 has also been generated. Although analysis of this mutant is rudimentary no obvious phenotypic differences between the mutant and wild-type strains were immediately evident.

Growth rate, colony morphology and 48 hour α -haemolysis were all identical between mutant and wild type strains. *C. jejuni* TlyA has 39.1% identity, 64.0% similarity to *H. pylori* 26695 TlyA and a 33.7% identity to the *S. hyodysenteriae* TlyA in a 240bp overlap but incorporates an additional 18 amino acids that have not been found on any other TlyA orthologues to date (Stabler, R.A., PhD Thesis, LSHTM).

1.6.3.4 Role of TlyA in Mycobacteria

Homologues of the TlyA family are widespread in the intracellular Mycobacteria, where it is found in both pathogenic and non-pathogenic members. *M. tuberculosis*, *M. leprae*, *M. avium* and *M. bovis* BCG all contain a TlyA homologue. It is also found in two non-pathogenic species *M. flavescens* and *M. gastri* and absent from the pathogenic strains *M. chelonae* and *M. kansasii* and the non-pathogenic *M. phlei*, *M. smegmatis* and *M. vaccae* (Wren *et al.*, 1998). The predicted proteins are highly conserved throughout the available sequences. For example, *M. bovis* TlyA is identical to *M. tuberculosis* TlyA, whereas the homologue from *M. smegmatis* is least conserved (76.9% identity, 90.3% similarity to *M. tuberculosis* TlyA). Results of osmotic protection assays indicate the *M. tuberculosis* and *M. bovis* TlyA cytolysins also exhibit a pore-forming mode of action. Inhibition of haemolytic activity using dextran molecules has estimated a pore of diameter between 1.14 and 2.0nm, comparable with other cytolysins. Although expected, the presence of these proteins on the bacterial cell surface has still not been clarified (Stabler, R.A. PhD Thesis, LSHTM). Introduction of the *M. tuberculosis tlyA* gene into *M. smegmatis* using a mycobacterial shuttle expression plasmid converted non-haemolytic cells into those exhibiting significant haemolytic activity. Similarly, inducible haemolytic activity was observed in sonicated bacteria when *M. tuberculosis* TlyA was expressed as a His₆-tagged fusion protein in *E. coli* (Wren *et al.*, 1998). Subsequent BLAST analysis has revealed a potential orthologue in *M. smegmatis* which was subsequently found to be non-haemolytic. Phenotypic differences could be due to disparities in the protein sequence or could be due to differences in regulation. TlyA homologues are also found in other actinobacteria e.g. *Corynebacterium diphtheriae* and *Streptomyces coelicolor*, suggesting that the gene was present in a common

ancestor and has been maintained in the majority of the actinomycetes (Stabler, R.A. PhD Thesis, LSHTM).

1.6.3.5 Role of LsaA in *L.intracellularis* infection

Previous studies on *L. intracellularis* have shown that all strains examined caused lysis of erythrocytes (Wyllie and Raulston, 2001) and LsaA, a TlyA homologue, is a possible candidate. LsaA has shown to be expressed during infection *in vitro* and *in vivo*. However, further examination of LsaA failed to demonstrate haemolytic activity in recombinant *E. coli* clones under aerobic, microaerophilic or anaerobic environments. The *L. intracellularis* ORF showed greatest similarity to homologues in two *Mycoplasma* species, *M. pulmonis* (53.3% identity) and *M. hyopneumoniae* (49.6% identity), both of which are phylogenetically distinct from *Lawsonia*. Homology to the TlyA from *B. hyodysenteriae*, *H. pylori* and *M. tuberculosis* (36.7%, 31.7-33.8% and 26.5% identity respectively) was in contrast low. This difference is possibly due to heterogeneity between functional domains of TlyA family members known to be haemolytic and LsaA. It is possible that gene products from this locus may differ in their specific activities (McCluskey *et al.*, 2002). Unfortunately, it is not yet known whether the TlyA homologue of *Rickettsia prowazekii*, the only other obligately intracellular bacterium known to possess this gene, confers haemolysis (Dale *et al.*, 1998). However, neutralization experiments using a monoclonal antibody against LsaA have shown a decrease in infectivity of over 50% when tested against two strains of *L. intracellularis* (NCTC 12657 and LR189/5/83) (McOrist *et al.*, 1997c). The fact that this effect is consistent between strains would indicate that this is not a side effect of steric hindrance and that, like other TlyA haemolysins, LsaA plays a role in the adherence of *L. intracellularis* to host cells. Available evidence suggests that TlyA family are important in virulence and homologues from *L. intracellularis* and *H. pylori* may have a role in cell interaction.

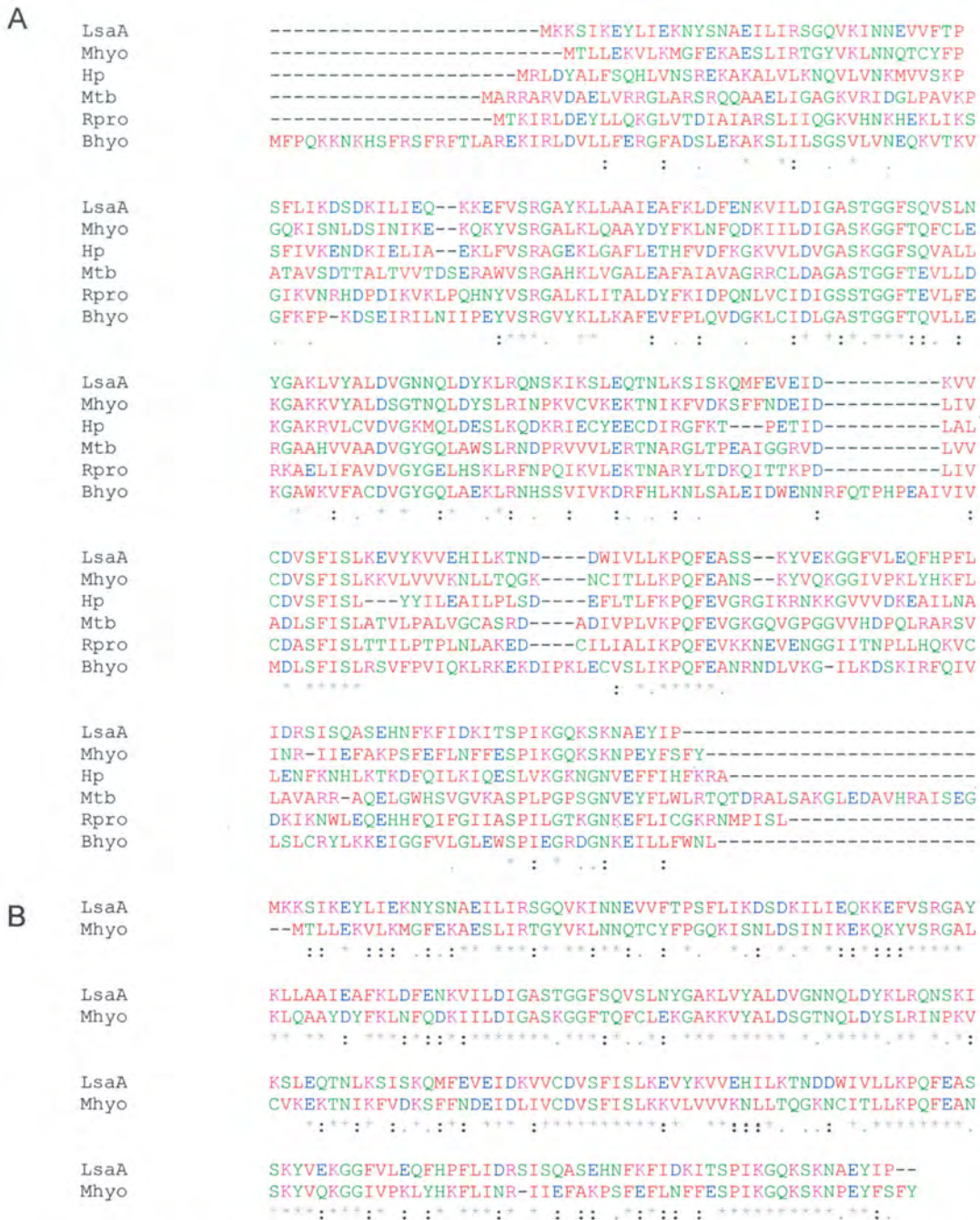


Figure 1.2: (A) Clustal W sequence alignment of *L. intracellularis* LsaA with homologues from *B. hyodysenteriae* (Bhyo), *M. tuberculosis* (Mtb), *R. prowazekii* (Rpro), *M. hyopneumoniae* (Mhyo) and *H. pylori* (Hp) for which phenotypes have been established. Alignment (B) focuses on homology between *L. intracellularis* LsaA and *M. hyopneumoniae* TlyA with which it shares greatest homology. Key: AVFPMILWRED Small + Hydrophobic; DE Acidic; RHK Basic; STYHCNGQ Hydroxyl + Amine + Basic. '*' indicates positions which have a single, fully conserved residue. ':' indicates conserved substitution '.' indicates semi-conserved substitution (Higgins *et al.*, 1994).

1.7.1 Mediators of bacterial attachment and entry into host cells

Attachment of bacteria to host cells is mediated via the large array of glycoproteins, glycolipids and proteoglycans that decorate the surfaces of mammalian cells. These glycoconjugates are molecules which mediate many cellular processes including cell-cell and cell-matrix adhesion, motility, growth, and signaling which, over time, have been exploited by many bacteria as means of attachment to host cells (Rostand and Esko, 1997) as well as triggering invasion.

A wide variety of different methods are used by bacteria to enter and adhere to receptors on the host cell surface. However, in respect of internalization, two major mechanisms have been recognized: the 'zipper' and 'trigger' processes. The 'zipper' process is defined by initial high-affinity binding of a bacterial surface protein adhesin with a transmembrane receptor molecule of the mammalian cell surface involved in cell adhesion. This interaction initiates the tight envelopment and subsequent internalisation of the bacterial cell body in a 'zipper' like process. This mechanism is exemplified by the interaction of the *Yersinia* outer membrane protein, Invasin, which binds with high affinity to a subset of the $\beta 1$ integrin family (Isberg and Barnes, 2001). Entry of *L. monocytogenes* into host cells appears to be mediated by a very similar mechanism to *Yersinia* (Finlay and Cossart, 1997) via cell surface proteins internalin (IntA) and IntB (Galliard *et al.*, 1991, Lingnau *et al.*, 1995, Dramsi *et al.*, 1995). The mammalian receptor for internalin A is E-cadherin, a Ca^{2+} -dependent cell adhesion protein located at the basolateral surface of epithelial cells (Schubert *et al.*, 2002, Lecuit *et al.*, 1997, Mengaud *et al.*, 1996). IntB is reported to target two alternative receptors: gC1q-R (Braun *et al.*, 2000) and Met (Shen *et al.*, 2000) and also interact with glycosaminoglycans (Marino *et al.*, 2002, Jonquieres *et al.*, 2001).

Alternatively, bacteria may enter by the 'trigger' mechanism in which they induce massive cytoskeletal changes in the mammalian cell underneath its site of interaction. This results in a ruffling process that internalises the bacterial body in a macropinocytotic vacuole (Finlay and Cossart, 1997). The latter 'triggering' mechanism is employed by both *Salmonella* and *Shigella* to gain entry into the host

cell. In both cases secretion of bacterial factors into the host cell by a type III secretion system cause the small actin regulatory GTPases to produce actin-rich surface extensions, known as filopodia and lamellipodia, resulting in macropinocytosis and virtual passive entry of the bacteria (Donnenberg, 2000). In the case of *Shigella* actin polymerization is stimulated by the Cdc42 and Rac activation of WASP family proteins and the Arp2/3 complex (Ridley *et al.*, 2003). Upon contact with host cells, the *Shigella* proteins IpaA-DA complex containing IpaB and IpaC, secreted via the type III secretion apparatus, is inserted into the host cell membrane. Although IpaB is known to interact with the CD44 hyaluronic receptor (Skoudy *et al.*, 2000), it is not clear if this interaction by itself leads to outside-in signaling (Tran Van Nhieu *et al.*, 2005). IpaC, however, has been shown to induce actin polymerization in a cell-permeabilised assay or after micro-injection into host cells (Tran Van Nhieu *et al.*, 2000). Antibodies directed against the carboxyl terminus of IpaC block IpaC- and *Shigella*-induced actin polymerization. These findings indicate that IpaC could constitute the major effector responsible for the activation of Cdc42 and Rac during bacterial uptake (Kueltz *et al.*, 2003).

Cytochalasin D has been shown to have an inhibitory effect (approximately 60%) on the internalisation of *L. intracellularis* indicating that cell entry is likely to be an actin dependant process involving cell microfilament activity (Lawson *et al.*, 1995) as seen in *Salmonella* and *Shigella* entry mechanisms. However, attachment and entry of *L. intracellularis* into enterocytes is dependent on host cell viability but not bacterial viability (Lawson *et al.*, 1995). This is converse to the situation in *Shigella* and *Salmonella* species, which require host cell activity as well as metabolically active bacteria (Finlay and Falkow, 1997), but similar to that of *Chlamydia* and *Yersinia* which do not require bacterial viability for cell entry (Moulder, 1985).

Bacterial internalisation therefore occurs through adhesin-receptor (zipper) or effector induced (trigger) mechanisms. It is not yet clear which mechanism *L. intracellularis* employs, however, re-organisation of the actin cytoskeleton, as for the above organisms, is a necessary step. Since *Salmonella* and *Shigella* employ the 'trigger' mechanism and *Yersinia* employs a 'zipper' mechanism it is possible that

Lawsonia intracellularis also uses the latter thus its entry is dependant upon high affinity adhesin-receptor interactions.

1.7.2 Entry of the obligate intracellular *Chlamydiae*

Adherence and entry to host cells is a vital stage in the life cycle of many pathogens, however, it is a vital prerequisite for survival and successful replication of those of an obligate intracellular nature. The best characterized of the obligate intracellular pathogens are the *Chlamydiae*. Typically, *Chlamydiae* are observed attached to the host cell near the base of microvilli from which site they are actively endocytosed by the host cell in tight endocytic vesicles (Reynolds and Pearce, 1999). Electron microscopy work has led to the establishment of two arguments for chlamydial mechanisms of entry. Firstly, a phagocytic, zipper-like mechanism requiring direct circumferential contact between bacterial adhesins and host cell receptors (Finlay and Cossart, 1997). Secondly, receptor-mediated endocytosis followed by uptake into clathrin coated vesicles (Hodinka *et al.*, 1988). However, the bacterium at this stage has been calculated to be larger than the size of physiological molecules which would normally utilise the latter mechanism and a study of *C. caviae* and *C. trachomatis* entering McCoy cells found that only a small proportion of either organism occurred in coated vesicles. Instead clathrin-coated cytoplasmic membrane was generally detected as small vesicles which had been internalised ahead of the *Chlamydiae* (Reynolds and Pearce, 1990). However, much conflicting evidence surrounds the two arguments (Sandvig *et al.*, 1987, Reynolds and Pearce, 1990) and it seems probable that *Chlamydiae*, dependant on the strain, host cell and other circumstances may be able to enter cells by either route (Prain and Pearce, 1989). Similarly, *Chlamydial* surface proteins used in adherence have also been shown to vary between species (Ting *et al.*, 1995, Zhang and Stephens, 1992, Su *et al.*, 1996, Stephens *et al.*, 2001, Raulston *et al.*, 2002), as has the extent of use of heparan sulphate as host cell receptor (Chen and Stephens, 1997, Kuo *et al.*, 1973, Carabeo and Hackstadt, 2001). Nonetheless, the necessity of attachment and entry to host

cells for this group of pathogens is well demonstrated by the multifaceted mechanisms exhibited.

1.7.3 Entry of *L. intracellularis* into host cells

Until the neutralisation experiments showing that the monoclonal antibody, VPM53, against LsaA is capable of reducing attachment of *L. intracellularis* to host cells, nothing was known about the molecular interactions that occur to induce uptake of *L. intracellularis* into intestinal epithelial cells. There is no evidence to date of fimbrial adhesions or of members of the intimin/invasion family of adhesions (Lawson *et al.*, 1995, McOrist *et al.*, 1997c). Treatment of host cells with heparin has been shown to have no effect on the binding of *L. intracellularis* (Lawson and Gebhart, 2000) suggesting that the cell surface receptors recognised by *L. intracellularis* are not proteoglycan in nature. These ubiquitous cell surface proteins are known to be utilised as receptors by a wide range of bacteria and viruses (Wadstream and Ljungh, 1999) including the obligate intracellular bacteria *Chlamydia*. Similarly, no difference was observed in the ability of *L. intracellularis* to enter enterocytes in the presence of bovine plasma fibronectin or the peptide Arg-Gly-Ser. However, there was an increase in cellular uptake of *L. intracellularis* in presence of Arg-Gly-Asp (RGD) (McOrist *et al.*, 1997c). The importance of the RGD peptide sequence in adherence was first described in the 1980s by Pierschbacher and Ruoslahti (Pierschbacher and Ruoslahti, 1984) and its main function is the binding of integrins to extracellular matrix proteins such as fibronectin. Many organisms exploit the RGD motif for binding and invasion into target cells, e.g. either directly, via the envelope E protein of flaviviruses or the capsid protein of the foot and mouth disease VP1 virus, or indirectly, via the *S. aureus* fibronectin binding proteins (FnBPs) which bind to the $\alpha_5\beta_1$ integrins on the cell surface via fibronectin as a bridging molecule (Neff and Baxt, 2001, Lee and Lobigs, 1999) The ability of soluble RGD to enhance uptake of bacteria, whether or not it is the actual ligand, is unexpected but has been documented previously. For instance, non-specific phagocytosis of Group B streptococci (Hill *et al.*, 1993), *Pseudomonas aeruginosa* (Kluftinger *et al.*, 1989) and *Streptococcus pyogenes* (Cue and Cleary, 1997), have all been enhanced by the

presence of soluble fibrinogen and RGD peptides. The exact mechanism of enhancement is not clear but Cue and Cleary (1997) have suggested that ligand binding may promote conformational changes in the bacterial adhesin making it more prone to bind the appropriate epithelial surface receptor (Cue and Cleary, 1997).

An important step in understanding the pathogenesis of *Lawsonia* infection is to define the mechanism(s) whereby it gains access to the host cell and the adhesin(s) and receptor(s) involved. To date LsaA is the only candidate as a possible adhesin and this protein forms the focus of investigations.

1.8 Aim of Research

The main aim of the thesis was to establish a role for the immunodominant surface antigen LsaA in the pathogenesis of *L. intracellularis*. Members of the TlyA family of haemolysins have proved in the past to be important virulence factors in several pathogenic bacteria and the TlyA family members examined thus far have been implicated in attachment/adherence to host cells. As an obligate intracellular bacteria attachment and entry into host cells is a prerequisite for infection of host cells by *L. intracellularis*. Therefore the elucidation of the role of the LsaA in pathogenesis of *L. intracellularis* infection will focus initially on its putative role as an adhesion.

Furthermore the identification of further immunodominant antigens is hoped to shed further light on *L. intracellularis* pathogenesis and lead to new and exciting paths of research in the future.

Chapter 2

MATERIALS AND METHODS

CHAPTER 2

Materials and Methods

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2.1 Source of materials

All chemicals and reagents were purchased from Sigma-Aldrich (Dorset, UK) unless stated otherwise.

2.2 Bacterial strains and culture conditions

2.2.1 K12 Derivatives

2.2.1.1 *E. coli* BL21 (DE3)pLysS

E. coli BL21 (DE3)pLysS (F'*ompThsdS_B(r_B⁻m_B⁻)galdcn*(DE3)pLysS(Cam^R)) (Promega) was used for the IPTG inducible expression of poly-histidine tagged recombinant fusion proteins. This strain is deficient in both *lon* and *ompT* proteases resulting in superior isolation of intact recombinant proteins. It utilises the T7 RNA promoter to control recombinant protein expression. The strain also carries a low level expression plasmid (pLysS) that expresses the T7 lysozyme gene at nominal levels. T7 lysozyme binds to T7 RNA polymerase, thus inhibiting transcription by this enzyme. Upon IPTG induction, over-production of the T7 RNA polymerase effectively shuts down any low level inhibition by T7 lysozyme.

2.2.1.2 One Shot[®] TOP 10 Chemically Competent *E. coli*

One Shot[®] TOP 10 Chemically Competent *E. coli* (F'*mcrA*Δ(*mrr-hsdRMS-mcrBC*)Φ80 *lacZ*ΔM15Δ*lacX74**recA1* *araD139*Δ(*ara-leu*)7697*galUgalK**rpsL*(Str^R)*endA1**nupG*) (Invitrogen) are commercially available competent *E. coli* cells that were used for general cloning purposes.

2.2.1.3 XL10-Gold[®] Ultracompetent *E. coli* Cells

XL-10-Gold[®] ultracompetent cells (Tet^r Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1* *supE44* *thi-1* *recA1* *gyrA96* *relA1* *lac* Hte [F' *proAB* *lacI^q*ZΔM15 *Tn10* (Tet^r) *Amy* Cam^r] (Stratagene) were created for the transformation of large DNA molecules with high efficiency and are ideal for constructing plasmid DNA libraries because they decrease size bias and produce larger, more complex plasmid libraries. XL-10-Gold[®] ultracompetent cells are deficient in all known restriction systems [Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173] are hence are efficient for cloning using methylated DNA.

2.2.1.4 DH5 α

Used as a negative control in adherence assays (Section 2.45). (F'phi80*lacZ* delta(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17* (rk-, m k+) *phoA supE44 lambda-thi-1 gyrA96 relA1/F' proAB+ lacIqZdeltaM15 Tn10(tet^r)*).

2.2.2 *Helicobacter pylori*

2.2.2.1 SS1

Virulent wild-type strain. *H. pylori* SS1 (Sydney strain) colonises standardised mouse model producing gastritis pathology (Lee *et al.*, 1997).

2.2.2.2 RS7

Insertional deletion mutant of *tlyA* gene derived from wild-type strain SS1 (Martino *et al.*, 2001).

2.2.3 *Lawsonia intracellularis*

2.2.3.1 LR/189/S22

L. intracellularis strain LR/189/S22 cultured in INT-407 intestinal epithelial cell monolayers as described previously (Lawson *et al.*, 1993). LR189 is one of several wild-type virulent strains of *L. intracellularis* used for experimental challenge studies to successfully reproduce lesions of proliferative enteropathy in pigs (Smith & McOrist 1997) and mice (Smith *et al.*, 2000).

2.2.3.2 DK15540C

This strain was donated by Boeringer Ingelheim Animal Health and was isolated from a field case in Denmark.

2.3 *Saccharomyces cerevisiae* AH109

Supplied for use with Matchmaker GAL4 Two Hybrid System 3 (BD Biosciences). AH109 genotype: MATa, *trp1-901, leu2-3,112, ura3-52, his3-200, gal4 Δ , gal80 Δ ,*

LYS2::GAL1_{UAS}-GAL1_{TATA}-HIS3, GAL2_{UAS}-GAL2_{TATA}-ADE2, URA3::MEL1_{UAS}-MEL1_{TATA}-lacZ.

2.4 Culture Conditions

2.4.1 *E. coli*

E. coli K12 derivative strains were incubated aerobically on Luria-Bertani (LB) medium agar plates (Section 2.10.1) or in LB broth with shaking at 200 r.p.m. Antibiotics were added as appropriate.

2.4.2 *H. pylori*

H. pylori were grown on DENT agar plates (Section 2.10.3). *H. pylori* were incubated in a microaerophilic atmosphere (85% N₂, 10% CO₂, 5% O₂) generated using the Campygen system (Oxoid) at 37°C.

2.4.3 *L. intracellularis*

L. intracellularis was maintained in the INT-407 cell line in which it has previously been shown to replicate successfully. Tracs (plastic bijoux, Bibby sterilin 129AX/1, containing 13mm diameter coverslips) were seeded with INT-407 cells at a concentration of 1×10^5 to reach 40% confluence overnight prior to infection with *L. intracellularis*. Eighteen hours later the medium was aspirated from the tracs and replaced with media inoculated (1/20) with a frozen aliquot of *L. intracellularis* LR/189/S22. The tracks were transferred to an anaerobic jar and, evacuated to 500mmHg, replaced with hydrogen, and maintained in a microaerophilic atmosphere, 8% O₂, 8.8% CO₂.

2.5 Cell Lines

2.5.1 IEC-18

The IEC-18 cell line (European cell culture collection number 88011801) was derived from normal epithelial cells of the rat ileum and supports growth of *L. intracellularis* without apparent vacuolation or disruption to the normal cell cycle (Lawson *et al.*,

1993). The IEC-18 cells were grown in Dulbecco's modified Eagles medium (DMEM) with 10% (v/v) FBS (plus 1% l-glutamine and 1% non-essential amino acids).

2.5.2 Human gastric adenocarcinoma (AGS)

The AGS cell line (European cell culture collection number 89090402) was derived from an adenocarcinoma of the stomach of a 54 year old Caucasian female with no prior anti-cancer treatment. The AGS cells were grown in Ham's F12 (Ham-F12) with 10% (v/v) FBS (plus 1% l-glutamine and 10,000 units/ml penicillin and 10 mg/ml streptomycin).

2.5.3 INT-407

The INT-407 (Henle407) cell line (European cell culture collection number 85051004) was derived from the jejunum and ileum of a 2 month old Caucasian embryo. The INT-407 cells were grown in minimal essential Eagle's medium (EMEM) with 10% (v/v) FBS (plus 1% l-glutamine and 1% non-essential amino acids).

2.6 Antibiotic supplements

Antibiotic stock solutions were prepared with sterile double distilled water or ethanol and filter sterilised.

Antibiotic	Stock Concentration	Working Concentration
Ampicillin	100mg/ml ⁻¹	50 µg ml ⁻¹
Chloramphenicol	34mg/ml ⁻¹	35 µg ml ⁻¹
Kanamycin	30mg/ml ⁻¹	50 µg ml ⁻¹ (<i>E.coli</i>) 20 µg ml ⁻¹ (<i>H.pylori</i> RS7)

Table 2.1: Antibiotic stock and working concentrations

2.7 Preparation of *E. coli* BL21 (DE3) pLysS Competent Cells

A single colony was used to inoculate 20ml of LB broth in a 250ml flask. The culture was grown to A_{600} 0.2-0.8 before adding to a 2L flask containing 200ml of LB. When the cells reached an OD of A_{600} 0.6 the flask was placed in ice to cool rapidly. Once cells had cooled they were centrifuged (3,700 x g, 4°C) for 10 min and the pellet resuspended in 20ml of cold TFBI by gently shaking on ice. Cells were re-spun (3,700 x g, 8 min) and resuspended in 20 ml of cold TFBII by gentle shaking on ice. Cells were then aliquoted into 0.5ml pre-chilled microcentrifuge tubes and stored at -70°C.

TFBI: 30mM KAc, 10mM CaCl₂, 100mM KCl, 15% v/v Glycerol. Made up to 900ml with dH₂O and following autoclaving added 100ml of 500mM MnCl₂ to a final concentration of 50mM.

TFBII: 75mM CaCl₂, 10mM KCl, 15% v/v Glycerol. Made up to 900ml with dH₂O and added 100ml of 100mM Na-MOPS (pH7.0) before autoclaving.

2.8 Transformation

2.8.1 *E.coli* heat shock transformation

Frozen competent cells were taken from -70°C and thawed until just defrosted on ice. 100µl of the competent cells were added to a sterile glass tube on ice. 5-10 µg of DNA was added to the tube and the mixture incubated on ice for 30 min. Cells were heat shocked at 42°C for 60 secs before adding 1ml of LB and growing the cells at 37°C for at least 2 hours. 100µl of the transformation reaction was then spread onto agar plates containing the appropriate antibiotics and plates were left overnight at 37°C

2.8.2 Transformation of *H.pylori*

2.8.2.1 Electroporation

H.pylori tlyA knockout strain RS7 was harvested using a sterile disposable 10µl loop from a 24 hour DENT plate and resuspended in 10ml ice-cold EBF buffer (15% v/v

glycerol, 10%w/v sucrose). The cells were centrifuged at 4,000 x g for 10 min, resuspended in 1 ml EBF buffer, re-centrifuged at 14,000 x g for one minute and resuspended in 250µl EBF buffer. For each transformation reaction 1.0 – 5.0 µl of DNA equivalent to 1.0 – 5.0µg, was added to 50µl RS7. This was mixed by pipetting and incubated on ice for 10 min. The cells were transferred to an ice cold electroporation cuvette and electroporated at 2.5 kV, 25 Fdµ and 200Ω (Easyject Plus, Equibio), ensuring that the time constant remained between 3.0 and 5.0ms. Pre-heated BHI Broth (10% FBS) was added immediately to recover the cells. The cells were then spread onto non-selective DENT plates and incubated under normal microaerophilic conditions for 48 hours. The cells were harvested using a sterile disposable 10 µl loop, re-suspended in 0.5 ml broth aliquots and 200µl spread evenly onto DENT plates supplemented with appropriate antibiotics and incubated under microaerophilic conditions for three to five days.

2.8.2.2 Natural Transformation

Plate-grown *H. pylori* RS7 were resuspended in BHI broth (Oxoid) to an OD₆₀₀ of 0.6. 1µg of plasmid DNA (either directly isolated from *E. coli* or treated with *H. pylori* cell free extract (as described in Section 2.8.2.2.1 and 2.8.2.2.2) was added and incubated for 3 h at 37°C in a CO₂ incubator. The cells were then spun down (4,000 x g, 10 min) and resuspended in 100µl of pre-warmed BHI broth. The entire 100µl was spread onto DENT plates (Section 2.10.3) with kanamycin and incubated in a microaerophilic atmosphere for 24h. After 24h the cells were scraped from the plate and replated on blood agar plates containing chloramphenicol and kanamycin.

2.8.2.2.1 Bacterial extract preparation

H. pylori cell free extract (CFE) was prepared as described by Donahue et al. (2000). Plate-grown *H.pylori* were resuspended in five volumes (ml) of extraction buffer [20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 5mM Na₂EDTA, 1mM dithiothreitol (DTT) protease inhibitor cocktail (Bacterial, Sigma)] g⁻¹. Bacterial cell suspensions were sonicated on ice and then centrifuged at 15,000 x g for 5 min (4°C). Supernatants were removed and used immediately or after one freeze-thaw cycle (-70°C) for *in vitro* plasmid DNA modification reactions (Donahue et al. 2000).

2.8.2.2.2 Treatment of plasmid DNA with *H. pylori* Cell Free Extract (CFE).

Plasmid DNA (12 μ g), was combined with *H. pylori* SS1 CFE (300-400 μ g of protein) in a 50 μ l reaction containing 20mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 5mM Na₂EDTA (pH 7.9), 1 mM DTT and 200 μ M S'adenosylmethionine (SAM) (NEB). All samples were incubated at 37 $^{\circ}$ C for 1 hour and then extracted with phenol chloroform. Plasmid DNA was precipitated with ethanol and dissolved in 10mM Tris-HCl (pH 7.5) to a final concentration of 0.5mg/ml⁻¹ (Donahue et al. 2000).

2.8.3 Small Scale yeast transformation.

Several colonies, 2-3mm in diameter, of AH109 grown on YPD agar were used to inoculate 1 ml of YPDA or SD medium (BD Biosciences) and vortexed vigorously for 5 min to disperse any clumps. This was transferred to a flask containing 50ml of YPD or SD medium and incubated at 30 $^{\circ}$ C for 16-18 hr with shaking at 250 rpm until the culture reached stationary phase (OD₆₀₀>1.5). The overnight culture was used to inoculate 300 ml of YPD to an OD₆₀₀ of 0.2 – 0.3. The culture was then grown to for 3 hr at 30 $^{\circ}$ C with shaking (230 rpm) at which point the OD₆₀₀ should be 0.4 – 0.6. Cells were centrifuged at 1,000 x g for 5 min at room temperature the supernates were discarded and the pellets resuspended in sterile TE (0.1 M Tris-HCl, 10mM EDTA, pH 7.5) or H₂O (final volume 25 – 50 ml). Cells were centrifuged (1,000 x g, 5 mins) at room temperature and the pellets resuspended in 1.5 ml of sterile 1 x TE/1x LiAc (0.1M LiAc, 0.1 M Tris-HCl, 10mM EDTA, pH 7.5). In a fresh tube 0.1 μ g of plasmid DNA and 0.1 mg of herring testes carrier DNA were mixed with 100 μ l of yeast competent cells and the mixture vortexed well. 0.6 ml of sterile PEG/LiAc (40% (v/v) PEG 4000, 0.1M LiAc, 0.1 M Tris-HCl, 10mM EDTA, pH 7.5) solution was added to each tube and vortexed at high speed for 10 sec. The transformation reaction was then incubated at 30 $^{\circ}$ C for 30 min with shaking at 200 rpm. 70 μ l of DMSO was added and mixed well by inversion. Cells were heat shocked for 15 min in a 42 $^{\circ}$ C water bath before chilling cells on ice for 1-2 min. Cells were collected by centrifugation (14,000 x g) and resuspended in 0.5ml of sterile TE and plated onto appropriate SD agar dropout plates (Section 2.10.2).

2.9. Preservation of bacterial strains

2.9.1 *E. coli*

A single colony was inoculated into 10 ml of LB broth with selective antibiotics and grown overnight at 37°C at 200r.p.m. 0.85ml of the culture and 0.15 ml of sterile 100% glycerol was pipetted into a 2 ml cryotube. This was briefly vortexed and the clone stored at -80°C until required.

2.9.2 *H. pylori*

H.pylori strains to be stored were grown for 24 hours on DENT plates with selective antibiotics. The bacteria were removed using a sterile 10µl loop and resuspended in 1ml of Brain Heart Infusion (BHI) broth (Oxoid) containing 15% v/v glycerol and 10% fetal calf serum. The bacteria were mixed by careful pipetting and stored at -80°C until required.

2.10 Preservation of Yeast strains

A sterile inoculation loop was used to scrape an isolated colony from a YPD agar plate. The cells were resuspended in 200-500µl of YPD medium (BD Biosciences) in a sterile 1.5ml microcentrifuge tube. The tube was vortexed vigorously to disperse the cell and sterile 50% glycerol added to a final concentration of 25% before storing at -70°C.

2.11 Agar Plates

2.11.1 Preparation of LB ampicillin/chloramphenicol/kanamycin agar plates

To prepare antibiotic plates, 500ml of LB agar was melted using a microwave at low power for 20 min. Appropriate antibiotics (see Table 2.1) were added after the agar cooled to below 55°C. Approximately 25ml of LB agar was poured into 100ml petri dishes and allowed to set at room temperature. The plates were dried for 10-15 min and stored at 4°C in the dark for up to 1 month.

2.11.2 Preparation of SD Dropout Agar Plates

SD Dropout agar plates were prepared by combining the appropriate amount of Minimal SD Agar Base (BD Biosciences) with the appropriate amount of the required Dropout Supplement (BD Biosciences) and making up to the required volume with dH₂O. The

agar was then autoclaved (121°C, 15 min) and allowed to cool before pouring plates. The plates were dried for 10-15 min and stored at 4°C in the dark for up to 1 month.

2.11.3 Preparation of DENT plates

DENT plates were prepared by melting 500ml of LB agar as before. Upon cooling to 50°C 25ml of defibrinated horse blood (Oxoid), *Helicobacter pylori* selective supplement (DENT) (Oxoid) and appropriate antibiotics (see Table 2.1) were added. The plates were dried for 10-15 min and stored at 4°C in the dark for up to 1 month. DENT supplement consisted of 5mg vancomycin, 2.5mg cefsulodin, 2.5mg trimethoprim lactate and 2.5mg amphotericin B per 500ml of LB agar base.

2.12 Plasmid Table

Plasmids used and produced in this study are detailed in Table 2.2 below.

Chapter	Plasmid	Description	Use	Source
3	pRSETA/LsaA	Amp ^R N-terminal (His) ₆ -tagged expression plasmid	Expression of tagged LsaA fusion protein	(McCluskey <i>et al.</i> , 2002)
5	pHel2*	<i>E. coli</i> / <i>H. pylori</i> shuttle vector	Complementation of <i>H. pylori</i>	(Heuermann and Haas, 1998)
5	pHel2/TlyA _{HP}	pHel2 containing <i>H. pylori tlyA</i> insert including 100bp upstream region	Re-complement <i>H. pylori</i> SS1 <i>tlyA</i> knockout, RS7, with wild-type gene.	This study
5	pHel2/LsaA	pHel2 containing <i>LsaA</i> insert including 100bp upstream region	Complementation of <i>H. pylori</i> SS1 <i>tlyA</i> knockout, RS7, with LsaA	This study
6	pEGFPC1	GFP Mammalian Expression Vector	Encodes a variant of wild-type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells.	Invitrogen
6	pEGFPC1/TlyA _{HP}	Expression of GFP- <i>H. pylori</i> TlyA fusion protein	Intracellular localisation of <i>H. pylori</i> TlyA	This study

6	pEGFPC1/LsaA	Expression of GFP- LsaA fusion protein	Intracellular localisation of LsaA	This study
4	pGBKT7	Eukaryotic vector for expression of proteins fused to amino acids 1-147 of the GAL4 DNA binding domain	Cloning of LsaA	Clutch
4	pGADT7	Eukaryotic vector for expression of proteins fused to amino acids 768-881 of the GAL4 activation domain	Cloning of INT-407 coda library	Clutch

Table 2.2: Plasmids used in this study

*Shuttle vector pHel2 was kindly provided by R. Haas (Pettenkofer Institute, Munich, Germany).

2.13 Primers

All primers were purchased from MWG Biotech and are detailed in Table 2.3.

Primer Name	Orientation	Restriction site Incorporated (underlined)	Primer Sequences	Function	Target
GFP300For	Forward	-	ttgattgtaccacaacatctaagcgta	Amplification of GFP fusion protein	GFP
Hp TlyA Rev	Reverse	KpnI	5' <u>cggtac</u> cttagctcgcttgaatg gata	Amplification of H. pylori TlyA	<i>H. pylori</i> TlyA
HpTlyA_C1Bgl	Forward	BglI	5' <u>gcagat</u> ctatcgcttagattacgcct tattcagt	Amplification of H. pylori TlyA	<i>H. pylori</i> TlyA
HpTlyA300Rev	Forward	-	ttagccctttaaagcgccacttga	Amplification of H. pylori TlyA / GFP fusion	<i>H. pylori</i> TlyA
Hp100	Forward	BamHI	5' <u>cgggat</u> ccgattatttagcggttcaa ga	Amplification of H. pylori TlyA incorporating 100bp upstream region	<i>H. pylori</i> TlyA
Hp250	Forward	BamHI	5' <u>cgggat</u> ccgaggctatattcttatg a	Amplification of H. pylori TlyA incorporating 250bp upstream region	<i>H. pylori</i> TlyA
Hp500	Forward	BamHI	5' <u>cgggat</u> ccctctcaacacttctggg t	Amplification of H. pylori TlyA incorporating 500bp upstream region	<i>H. pylori</i> TlyA

LsaA Rev	Reverse	KpnI	5' <u>cgggtaccgctt</u> gtgatatgctcga	Amplification of LsaA	<i>LsaA</i>
LsaA_C1Bgl	Forward	BglI	5' <u>gcagatctatg</u> aaaaaaaaagcattaaa gaataactta	Amplification of LsaA	<i>LsaA</i>
LsaA_Library For	Forward	EcoRI	5' <u>cgggaattcatg</u> aaaaaaaaagcattaaa gaa	Amplification of LsaA	<i>LsaA</i>
LsaA_BamHI Rev	Reverse	BamHI	5' <u>cgggatccgctt</u> gtgatatgctcga	Amplification of LsaA	<i>LsaA</i>
LsaA300Rev	Forward	-	ttgattgtaccacaacatctaaaagcgta	Amplification of LsaA / GFP fusion	<i>LsaA</i>
Li100	Forward	BamHI	5' <u>cgggatcccttctt</u> taataattttcac	Amplification of LsaA incorporating 100bp upstream region	<i>LsaA</i>

Table 2.3 Primers used in study.

2.14 RNA Extraction

RNA was extracted from bacterial and mammalian samples using the RNeasy mini/maxi kit from Qiagen (Crawley, UK).

2.14.1 RNA extraction using RNeasy® mini kit

2.14.1.1 Mammalian cells

INT-407 cell monolayers grown in 24 well plates were lysed directly by the addition of 300 μ l of RLT buffer to each well and the cell suspension mixed by pipetting. Two wells of corresponding sample were pooled and the sample homogenised by pipetting the lysate directly onto a QIAshredder spin column placed in a 2ml collection tube and centrifuged for 2 min at maximum speed in a microcentrifuge tube. One volume (600 μ l) of 70% ethanol was added to the homogenised lysate and mixed well by pipetting. The sample was then applied to an RNeasy mini column placed in a 2ml collection tube and centrifuged briefly for (8000 x g, 15 sec). The flow-through was discarded and 700 μ l buffer RW1 added to the RNeasy column. The samples were centrifuged briefly (8000 x g, 15 sec) and the column placed in a fresh 2ml collection tube. The column was washed with 500 μ l of buffer RPE (8,000 x g, 15s). Another 500 μ l of buffer RPE was added to the column which was then centrifuged (8,000 x g, 2 min) to dry the silica-gel membrane. The column was transferred to a new 1.5ml collection tube, 30 μ l of RNase-free water added directly to the silica-gel membrane and the column centrifuged for 1 min at 8,000 x g. The first elute was reapplied to the membrane and the column recentrifuged (8,000 x g, 1 min) to maximise the yield.

2.14.1.2 Bacterial Cells

No more than 1×10^9 bacteria were harvested by centrifugation (5000 x g, 5min, 4°C). The supernatant was discarded and all remaining media removed by aspiration. The bacterial pellet was resuspended in 100 μ l of Lysis buffer (400 μ g lysoszyme/ml TE buffer) and incubated at room temperature for 3 – 5 min. 350 μ l of RLT buffer was added to the sample, which was vortexed vigorously to mix. Any insoluble material still remaining was removed by centrifugation for 2 min at maximum speed in a microcentrifuge and only the supernatant used in subsequent steps. 250 μ l of ethanol (96-100%) was added to the lysate and mixed thoroughly by pipetting. The sample was then applied to an RNeasy mini column placed in a 2ml collection tube and

centrifuged briefly (8000 x g, 15 sec). The flow-through was discarded and 700 μ l buffer RW1 added to the RNeasy column. The samples were centrifuged briefly (8000 x g, 15 sec) and the column placed in a fresh 2ml collection tube. The column was washed with 500 μ l of buffer RPE (8,000 x g, 15s). Another 500 μ l of buffer RPE was added to the column which was then centrifuged (8,000 x g, 2 min) to dry the silica-gel membrane. The column was transferred to a new 1.5ml collection tube and 30 μ l of RNasee-free water added directly to the silica-gel membrane and the column centrifuged for 1 min at 8,000 x g. The first elute was reapplied to the membrane and the column re-centrifuged (8, 000 x g, 1 min) to maximise the yield. RNA was stored at -70°C

2.14.1.3 Removal of DNA contamination from RNA samples

Removal of DNA contamination in RNA samples was performed using the RNase-Free DNase Set (Qiagen, Crawley, UK) using either the On column DNase digestion or the DNase digestion prior to RTPCR protocol.

2.14.1.3.1 On column DNase digestion

Instead of continuing with the RW1 buffer step 350 μ l of buffer RW1 was added to the RNeasy mini column and centrifuged for 15s at 8000 x g to wash. The flow through was discarded. 10 μ l of DNase I stock solution (1500 Kunitz units of DNase I dissolved in 550 μ l of RNase free water) was added to 70 μ l buffer RDD and the solution mixed gently by inverting the tube. The Dnase I mixture was pipetted directly onto the RNeasy silica-gel membrane and left at room temperature for 15 min. The column was washed with 350 μ l of buffer RW1 (8000 x g, 15s) and the flow through discarded. The relevant protocol was continued at the first buffer RPE wash step.

2.14.1.3.2 DnaseI digestion prior to RTPCR

The following was mixed in a microcentrifuge tube; 1-2 μ g RNA 2 μ l 10x DNase buffer (500mM Tris-HCl, pH8.0; 50mM MgCl₂; 10mM DTT); 10 units RNase inhibitor; 0.5 Kunitz units of RNase free DNase I and the volume made up to 20 μ l with RNase free water. The tube was then incubated at 37°C for 30 min. 2 μ l of EDTA was added and the

reaction and the sample incubated for 5 min at 65°C to inactivate the DNaseI prior to RTPCR.

2.14.1.5 Extraction of poly A⁺ mRNA from INT 407 cells

Poly A⁺ mRNA was extracted from up to 1mg of total RNA using the Oligotex® mRNA mini kit (Qiagen, Crawley, UK). Total RNA was added to an RNase free 1.5ml microcentrifuge tube and the volume adjusted to 250µl. 250-500µl of buffer OBB and between 15µl and 55µl of Oligotex suspension, depending on the amount of starting material was added to the tube. The tube was then incubated for 3 min at 70°C to allow annealing of the poly A⁺ mRNA tails to the oligo dT resin, and then cooled to room temperature for 10 min. The oligotex:mRNA complex was pelleted (2 min, 14,000g) and the supernatant carefully removed. The pellet was resuspended in 400µl buffer OW2 and the sample loaded into a spin column placed in a 1.5ml collection tube. The column was centrifuged for 1 min at 14,000g and the column was then transferred to a new 1.5ml collection tube. 400µl of buffer OW2 was applied to the column which was then centrifuged (1 min, 14,000 x g) before discarding the flow-through and transferring the column to a new 1.5ml collection tube. 20µl of pre-warmed buffer OEB (70°C) was pipetted onto the membrane and the suspension pipetted up and down several times. The column was then centrifuged at 14,000g for 1 min to elute the poly A⁺ mRNA. The eluate was reapplied to the column, which was briefly reheated to 70°C and the resuspension and centrifugation steps repeated in order to maximise the yield of poly A⁺ mRNA.

2.14.2 RNA extraction using RNeasy® maxi kit from Qiagen (Crawley, UK)

2.14.2.1 Mammalian cells

To acquire a high yield of semi-confluent INT-407 RNA for poly A⁺ RNA extraction monolayers were grown in 225ml tissue culture flasks and trypsinised before addition of RLT buffer. Briefly, medium was aspirated, cells were washed with phosphate buffered saline (PBS; 137mM NaCl, 27mM KCl, 8mM Na₂HPO₄, 15mM KH₂PO₄, pH7.2) and trypsin/EDTA added to trypsinise cells. Once cells had detached from the tissue culture flask, medium was added and the cells removed to an RNase free polypropylene tube.

Cell numbers were estimated using a haemocytometer. 5×10^8 cells were then pelleted (3,000g, 5min) and 15ml of RLT buffer added to lyse the cells. Cells were then homogenised using a rotor-stator homogenizer for at least 45s until the lysate was uniformly homogeneous. One volume of 70% ethanol was added to the homogenized lysate and shaken vigorously. The sample was then applied to an RNeasy maxi column placed in a 50ml collection tube and centrifuged for 5 min at 3,000-5,000 x g. The flow-through was discarded and 15ml of buffer RW1 added to tube and the centrifugation repeated. The flow through was discarded and 10ml of buffer RPE added to the column. The column was centrifuged for 2 min at 3,000 – 5, 000 x g and the flow through discarded. A further 10ml of buffer RPE was applied to the column which was then centrifuged (5 min, 3,000 – 5,000 x g) to dry the silica-gel membrane. To elute the RNA the column was transferred to a new 50ml collection tube. 1.2ml of RNase-free water was pipetted onto the silica-gel membrane and the column allowed to stand for 1 min. The column was then centrifuged for 3 min at 3,000 – 5,000 x g. To achieve the maximum concentration of RNA for poly A⁺ mRNA extraction the eluate RNA was reapplied to column, incubated for 1 min, and the elution repeated (3min, 3,000 – 5,000 x g). Eluted RNA was quantified and either used directly for poly A⁺ mRNA extraction or stored at -70°C.

2.14.2.2 Extraction of poly A⁺ mRNA

Poly A⁺ mRNA was extracted from 1-3mg of total RNA using the Oligotex® mRNA maxi kit (Qiagen, Crawley, UK). Total RNA was added to an RNase free 1.5ml microcentrifuge tube and the volume adjusted to 650µl with RNase free water . 650µl of buffer OBB and between 85µl and 175µl of Oligotex suspension, depending on the amount of starting material were added to the tube. The tube was then incubated for 3 min at 70°C to allow annealing of the poly A⁺ mRNA tails to the oligo dT resin, and then cooled to room temperature for 10 min. The oligotex:mRNA complex was pelleted (2 min, 14,000g) and the supernatant carefully removed. The pellet was resuspended in 600µl buffer OW2 and the sample loaded into a spin column placed in a 1.5ml collection tube. The column was centrifuged for 1 min at 14,000g and the column was then transferred to a new 1.5ml collection tube. 20µl of pre-warmed buffer OEB (70°C) was pipetted onto the membrane and the suspension pipetted up and down several times.

The column was then centrifuged at 14,000g for 1 min to elute the poly A⁺ mRNA. The eluate was reapplied to the column, which was briefly reheated to 70°C and the resuspension and centrifugation steps repeated in order to maximise the yield of poly A⁺ mRNA.

2.14.3 Spectrophotometrical quantification of RNA

The concentration of RNA was determined by measuring the absorbance at 260nm (A_{260}) in a spectrophotometer. The buffer in which the RNA was diluted was used to zero the spectrophotometer and the ratio between the absorbance values at 260 and 280 nm recorded in order to estimate the RNA purity (pure RNA having a ratio of 1.9-2.1 in 10mM Tris-HCl, pH7.5).

An absorbance of 1 unit at 260nm corresponds to 40 μ g of RNA per ml of water.

Therefore RNA concentrations were estimated using the following equation:

$$\text{Concentration of RNA} = (40 \times A_{260} \times \text{dilution factor}) \mu\text{g/ml}^{-1}$$

$$\text{Total Yield} = \text{Concentration} \times \text{volume of sample in millilitres}$$

2.15 Reverse Transcriptase Polymerase Chain Reaction (RTPCR)

Performed using Omniscript Reverse Transcriptase Kit from Qiagen (Crawley, UK). Before use all solutions were thawed on ice, vortexed to mix, centrifuged, and then placed on ice. Recombinant RNasin[®] ribonuclease inhibitor (Promega, Southampton, UK) was diluted to a concentration of 10units/ μ l in ice cold 1 x RT buffer (supplied as 10 x concentrate). An RTPCR master mix was prepared as below:

10 x Buffer RT	2 μ l
dNTP mix (5mM each dNTP)	2 μ l
Oligo-dT primer (10 μ M)	2 μ l
RNasin (10units/ μ l)	1 μ l
Omniscript Reverse Transcriptase	1 μ l
RNase free water	variable
Template RNA	up to 2 μ g
Total Volume	20 μ l

Following addition of the template RNA the sample is vortexed very briefly to mix, centrifuged briefly and then placed at 37°C for 60 min. Aliquots of the reverse transcriptase reaction were either used directly for PCR or the entire reaction stored at -20°C.

2.16 Polymerase Chain Reaction (PCR)

PCR amplifications were carried out in a Thermal cycler JMBS 0.25 (Hybaid). Reaction mixes were set up in sterile 0.5ml tubes, consisting of 1 x DNA buffer, 25mM MgCl₂ and dNTPs each at 2mM. 0.1-0.5 μ m (100pmol/ μ l⁻¹) of each forward and reverse primer (MWG-Biotech UK Ltd, Milton Keynes) and various amounts of template DNA added and DNase free H₂O, to give a final reaction volume of 50 μ l. The reactions were placed on ice and 2.5U Taq DNA polymerase (Promega) added.

The amplification conditions (Table 2.4) were varied to suit the size of the expected product. The extension times were calculated as 60s/1Kb product. The annealing temperatures were set at 5°C lower than the lowest primer T_m .

Stage	Stage Name	Cycle Name	Temperature	Time	Cycles
1	Initial denaturation		94°C	5 mins	1
2	PCR	Denaturation	94°C	1 minute	35
		Annealing	various	1 minute	
		Extension	72°C	1-2 mins	
3	Final Extension		72°C	20 mins	1

Table 2.4: Polymerase chain reaction conditions

2.17 PCR Clean-up

The QIAquick PCR purification kit (Qiagen, Crawley, UK) was used for the removal of excess nucleotides, polymerases and buffer from PCR reactions and removal of buffers and enzymes from previous reactions, for example after restriction digests. To each volume of DNA five volumes of PB Buffer was added. This was carefully added to the top of a QIAquick column placed within a 2ml collection tube. The DNA was bound to the membrane by centrifugation at 14,000 x g for one minute. The eluate was discarded and the column washed by the centrifugation of 750µl of PE buffer (14,000 x g for one minute) through the column. The eluate was discarded and the DNA was eluted by adding 50 µl of dH₂O to the column and centrifuging at 14,000 x g for one minute.

2.18 DNA Gel Extraction

The removal of DNA from agarose gels was achieved using the QIAquick gel extraction kit (Qiagen, Crawley, UK). The selected region was first excised from the agarose gel using a scalpel and placed in a preweighed 1.5ml microtube and the gel slice weight calculated.

The gel slice was solubilised by the addition of three volumes of buffer QG and incubating at 50°C for 10 mins, vortexing occasionally. After the complete solubilisation

of the agarose one gel volume of 100% isopropanol was added. This mixture was carefully added to the top of a QIAquick column placed within a 2ml collection tube. The DNA was bound to the membrane by centrifugation at 14,000 x g for one minute. The eluate was discarded and the column washed by the centrifugation of 750 µl of PE buffer (14,000 x g, 1 min) through the column. The eluate was discarded and the DNA was eluted by adding 30µl of elution buffer (EB) to the column and centrifuging (14,000 x g, 1 min).

2.19 Agarose Gel Electrophoresis

2.19.1 Standard Conditions

1% to 1.2% of agarose w/v was solubilised in 1x TAE (0.04 M Tris-acetate, 0.001M EDTA) buffer by heating. The agarose was allowed to cool before the addition of 0.5% v/v ethidium bromide at a concentration of 10mg/ml. The mixture was then poured into an agarose gel forming tray containing a 'comb' well former. Once the agarose gel had set and the 'comb' removed, the gel was placed into a horizontal electrophoresis chamber, TBE was added until the level covered the gel.

DNA loading buffer was added to the samples to be analysed and a DNA marker was also prepared. The samples were loaded into the wells formed in the agarose gel and the samples electrophoresed at 100 V for 90 mins. The DNA was visualised using UV light and the images recorded photographically.

2.19.2 RNA

Gels for visualisation of RNA by ethidium bromide staining were prepared as for DNA but poured thinly (i.e, 2-10 mm). 2µl of RNA sample or RNA Ladder (NEB) was then boiled for 5 mins in a 65°C water bath in 0.5ml tube containing 3µl RNase free water and 5µl of RNA Ladder Sample Buffer (2 x TBE (pH 8.3), 13% ficoll (w/v) 0.01% bromophenol blue, 7M urea) prior to separation by agarose electrophoresis as described in section 2.19.1.

2.19.3 Alkaline Agarose Gel

1.4% (v/v) agarose was solubilised in 50mM NaOH, 1mM EDTA by heating. The mixture was then poured into an agarose gel forming tray containing a 'comb' well former. Once the agarose gel had set and the 'comb' removed, the gel was placed into a horizontal electrophoresis chamber, alkaline gel running buffer (30mM NaOH, 1mM EDTA) was added until the level covered the gel. The solidified gel was allowed to equilibrate for at least 30 mins in alkaline gel running buffer prior to electrophoresis. Samples were loaded in an equal amount of the alkaline agarose 2x loading buffer (200µl glycerol, 750µl water, 46µl saturated bromophenol blue, 5µl of 5mM NaOH). The gel was run at 100mA until the tracking dye has migrated about 2/3 of the way through the gel. The system was monitored throughout for heat, reducing the amperage if the temperature rose above 37°C. The gel was then soaked in several volumes of 7% TCA at room temperature for 30 mins or until the dye changes from blue to yellow. The gel was then dried under a weighted stack of paper towels for several hours.

2.20 Plasmid Purification

The removal of DNA from agarose gels was achieved using the QIAGEN plasmid mini-prep kit employing the modified alkaline lysis of method of Birnboim and Doly (1979) (Birnboim & Doly, 1979). Bacteria were lysed under alkaline conditions, and the lysate subsequently neutralised and adjusted to high-salt binding conditions in one step, ready for purification on the QIAprep silica-gel membrane. Cells from an overnight culture were pelleted (4000 x g, 10 min) and resuspended in 250µl of buffer P1 before transferring to a 1.5ml microcentrifuge tube. 250µl of buffer P2 was added then added to lyse the cells and the tube inverted 4-6 times to mix. The lysate was then neutralised and adjusted to high-salt binding conditions by addition of 350µl of buffer N3 and the tube, immediately and gently, inverted to mix. The sample was then centrifuged (17,900 x g, 10 min) in a micro-centrifuge and the supernatant applied to a QIAprep spin column placed in a collection tube and centrifuged (17,900 x g, 60 sec). The flow-through was discarded and the column washed with 750µl of buffer PE. Following which the flow-through was discarded and the tube centrifuged for an additional 1 min to remove any residual wash buffer. The QIAprep column was then transferred to a clean 1.5ml microcentrifuge tube and 50µl of buffer EB added to the centre of each column. The

columns were then allowed to stand for 1 min before centrifuging (17,900 X g, 1min) to elute DNA.

2.21 DNA manipulations

2.21.1 Restriction analysis of plasmid DNA

All restriction enzymes were obtained from New England Biolabs and subsequently from Promega. Digestions were carried out as recommended by the manufacturers.

2.21.2 Shrimp Alkaline Phosphatase (SAP) treatment of digested plasmids

In order to suppress self-ligation and re-circularisation of plasmid DNA 5'-phosphate groups were removed with alkaline phosphatase. SAP (Promega) (1unit/ μg DNA) was incubated with restriction digested buffer (in water or TE buffer) at 37°C for 15 mins in 1 x SAP reaction buffer in a final volume of 30–50 μl . SAP was then inactivated by heating at 65°C for 15 min.

2.21.3 Ligation

T4 DNA Ligase (Promega) was used for annealing fragments into plasmid vectors. A 1:3 molar ratio of vector to insert was used when cloning. Insert and vector DNA were combined in a 10 μl reaction containing 1 μl of 10 x T4 DNA Ligase Buffer and 0.1 – 1 Weiss units of T4 DNA Ligase. The reaction was placed at 4-8°C overnight.

2.22 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Two glass plates were clamped together vertically and a resolving gel composed of: N,N'-methylenebisacrylamide [30% (w/v): 0.8% (w/v)], 375mM Tris [pH8.8], 0.1% (w/v) SDS, 0.035% (w/v) ammonium persulphate and 0.0035% (v/v) N,N,N',N'-tetramethylethyldiamine (TEMED), was poured between them and overlaid with a small volume of 100% isopropanol to produce a smooth level surface. When polymerisation of the separating gel was complete and the layer of isopropanol had been poured off and the gel surface washed with dH₂O, a stacking gel composed of: 3% (v/v) acrylamide: N,N'-methylenebisacrylamide, 125mM Tris [pH6.8], 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate and 0.14% (v/v) TEMED was poured on top. A

Teflon comb was inserted into the stacking gel to form wells for the loading of samples. Following polymerisation of the stacking gel, the comb was removed and the assembled gel placed in a Biorad Mini-Protean II™ electrophoresis tank. Both reservoirs were then filled with electrophoresis buffer containing; 25mM Tris, 192mM glycine, 0.1% SDS. Once the separating and stacking gels were prepared, samples containing known quantities of protein were mixed 1:1 with SDS page loading buffer (288mMβ-mercaptoethanol, 2% SDS, 40% glycerol, 8mg bromophenol blue), boiled for 10 mins, then chilled on ice. Samples and prestained molecular weight markers (Biorad) were loaded into the wells of the gel using gel loading tips. Samples were electrophoresed at a constant voltage of 80V until the dye front had reached the bottom of the resolving gel.

2.23 SDS PAGE gel staining

2.23.1 Colloidal Blue

Gels were fixed in 40% methanol, 10% acetic acid for at least 1 hour. Before staining in colloidal coomassie solution (80% (v/v) colloidal coomassie (Genomic Solutions), 20% v/v methanol) for at least four hours. Gels were destained in 25% methanol for 1 hour.

2.23.2 Silver Staining

All silver staining was carried out using the Silverquest™ silver staining kit (Sigma). Following electrophoresis each mini-gel was washed briefly in deionised water before fixing in 100ml fixative solution (40% v/v methanol, 10% v/v acetic acid) for 20 mins to overnight. Gels were then washed in 30% ethanol for 10 mins before incubating in sensitizing solution (30% ethanol, 10% sensitizer) for 10 mins. This was followed by a 30% ethanol wash for 10 mins and then a wash in dH₂O. The gel was then incubated in staining solution (1% stainer) for 15 mins. Once staining was complete the staining solution is decanted and the gel is washed briefly in dH₂O for 20-60 secs. The gel was then incubated in developing solution for 4-8 mins until the desired intensity was attained when the reaction was stopped by the addition of stop solution directly into the developer.

2.24 Transfer to nitrocellulose

Immediately following SDS-PAGE, separated protein bands on gel were transferred onto Immobilon-P membranes (Millipore Corp., Bedford, Mass.) by electrophoretic blotting. The gel was pressed against a piece of nitrocellulose membrane which had been pre-soaked in transfer buffer containing 25mM Tris, 192mM glycine, 20% methanol and supported between two Whatmann 3MM papers and two sponge pads. This assembly was placed in a Biorad Mini Trans-Blot™ tank which was filled with transfer buffer. Transfer was carried out by applying a current of 240mA for 1h 45min, during which time the tank was cooled.

2.25 Native PAGE gels

Native PAGE was carried out as for SDS-PAGE described in section 2.22 excepting the exclusion of SDS and β -mercaptoethanol from all buffers and solutions which was replaced with dH₂O. The gel was run at 4°C and transferred to nitrocellulose as described in section 2.24 also at 4°C.

2.26 Western Blotting and protein detection.

Membranes were blocked overnight with PBS-Tween 20 (0.1%) + BSA 1% w/v or 5% w/v skimmed milk powder (Marvel[®]) at 4°C. Primary antibody was left on for an hour, except in the case of VPM53 and polyD which required an overnight incubation. The membranes were then washed with PBS-T (0.1%) for approximately 2 hours, changing the wash every 15 min. Secondary antibodies, horse radish peroxidase conjugated, were diluted to the appropriate concentration before incubating for an hour and the membranes washed as before. Membranes were then developed with 3,3'-diaminobenzidine (DAB) liquid substrate system (Vector laboratories Inc, Burlingame CA) or for greater sensitivity the enhanced chemiluminescence (ECL) system (Pierce, Rockford, IL). Gel images were captured using the AlphaImager system (Flowgen, Leicestershire, UK) and processed through Paintshop Pro6.

Antibody	Source (Catalogue No.)	Antigen
Primary		
VPM53	(McOrist <i>et al.</i> , 1987)	Monoclonal against LsaA
polyD	(McOrist <i>et al.</i> , 1987)	Polyclonal against <i>L. intracellularis</i>
Anti-Xpress™ Antibody	Invitrogen (R910-25)	Xpress™ epitope sequence –Asp-Leu-Tyr-Asp-Asp- Asp-Lys-
Anti-Xpress™-HRP Antibody (Invitrogen)	Invitrogen (R911-25)	Xpress™ epitope sequence –Asp-Leu-Tyr-Asp-Asp- Asp-Lys-
GAL4 DNA-BD Monoclonal Antibody	BD Biosciences (630403)	DNA-binding domain (BD) (a.a. 1-147) of the yeast GAL4 protein.
Anti-GFP Monoclonal Antibody	BD Biosciences (632375)	Green fluorescent protein (GFP) and its variants
Avadin peroxidase conjugate	Sigma (A7419)	Biotin
Anti-LsaA	Prepared by Diagnostics Scotland*	Polyclonal against rLsaA
Anti- <i>H. pylori</i> TlyA	Prepared by Diagnostics Scotland*	Polyclonal against <i>H. pylori</i> rTlyA
Anti- <i>H. pylori</i>	Dako (B0471)	Polyclonal against <i>H. pylori</i>

Monoclonal Anti-Biotin antibody produced in mouse	Sigma (B7653)	Biotin
Secondary		
Anti-mouse IgG (whole molecule)-FITC Ab produced in rabbits	Sigma (F9137)	Binds all mouse Igs
Anti-Rabbit IgG (whole molecule)-Peroxidase antibody produced in goat	Sigma (A6154)	Binds all rabbit Igs
Anti-Mouse IgG (whole molecule)-Peroxidase antibody produced in rabbit	Sigma (A9044)	Binds all mouse Igs
Anti-Pig IgG (whole molecule) –Peroxidase antibody produced in rabbit	Sigma (P8290)	Binds all pig Igs
Anti-Rabbit IgG (whole molecule) fluorescein isothiocyanate-conjugate produced in goats	Dako (B0471)	Binds all rabbit Igs
Alexa Flour [®] 488 Goat anti-Rabbit IgG	Molecular Probes (A-11008)	Binds all rabbit Igs. Labeled with photostable, green-fluorescent Alexa Flour 488 dye.

Table 2.5: Antibodies used during study. * Diagnostics Scotland, Castlelaw Building, Pentlands Science Park, Bush Loan, Penicuik, Midlothian, EH26 0PZ

2.27 Ammonium sulphate precipitation

Rabbit anti-sera produced against LsaA and the *H. pylori* TlyA protein was precipitated using ammonium sulphate. Saturated $(\text{NH}_4)_2\text{SO}_4$ was adjusted to pH 7.8 using 2M NaOH just prior to use. Saturated ammonium sulphate (2.5 ml) was slowly added to polyvalent antiserum (5 ml) with gentle stirring, allowing any precipitate to dissolve before further addition of antiserum. Once all the antiserum had been added, the solution was allowed to mix for a further two hours at room temperature. The solution was then centrifuged (14,000 x g, 30 mins) at room temperature. The supernatant was discarded and the precipitate made up to the original volume (5 ml) using saline (0.137 M NaCl). The precipitation was repeated three times. The final precipitate was resuspended in one half volume of PBS and precipitated immunoglobins dialysed against PBS at 4°C for 72 hrs with frequent changes of buffer. The precipitated IgG-PBS was stored at -20°C until required.

2.28 Production and purification of recombinant LsaA protein

2.28.1 Denaturing conditions

200ml of antibiotic-free LB broth was inoculated with a 10ml overnight culture of BL21/pRSETA::LsaA containing 35µg/ml chloramphenicol and 50µg/ml ampicillin. Growth was continued in an orbital shaker at 37°C until the OD_{600} reached between 0.4 and 0.6 when expression of recombinant LsaA (rLsaA) was induced via the addition of IPTG to a final concentration of 1mM. After a further 4-5 hours of growth at 37°C with continual shaking the culture was spun down (8000 x g, 8mins) and resuspended in 10ml of guanidium lysis buffer pH7.8, which was pre-warmed to 37°C. After the resuspension the culture was sonicated on ice using a probe sonicator (Ultrasonic Processor, Jenson Scientific Ltd.) with a micro-tip for 3 x 5 second pulses at 10u intensity with 5s cooling period between each burst. The sonicated culture was then centrifuged (3000 x g, 15 min) before decanting the supernatant to a fresh tube and storing at -20°C overnight. The following day resulting His-tag proteins were purified using the Xpress™ (Invitrogen) protein purification system according to the manufacturer's instructions. In brief, the resin inside the Pro-Bond purification columns was resuspended, the column fitted with a removable cap and placed in a 50 ml Falcon tube. The column was then

centrifuged (800 x g, 2 min) to allow aspiration of buffer. 7ml of dH₂O was then added to the column and the resin resuspended and the step repeated. Following aspiration of the second 7ml wash of dH₂O 7ml of denaturing binding buffer (8M urea, 20mM sodium phosphate, 500mM sodium chloride pH 7.8) was added and the resin resuspended before centrifuging. This step was repeated twice. After aspiration of the last 7ml aliquot of denaturing binding buffer 5ml of the sonicated lysate was added to the column, the resin resuspended and the whole assembly gently rocked for 10 mins to allow binding. Following centrifugation this step was repeated for the second 5ml lysate. The column was then washed twice with a further 4ml of denaturing binding buffer. Twice again with 4ml of denaturing wash buffer pH6.0 (8M urea, 20mM sodium phosphate, 500mM sodium chloride) and lastly twice with denaturing wash buffer pH5.3. Following aspiration of the last wash buffer the column was clamped in a retort stand, 5ml of elution buffer pH4.0 (8M urea, 20mM sodium phosphate, 500mM sodium chloride) added and the eluent was collected in 1ml fractions in 1.5ml Eppendorfs.

2.28.2 Optimisation of soluble protein expression.

100ml of modified LB broth was inoculated with a 10ml overnight culture of BL21/pRSETA::LsaA grown in the same media containing 35µg/ml chloramphenicol and 50µg/ml ampicillin. Expression cultures were grown at either 25°C or 37°C with agitation (200 r.p.m.) to early mid-log phase (A_{600} of 0.3). Protein expression was induced with IPTG (1mM) and samples (1ml) were taken at time zero and at four hourly intervals for approximately four hours. The resultant cells were collected by centrifugation (4,000 x g, 5 mins) and the pellet resuspended in 5 ml of native binding buffer (50mM NaPO₄, 0.5M NaCl, 10mM imidazole). The cells were frozen in liquid nitrogen and thawed in cold water before being briefly sonicated on ice to produce cell lysis. Cells were centrifuged (10,000 x g, 20 mins) and the supernatant, containing soluble cellular proteins, retained. The pellet, containing insoluble cellular proteins, was meanwhile resuspended in 5ml of native binding buffer. The samples were stored at 4°C until analysis by SDS-PAGE and western blotting.

2.28.3 Native conditions for rLsaA preparation

100ml of modified LB broth (10g/L tryptone, 5g/L yeast extract, 9g/L NaCl, pH6) was inoculated with a 10ml overnight culture of BL21/pRSETA::LsaA grown in the same media containing 35µg/ml chloramphenicol and 50µg/ml ampicillin. Growth was continued in an orbital shaker at 25°C until the OD₆₀₀ reached between 0.4 and 0.6 when expression of recombinant LsaA (rLsaA) was induced via the addition of IPTG to a final concentration of 1mM. After a further 4-5 hours of growth at 37°C with continual shaking the culture was spun down (8000 x g, 8mins) and resuspended in 8ml of native binding buffer (50mM NaPO₄, 0.5M NaCl, 10mM imidazole). The cells were frozen in liquid nitrogen and allowed to thaw in cold water followed by sonication on ice using a probe sonicator (Ultrasonic Processor, Jensons Scientific Ltd.) with a micro-tip for 3 x 5 second pulses at 10u intensity with a 10s cooling period between each burst. The culture was centrifuged (10,000 x g, 30 min) and the supernate containing soluble proteins used for Ni-NTA purification in the Xpress™ (Invitrogen) protein purification system according to the manufacturers instructions for native protein. Briefly, 2ml of Pro-Bond Resin was added to the 10ml purification column. The resin was settled by low-speed centrifugation and the supernatant aspirated. The resin was then resuspended in 6ml of dH₂O by alternately inverting and tapping the column. The resin was pelleted as described above, the dH₂O aspirated and replaced with 6ml of native binding buffer. The resin was resuspended in the native binding buffer, pelleted and the supernatant aspirated. This step was repeated twice. After aspiration of the last 6ml aliquot of native binding buffer 8ml of the sonicated lysate was added to the column. The column was agitated gently for 1-2 hours at 4°C to allow binding of the recombinant protein. The resin was then pelleted (800 x g, 1 min), and the supernate aspirated. The resin was washed with 8ml of native wash buffer (50mM NaPO₄, 0.5M NaCL, 20mM Imidazole), pelleted (800g, 1min) and the supernate aspirated. This step was repeated thrice more. Following aspiration of the fourth wash the column was clamped into a vertical position and the protein eluted via the addition of up to 35ml of Native Elution Buffer (50mM NaPO₄, 0.5M NaCL, 250mM Imidazole). Aliquots of 1ml were collected and stored at 4°C for further analysis.

2.29 Lowry protein determination

Protein standards of 0, 12.5, 25, 50, 100, 200 and 250 $\mu\text{g/ml}$ were prepared by diluting BSA in 0.1M sodium hydroxide (NaOH). The sample protein, in this case LsaA was diluted 1 in 5, 1 in 10 and 1 in 20 in 0.1M NaOH. 200 μl aliquots of standards and samples were added to 1ml eppendorfs in duplicate and triplicate respectively. 400 μl of freshly prepared copper reagent was added to each tube and incubated at 25 $^{\circ}\text{C}$ for 10 mins. 400 μl of Folin and Ciocalteu's phenol reagent, diluted 1 in 14 in dH_2O , was added to each tube and incubated at 25 $^{\circ}\text{C}$ for 15 mins. Each sample was transferred to a cuvette and the absorbance at 690nm measured on a spectrophotometer. A curve was generated using the standard samples of known concentration to allow calculation of the protein concentration in the test samples, an example of which is shown in Figure 2.1.

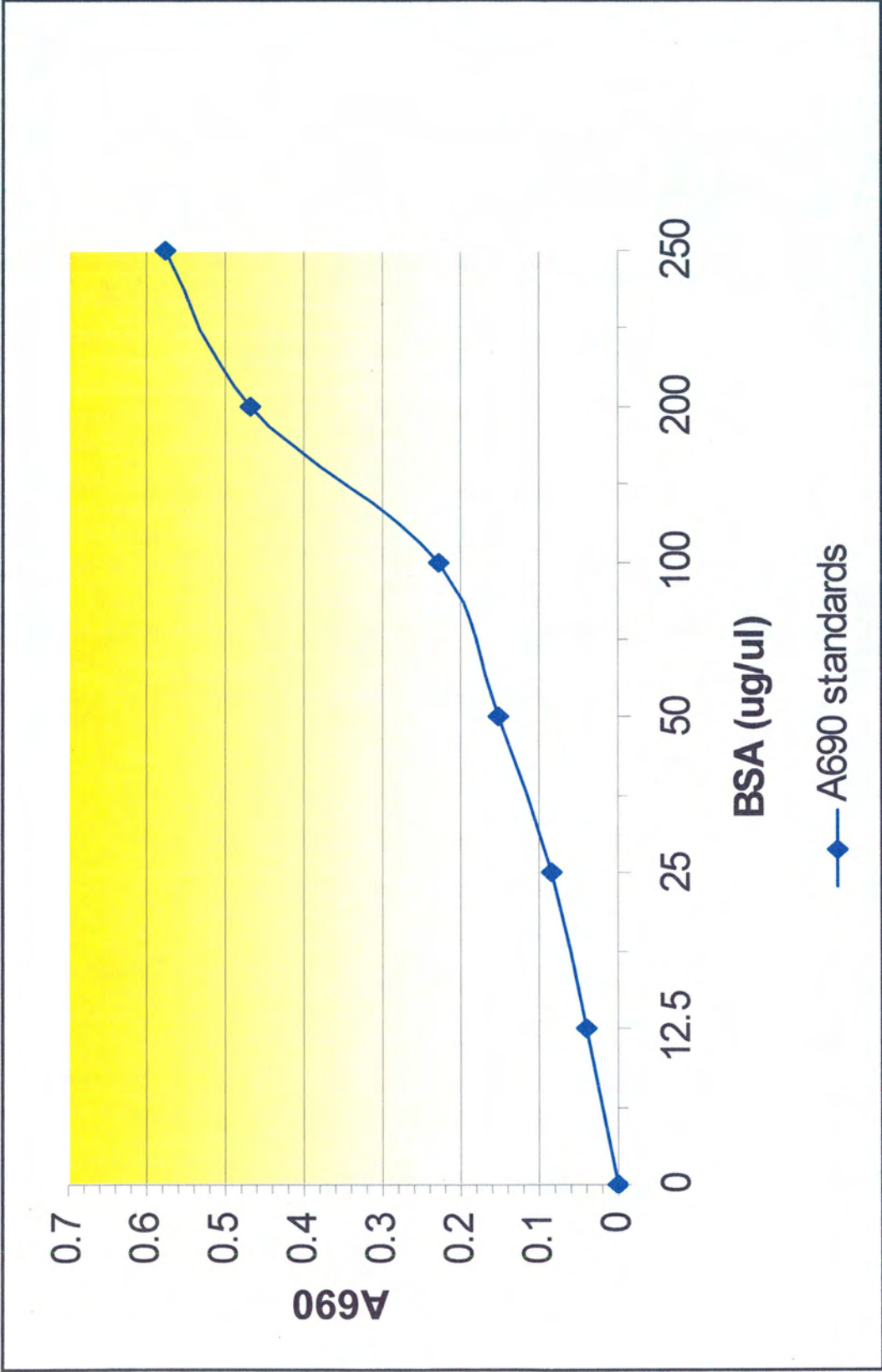


Figure 2.1: Example of Lowry assay graph

2.30 Dialysis of rLsaA

Dialysis tubing was boiled for 10 mins in a large volume of 2% (w/v) sodium bicarbonate and 1mM EDTA (pH8.0). Following a rinse in distilled water the tubing was boiled for a further 10 mins in 1mM EDTA (pH8.0). After cooling the tubing can be stored at 4°C. Before use the tubing was washed thoroughly in distilled water. All dialysis was carried out for at least 24 hours at 4°C with several changes of buffer and constant stirring.

2.31 Centricon[®] concentration of rLsaA

Centricon[®] Plus-20 centrifugal filter units (Millipore), with a molecular weight cut off of 10kDa, were used for concentration of recombinant LsaA according to the manufacturer's instructions. Briefly, a known volume of Ni-NTA purified recombinant native LsaA was decanted into the upper chamber of the filter unit and centrifuged (4,000 x g) at 4°C until the volume had decreased by approximately 75%. Concentrated protein was collected by inverting the Centricon[®] centrifugal filter unit into the collection cup provided and centrifuging briefly (4,000 x g) at 4°C.

2.32 Solubilisation of semi-confluent INT-407 and IEC-18 cells.

Cells were grown to semi-confluency in a 75ml tissue culture flask. Cells were then washed three times in ice-cold PBS before scraping into 0.5ml of ice-cold lysis buffer (50mM Tris-HCL pH7.6, 0.5mM EDTA, 0.5mM PMSF, 0.5% protease inhibitor cocktail + 1% TritonX-100). Cells were pipetted up and down extensively for 10 mins before centrifuging (13,000 x g) for 10 mins in a microcentrifuge at 4°C and the supernatant retained.

2.33 Affinity Chromatography

20ml of LsaA (0.75 mg/ml) was dialysed overnight in approximately 1 litre of coupling buffer (0.1 M NaHCO₃ (pH8.3) containing 0.5M NaCl). The following day approximately 2g of CN-Br activated sepharose (Amersham Pharmacia Biotech, Sweden) was allowed to swell in 1mM HCL before washing through a glass-sintered filter initially with 1mM HCL and then coupling buffer. Coupling solution containing the ligand and gel were then mixed in a falcon tube in an end-over-end stirrer for 1 hour

at room temperature. Excess ligand was then washed away. To block any remaining active sites the slurry is then left to stand for 2 hours in 0.1M Tris HCl pH8.0 before packing into a 5ml syringe barrel. Unbound ligand was washed off with washes of alternating pH (0.1M acetate pH 4.0 followed by 0.1M Tris-HCL pH8.3 containing 0.5M NaCl). The slurry is equilibrated with PBS. 1ml sub-confluent INT-407 membrane-enriched cell lysate was left in the column overnight at 4°C to facilitate binding. Approximately 10ml PBS and then 10ml PBS containing 1% Triton X-100 was passed through the column and collected in 200µl fractions. Once the protein content of these fractions was undetectable 1M NaCl was passed through the column and 200µl fractions collected until protein content was again negligible. Fractions are analysed by SDS-PAGE and ECL Western blotting detection kit (Pierce, Rockford, IL.).

2.34 Biotin labelling of cell membrane proteins

A semi-confluent flask of INT-407s was washed with ice-cold PBS sulfosuccinimidyl 6-(biotinamido)hexanoate (Sulfo-NHS-LC-biotin) as recommended by the manufacturer (Pierce, Rockford, IL). Cells were washed with PBS and ice-cold extraction buffer (PBS containing 1mM MgCl₂, 0.05mM CaCl₂, 200mM Octyl-B-D-glucopyranoside (Fisher Scientific UK Ltd, Loughborough), 1mM NaVO₃ and 10 ml protease inhibitor cocktail added. Following incubation for 1 hour at room temperature the cells are scraped into the extraction buffer and centrifuged (13,000 x g) for 15 mins in a microcentrifuge at 4°C and the supernatant retained.

2.35 TCA Protein Precipitation

100% TCA was added to the protein sample to give a final concentration of 10%TCA (v/v) and the solution incubated overnight at 4°C. Following centrifugation at 15,000 x g (4°C) for 30 mins the pellets were dried for 30 mins – 1 hour or until no trace of liquid remained. Pellets were then resuspended in a suitable volume of Tris-HCl pH8.0.

2.36 Cation Exchange Chromatography

Cation exchange HPLC was performed using a TSK-DEAE-5PW (8 x 75mm) column pre-equilibrated with 20mM Na₂HPO₄(pH 7) buffer. Approximately 8ml of rLsaA, which had been dialysed against 20mM Na₂HPO₄(pH 7), was applied to the column in a series of 1ml injections. Sufficient time was allowed after the final injection for non-absorbed material to pass through the column and for the baseline to stabilise. Absorbed proteins were eluted from the cation exchange matrix over a period of 30 minutes by the application of a stepped NaCl gradient at a flow rate of 0.5ml/min. Fractions of 1ml were collected and examined for the presence of rLsaA by SDS-PAGE and immunoblotting.

2.37 Binding of biotinylated *L.intracellularis* proteins to glutaraldehyde-fixed mammalian cells

Intact *L. intracellularis* (DK15540C) were labelled with sulfosuccinimidyl 6-(biotinamido)hexanoate (NHS-LC-biotin) as recommended by the manufacturer (Pierce, Rockford, IL). The biotinylated organisms were suspended in solubilisation buffer (35mM Tris [pH8.2], 0.25M NaCl, 16mg of sodium deoxycholate per ml, 0.1% sodium dodecyl sulphate (SDS), 1mM phenylmethylsulfonyl flouride) and incubated for 30 min at 37°C with gentle mixing. The suspension was centrifuged at 100,000 x g for 1h at 4°C to obtain a supernatant fraction of biotinylated-detergent-soluble proteins (BDSP). Semi-confluent INT-407 cells (2 x 10⁵/well) were cultured for 20-24hrs in a 24 well plate. Cells were washed twice in PBS before fixing for 10 min with 2.5% (v/v) glutaraldehyde at room temperature. Varying concentrations of DSBP were added to the glutaraldehyde fixed cells for 1h at 37°C with gentle rocking and washed three times with PBS containing 0.5% Tween 20. The BDSP, which selectively bound to glutaraldehyde fixed cells, were resuspended in 1 x SDS loading buffer and separated by SDS-polyacrylamide gel electrophoresis.

2.38 2D Gel electrophoresis (2D-PAGE)

The method used for 2-DGE was based on that recommended by Amersham Bioscience. Commercially available precast gels were used for both the first and second dimensions which were carried out using an IPGphor (first dimension) and either a flat-bed Multiphor or a vertical Ettan DALTsix system (second dimension). Commercially available first dimension strips used are 7 cm 3-10 NL and 24 cm 3-10 NL which were then run on either a 12.5% ExcelGel (7 cm strips) or a 12.5% Ettan DALT Gel (24 cm strips).

Bacterial pellets were resuspended in lysis buffer (8M Urease, 4% (w/v) CHAPS, 40mM Tris, Protease inhibitor cocktail (Boehringer Mannheim) and briefly sonicated on ice. Samples were then centrifuged at 20,000 x g for 30 mins at 4°C. Samples were cleaned up using the 2-D Clean-Up kit (Amersham Biosciences) according to the manufacturer's instructions.

Following the clean-up process protein pellets were resuspended in an appropriate volume of rehydration solution (8M urea, 2% (w/v) CHAPS, 0.2% (w/v) DTT, 1% (v/v) IPG buffer and 0.002% bromophenol blue) for the strip being used. In the first dimension IPG strips were rehydrated for a minimum of 12 hours either by using the DryStrip reswelling tray or the IPGphor strip holders. Once this time had elapsed, the current was automatically applied to the IPGphor strip holders whereas the strips from the DryStrip reswelling tray first need to be transferred to cup loading strip holders and then placed on the IPGphor.

The method of rehydration used depended on the first dimension strip. For 7 cm strips that are used to give a snap-shot of the protein present, the IPGphor strip holders were used, however, for large analytical gels, the DryStrip reswelling tray method was used. Seven centimetre strips are focused to ~20,000 volt hours (Vhr) while 18 cm strips were focused to ~80,000 Vhr. At this point strips were stored at -80°C until required for second dimension analysis.

For the second dimension, the first dimension strips were removed from -80°C storage and allowed to warm up to room temperature. The strips then underwent a two step equilibration process with equilibration buffer (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% bromophenol blue). Strips were incubated at

room temperature for 20 minutes on a shaking platform with 5 – 10 ml of equilibration buffer containing 4% (w/v) iodoacetamide. The equilibration buffer was then discarded and replaced with the same volume of fresh equilibration buffer containing 1% DTT and incubated as previously.

First dimension strips were then removed from the equilibration buffer and carefully blotted to remove excess traces of equilibration buffer before being placed onto the ExcelGel for electrophoresis. Conditions for the ExcelGel 12.5% are 600 V, 20 mA, 40 W for 30 minutes after which time, the first dimension strips were removed from the gel. Electrophoresis was then continued at 600 V, 40 mA, 40 W until the tracking dye had migrated to the anodic buffer strip. For the Ettan DALTEGel 12.5% conditions were (1000V, 20 mA, 40 W for the first 30 minutes when the first dimension strips were removed and electrophoresis is continued at 1000 V, 40mA, 40 W).

2.39 MALDI-ToF mass spectrometry

Bands of interest were excised from 1-D or 2-D gels. Proteins were destained and reductively alkylated using DTT and Iodoacetamide. The gel pieces were then digested overnight with Trypsin (Promega Porcine trypsin) at 37°C. Digests were analysed on a Voyager DE-PRO MALDI-ToF mass spectrometer (Applied Biosystems), scanning the 600 to 5000 kilodalton region in reflectron mode producing monoisotopic resolution. The spectra generated were mass calibrated using known standards and the peaks deisotoped. Masses obtained were then database searched using the MASCOT search engine and the NCBI nr and Swissprot databases. Searches were conducted using 50 ppm and 100ppm mass tolerance windows.

2.40 Infection Inhibition Assays

Tracks were seeded with 40% semi-confluent INT-407s overnight. The following morning cells are treated with either trypsin 200µg/ml⁻¹, proteinase K 200µg/ml⁻¹. All reagents were made up in Dulbeccos phosphate buffered saline (DPBS) before addition to the monolayer for 90 mins at 37°C. Following which cells were washed in PBS and an aliquot of frozen *L. intracellularis* LR189 (10⁷/ml) was used for infection. Following an overnight incubation tracks were immunostained using an immunoperoxidase technique

and a mouse monoclonal antibody (VPM53). The coverslip was removed from the Trac bottle and washed in PBS for 5 mins, then placed in acetone for 60 sec. Once removed and dried they were fixed to a glass slide using a drop of Loctite™ glass bond glue. Staining was carried out in a moisture chamber: firstly slides were washed in PBS for 5 mins, then for 2x10 minute washes with PBS-0.5% azide (to block endogenous peroxidase). Next a 5 minute wash with PBS, preceded 10 mins with IPX buffer (0.08% Tween 80, 0.1% BSA in PBS). The slides were incubated with the primary mouse monoclonal antibody (VPM53), diluted 1:200 in IPX buffer, and incubated with the secondary antibody (goat anti-mouse HRP) at room temperature for 1 hour. Slides were then rinsed in IPX buffer and PBS (for 5 mins each) before the DAB substrate (3,3'-diaminobenzidine, Vector Laboratories) was added for 5 min. Finally the slides were counterstained with haematoxylin and mounted in DPX (BDH Laboratory supplies, Poole, England). *L. intracellularis* stained deep brown, and the nuclei of the epithelial cells blue/purple.

2.41 Transfection of INT-407 cells with pEGFPC1 constructs

50µl of plasmid DNA and 30µl of DOTAP (Roche) mixed with 70µl sterile PBS were combined and incubated at room temperature for 10-15 min. The mixture was then resuspended in 2ml of INT-407 media and 200µl added to the each well of an 8 well chamber slide (Nalge Nunc International) containing 40% semi-confluent INT-407. The transfection media was left on the cells for approximately 6 hours before changing to fresh media. Following an overnight incubation the media was aspirated and cells washed in PBS before staining and visualisation.

2.42 Cell Staining for Confocal Microscopy

2.42.1 GFP

Cells which had been cultivated in an 8 well chamber slide were gently washed 2- 3 times with PBS before fixing with ice-cold 2% paraformaldehyde, 0.2% Triton X-100 for 20 mins. The fixative was removed and cells were washed with PBS. The monolayer was then covered in 150µl Phalloidin-conjugated TRITC (5µg/ml), and left in the dark at

room temperature. After twenty mins cells were washed with PBS and 100 μ l TOPRO-2 (1/1000 in PBS) (Molecular Probes) was added to each well and the slide incubated in the dark at room temperature for 10 mins. The slide was then given a final PBS wash before removing the wells. A drop of fluorescent mounting media (DAKO) was added to cover the wells before the coverslip was glued in position and the slide left to set at 4°C for at least an hour. Images were acquired using a Leica TCSNT confocal system (Leica Microsystems, GmbH, Heidelberg, Germany), equipped with an Argon/Krypton mixed gas laser, allowing 3-channel detection of fluorophores. Multi-channel images were acquired either simultaneously, or sequentially. A x63 Plan Apo oil immersion lens (NA 1.32) was routinely used, often in conjunction with the digital zoom capability of the confocal system.

2.42.2 *L.intracellularis* infected cells

Following fixing, slides were incubated with VPM53 (1/200) for 30 min at 37°C before washing with PBS. Slides were then incubated with rabbit anti-mouse FITC conjugate (1/25) for 1 hr at room temperature. Staining with phalloidin and TOPRO-2 was then continued as detailed in section 2.40.1.

2.43 Yeast protein extraction

2.43.1 Preparation of yeast cultures for protein extraction

For each transformed strain to be assayed in a Western Blot, a 5ml culture was prepared in the appropriate SD selection medium (Clontech). The medium was inoculated with a single isolated colony (1-2 mm in diameter). A 10 ml culture of an untransformed yeast strain AH109 was also prepared in the appropriate SD medium (SD -Ura). Cultures were grown overnight at 30°C with shaking (250 rpm). The following day overnight cultures were vortexed to disperse cell clumps and the entire overnight culture used to inoculate separate 50ml aliquots of YPD medium. The cultures were grown at 30°C (200-250 rpm) until the OD₆₀₀ reached 0.4 – 0.6. Total number of OD₆₀₀ units for each culture was obtained by multiplying the OD₆₀₀ (of a 1ml sample) by the culture volume. The culture was then quickly pre-chilled by pouring into 100 ml centrifuge tubes filled

halfway with ice. Tubes were immediately centrifuged at 4°C (1,000 x g, 5 min) and the cell pellet resuspended in 50ml of ice-cold H₂O. The pellet was then recovered (1,000 x g, 5 min) and immediately frozen in liquid nitrogen before storing at -70°C until required.

2.43.2 Preparation of Yeast Protein Extracts

Cell pellets were thawed by resuspending in pre-warmed (60°C) cracking buffer (8M Urea, 5% (w/v) SDS, 40mM Tris-HCl [pH 6.8], 0.4% (w/v) bromophenol blue, 0.88% (v/v) β-mercaptoethanol, pepstatin A 0.1 mg/ml, leupeptin 0.03mM, Benzamidine 145 mM, Aprotinin 0.37 gm/ml, 0.01mM PMSF*). Each cell suspension was then transferred to a 1.5 ml screw-cap microcentrifuge tube containing 80μl of glass beads per 7.5 OD₆₀₀ units of cells. Samples were heated at 70°C for 10 min before vortexing vigorously (1 min). Debris and unbroken cells were pelleted in a microcentrifuge at 4°C (12,000 x g, 5 min) and the supernatants transferred to a fresh 1.5 ml microcentrifuge tube on ice. Pellets were treated as follows: Placed in boiling water bath (100°C) for 3 – 5 min, Vortexed vigorously for 1 min, unbroken cells were pelleted (12, 000 x g, 5 min) at 4°C and the supernate removed. The supernate was either boiled and immediately loaded onto an SDS PAGE gel or stored at -70°C for later analysis.

* Due to the short degradation of PMSF in aqueous solution (half-life of approx. 60 mins) an additional aliquot of PMSF stock solution (1mM in isopropanol) was added to the samples after 15 min and approximately every 7 min thereafter until the sample was boiled.

2.44 cDNA Library Synthesis

2.44.1

The cDNA synthesis kit (Stratagene) is optimised for 5µg of poly(A)⁺ RNA. All non-enzymatic first and second strand components were thawed briefly and vortexed before placing on ice.

2.44.2 First Strand cDNA synthesis

The following reagents were added in order to an RNase-free tube: 5µl 10x first strand buffer, 3µl first strand methyl nucleotide mixture, 2µl linker-primer (1.4µg/ml-1) 12.5µl diethylpyrocarbonate (DEPC)-treated water, 1µl RNase block ribonuclease inhibitor (40U/µl). The reaction was gently mixed and 5 µg of poly(A)⁺ mRNA added. For the control, the above annealing reaction was used with 25µl (5µg) of test RNA and 12.5µl DEPC-treated water. The primer was allowed to anneal to the template for 10 min at room temperature (RT) before the addition of 1.5µl of MMLV-RT (50U/µl), to give a final reaction volume of 50µl. The sample was mixed gently and centrifuged. 5µl of this first-strand synthesis reaction was transferred to a tube containing 0.5µl [α -³²P]dGTP (800Ci/mmol) and this constituted the first strand synthesis control reaction. Both reactions were incubated at 37°C for 1hr: the non-radioactive first strand synthesis reaction was placed on ice and the radioactive control kept at -20°C.

2.44.3 Second strand cDNA synthesis

The following components were added in order to the 45 µl non radioactive first strand synthesis reaction on ice: 20µl 10x second strand buffer, 6µl second strand deoxyribonucleotide (dNTP) mixture, 114µl sterile water, 2µl [α -³²P]dGTP (800Ci/mmol). To this second strand synthesis reaction 2µl of RNase H (1.5U/µl) and 11µl DNA polymerase I (9.0U/µl) were added. The reaction was gently mixed, centrifuged and incubated at 16°C for 2.5h. After the second strand synthesis, the reaction was placed on ice immediately. The low incubation temperature was critical in preventing the formation of unclonable hairpin structures.

To the second strand synthesis reaction 23µl blunting dNTP mix and 2µl of (5U/µl) *Pfu* DNA polymerase were gently mixed and centrifuged following incubation at 72°C for 30 mins. 200µl phenol-chloroform [1:1(v/v)] was added and mixed by vortex. The

reaction was centrifuged for 2 mins at room temperature and the upper aqueous layer containing the cDNA transferred to a fresh tube, carefully avoiding removal of any interphase. An equal volume of chloroform was added and mixed. The cDNA was precipitated overnight at -20°C with $20\mu\text{l}$ 3M sodium acetate and $400\mu\text{l}$ of 100% (v/v) ethanol, followed by centrifugation at maximum speed for 60 mins at 4°C . The supernatant was discarded and the cDNA pellet gently washed with $500\mu\text{l}$ 70% (v/v) ethanol and lyophilised until dry. the pellet was resuspended in $9\mu\text{l}$ *EcoRI* adapters and incubated at 4°C for at least 30 mins. $1\mu\text{l}$ of this second strand synthesis reaction was transferred to a fresh tube and this represented the second strand synthesis control reaction.

2.44.4 Resolving cDNA on alkaline and non-denaturing acrylamide gels

cDNAs can be resolved by electrophoresis on an alkaline gel to determine their size range. The first and second strand cDNA synthesis reactions prepared in section 2.44.2 and 2.44.3, including controls, were resolved on a 1% alkaline agarose gel (1-3kb cDNA size range). Due to the low buffering capacity of these gels the reactions were electrophoresed at 100mA for approximately 3.5h. The gel was dried using a gel drier and exposed to x-ray film over night at -20°C .

Alternatively, the size fractionated cDNAs prepared in section 2.44.8 were electrophoresed at 100V for 1 hour on a 5% (w/v) non-denaturing acrylamide gel and exposed to x-ray film over-night at -70°C .

2.44.5 Ligation of *EcoR* I adapters

The following components were added to the blunted cDNA and *EcoRI* adapters: $1\mu\text{l}$ 10x ligase buffer, $1\mu\text{l}$ 10mM rATP and $1\mu\text{l}$ T4 DNA ligase ($4\text{U}/\mu\text{l}^{-1}$) and incubated overnight at 8°C The ligase was heat inactivated at 70°C for 30 min, after which the reaction was centrifuged for 2 secs before being cooled at RT for 5 min.

2.44.6 Phosphorylation of *EcoR* I ends

The *EcoRI* adapter ends were phosphorylated by the addition of: 1 l 10x ligase buffer, $2\mu\text{l}$ 10mM rATP, $6\mu\text{l}$ sterile water and $1\mu\text{l}$ T4 polynucleotide kinase ($10.0\text{U}/\mu\text{l}^{-1}$) and

incubated for 30 min at 37°C. The kinase was heat inactivated for 30 min at 70°C, after which the reaction was centrifuged for 2 sec before being cooled at RT for 5 min.

2.44.7 *Xho* I Digestion

The cDNA was digested with *Xho* I restriction endonuclease with the addition of 28µl *Xho* I buffer and 3µl *Xho* I (40U/µl⁻¹), followed by incubation for 1.5h at 37°C, 5µl of 10x STE buffer (1M NaCl, 200mM Tris-HCL pH 7.5, 100mM EDTA) and 12µl 100% ethanol were added and the cDNA precipitated overnight at -20°C. Following precipitation, the cDNA was centrifuged for 60 min at 4°C, and the pellet dried completely before resuspension in 14µl 1x STE buffer, 3.5µl of the column loading dye was added to the sample prior to size fractionation.

2.44.8 cDNA size fractionation

The cDNA was size fractionated using a drip column containing sepharose CL-2B gel filtration medium assembled according to the manufacturer's instructions. The column was washed with 10ml of 1xSTE buffer, ensuring a steady flow rate was maintained and preventing the column drying out. When approximately 50µl of STE buffer remained above the surface of the resin, the cDNA sample was loaded gently onto the column bed. Once the sample entered the sepharose CL-2B gel filtration medium, 3ml of 1xSTE buffer were added to the reservoir.

In order to gauge sample elution from the column, the progress of the dye front was monitored. As the cDNA sample eluted through the column, the dye gradually migrated through the resin. A minimum of 12 fractions, each containing 3 drops (~ 100µl) were collected. To ensure the cDNA had been successfully eluted, the fractions were monitored for the presence of radioactivity and collected until the unincorporated nucleotides were eluted.

Before progress of the fractions and recovery of the size-fractionated cDNA, 8µl of each fraction was saved for analysis by DNA polyacrylamide gel electrophoresis (section): to assess the effectiveness of the size fractionation and determine which fractions could be used for ligation.

2.44.9 Processing the cDNA fractions

To recover the size selected cDNA, the fractions collected from the drip column were extracted with phenol-chloroform and precipitated with ethanol to remove contaminating proteins carried over from previous steps. To ensure the cDNA had been recovered, the level of radioactivity present in the pellet was monitored.

The pellet was carefully washed with 200 μ l 80% (v/v) ethanol, centrifuged for 2 min at RT and air dried for a maximum of ~ 5 min. Recovery of the cDNA was verified by radioactive monitoring.

2.44.10 Quantification of DNA: Ethidium bromide plate assay

DNA can be quantified by UV visualisation on ethidium bromide (EtBr) agarose plates [0.8% (w/v) agarose / Tris-acetate media / EtBr (10mg/ml⁻¹)], using a DNA sample of known concentration as a standard.

Several dilutions of a DNA sample of known concentration (1Kb DNA ladder) were prepared in 100mM EDTA ranging from 10-200 ng/ μ l⁻¹. 0.5 μ l of the cDNA was spotted adjacent to the standards, and the samples absorbed into the plate for 10-15 min at RT. The spotted sample of unknown concentration was then compared with the standards and quantified.

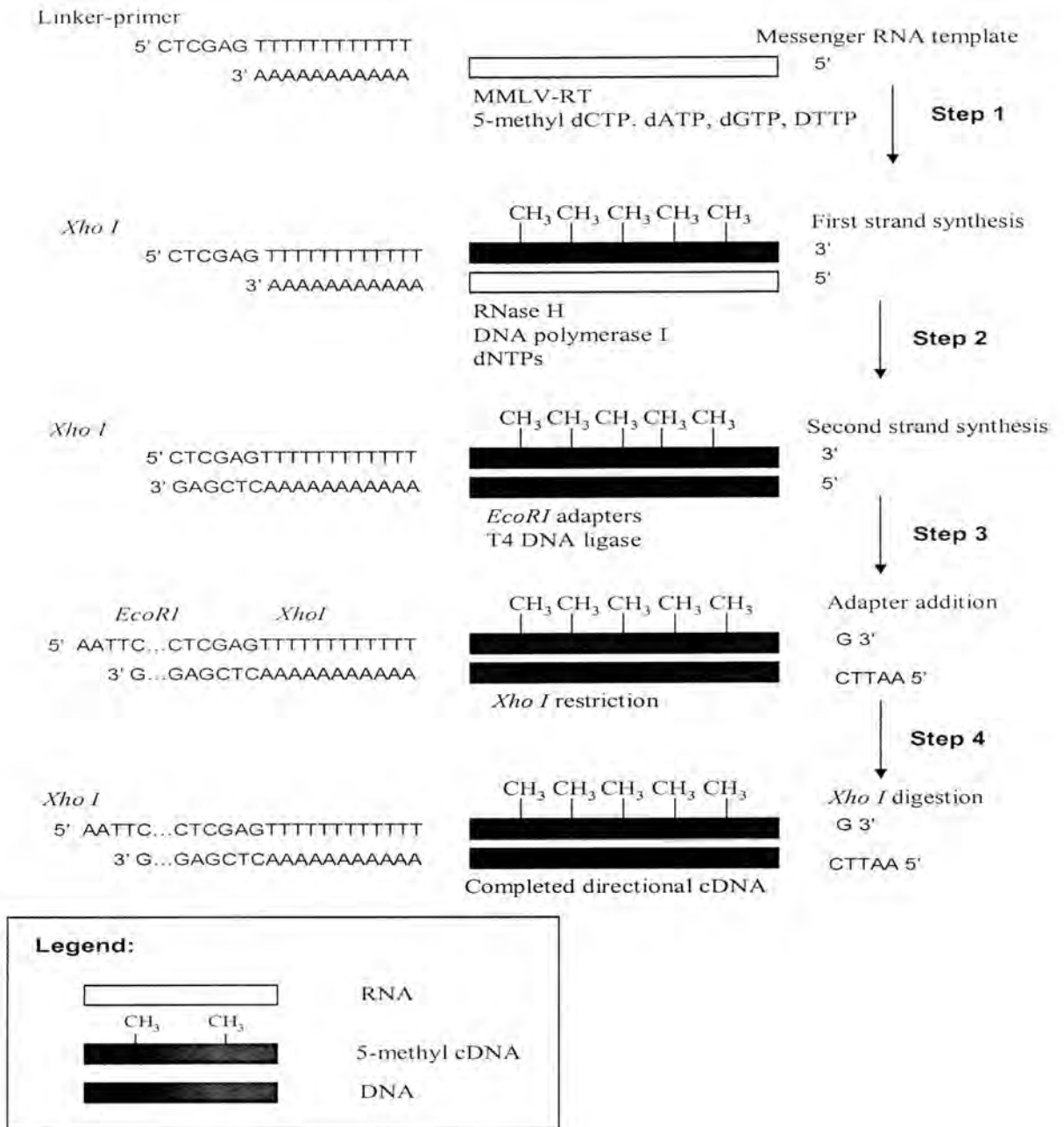


Figure 2.2: Flow chart of cDNA synthesis: During the first-strand synthesis an oligo (dT) linker-primer containing the *Xho I* restriction site anneals to the messenger RNA, which is reverse transcribed using MMLV-RT and 5-methyl dGTP. RNA fragments are 'nick translated' by DNA polymerase I during second strand synthesis, resulting in double stranded cDNA. The blunt ends of the cDNA fragments are ligated with *EcoR I* adapters and subsequent digestion with *Xho I*, releases the *EcoR I* adapters and residual primer-linker from the 3' end of the cDNA.

2.44.11 Non-Denaturing DNA Polyacrylamide Gel Electrophoresis

Two glass plates were clamped together vertically and a resolving gel composed of; N,N'-methylenebisacrylamide [30% (w/v): 0.8% (w/v)], 17.7mM Tris-Borate, 0.39mM EDTA (pH 8.3), 1.64% (w/v) ammonium persulphate and 0.0082% (v/v) N,N,N',N'-tetramethylethyldiamine (TEMED), was poured between them and a Teflon comb was inserted to form wells for the loading of samples. Following polymerisation the comb was removed and the wells rinsed with 1 x TBE (0.09M Tris-Borate, 0.01M EDTA [pH 8.3]). Assembled gel placed in a Biorad Mini-Protean II™ electrophoresis tank. Both reservoirs were then filled with electrophoresis buffer containing; 1 x TBE. Samples were mixed 1:1 with loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol) and loaded into the wells of the gel using gel loading tips. Samples were electrophoresed at a constant voltage of 80V until the dye front had reached the bottom of the gel. The gel was then removed from the assembly and dried using a flat bed gel drier. DNA was visualised by exposing the dried gel to film overnight at -70°C before developing.

2.45 Quantification of *H. pylori* adherence to AGS cells.

Plate grown *H. pylori* were resuspended in BHI broth to an OD_{600} of 0.7 approximately equating to 1×10^7 cfu/ml⁻¹. AGS cells were counted using a haemocytometer and seeded at a density of 1×10^5 to give a ratio of 100:1 (bacteria:cells). *H. pylori* were incubated with AGS cells at 37°C for 1 hour. Non-adherent bacteria were removed by washing with PBS.

2.45.1 Urease Assay

50 μl of PBS containing 300mM urea, 0.5% (w/v) phenol red was added to each well of a 96 well plate which was then placed in a 37°C incubator for 20 or 50 mins. Following the prescribed time period the medium was transferred to a fresh 96 well plate and the OD_{562} recorded using a plate reader (ELx808_{1U} Ultra Microplate Reader BIO-TEK Instruments Inc.).

2.45.2 Flow cytometry

AGS cells were removed from flasks by using nonenzymatic detachment (Accutase; Innovative Cell Technologies, La Jolla, Calif.) and resuspended in PBS at a known density using a haemocytometer. *H. pylori* were treated as follows:

2.45.2.1 Anti-*H. pylori* antibody labelling.

Bacteria were washed once and subsequently incubated with a (1:5) dilution of rabbit anti-*H. pylori* antibody in PBS on ice for (30 min). After being washed with PBS the cells were then incubated for an additional 30 min on ice in a (1:20 in PBS) dilution of anti-rabbit FITC-conjugate.

2.45.2.2 SYTO 9 labelling

A known number of *H. pylori* (Section 2.47) were resuspended in 0.85% NaCl and incubated with 0.5 μ M SYTO 9 (Molecular Probes) for 15-30 minutes in the dark.

Following labelling bacteria were washed in PBS and added to a predefined number of AGS cells.

Following addition of *H. pylori* to AGS cells, in a 10:1 ratio, bacteria were allowed to adhere at 37°C (150 r.p.m.) for one hour. Following which non-adherent bacteria were removed with several washes of 15% sucrose (15ml) and a final wash of PBS before resuspending in 0.5ml of FACS buffer or 1 ml of 1% formaldehyde for flow cytometric analysis. A FACSCalibur flow cytometer (Becton Dickinson, Mountain View, Calif.) was used to measure bacteria adhering to AGS cells and the sample was gated to exclude cell debris and unbound bacteria. Data acquisition and analysis was performed with CellQuest software (Becton Dickinson, Mountain View, Calif.).

2.46 Determination of viable mammalian cell numbers

Estimation of the number of viable cells was made using 0.4% (w/v) trypan blue dye and a Neubauer haemocytometer (Hawksley, England). Live cells exclude the blue dye and appear clear whilst dead cells stain blue. The average number of cells per large square (mm²) of the haemocytometer was estimated under phase contrast, using an inverted

microscope (Leica, Portugal). The number of cells/ml⁻¹ in the original suspension was calculated using the following equation:

$$\text{Viable cells ml}^{-1} = \text{cell count} \times \text{dilution factor} \times 10^4$$

10⁴ (Factor to adjust for the volume between the coverslip and haemocytometer chamber)

2.47 Viability counts of *H. pylori*

Viability counts were carried out following 48 hrs growth on blood agar plates. Cultures resuspended in BHI broth at a known OD₆₀₀ were used to make 10-fold serial dilutions and plated onto blood agar plates. Cfu were counted at a range of OD₆₀₀ densities and used to plot a graph of OD₆₀₀ vs. cfu.

Chapter 3

PRODUCTION AND FUNCTIONAL ANALYSIS OF RECOMBINANT LsaA

CHAPTER 3

Production and functional analysis of recombinant LsaA

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3.1 Introduction

The *LsaA* ORF is 702bp and, in common with other *L.intracellularis* genes and other members of the TlyA family, is AT rich (approximately 73% AT). The *LsaA* ORF has been sequenced and *LsaA* is adjacent to a homologue of *deoB*, *nusB* (upstream) and *nifS* (downstream). Sequences of *deoB* and *nifS* are incomplete whilst *nusB* is partial, possibly a pseudogene (McCluskey *et al.*, 2002). *NifS* and *DeoB* are both central to the metabolic pathway and their loss may be representative of an adaptation of *L. intracellularis* to an intracellular lifestyle. The *NusB* protein is involved in the regulation of rRNA biosynthesis by transcriptional antitermination (Huenges *et al.*, 1998).

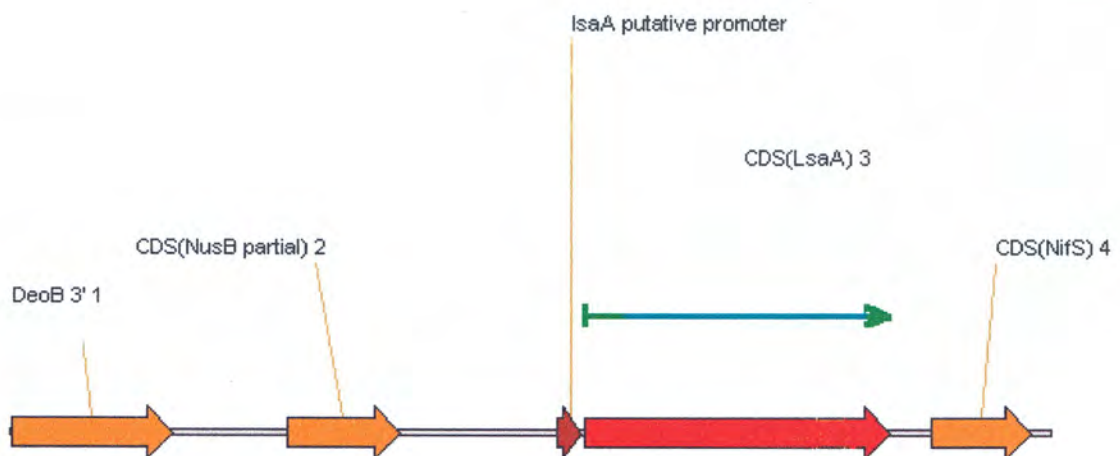


Figure 3.1: *Lawsonia intracellularis* *LsaA* locus containing four open reading frames. *LsaA* putative promoter is highlighted (Alberdi *et al.*, manuscript in preparation), and appears to be located immediately upstream from the *LsaA* start codon. Regions upstream and downstream from this putative Orf showed similarities to other bacterial sequences (*deoB*, *nusB* and *nifS*), however, none of which represent possible complete open reading frames (McCluskey, Hannigan *et al.*, 2002).

Analysis of the upstream DNA sequence of both *LsaA* and the 16s gene from *L.intracellularis* found no evidence of the typical *E.coli* -35 (TATAAT) and -10 (TTGACA) sequences associated with the start of transcription for principal sigma factor (δ^{70})-dependent promoters (Hawkey and McClure, 1983). Although the

expression of LsaA may not be under δ^{70} control it would appear to be under the control of a different δ factor which binds to alternative -10 and -35 DNA sequences. The -10 transcription initiation site predicted for LsaA is equally AT rich as the *E. coli* Pribnow box. In the latter case, the low G+C content is thought to facilitate strand dissociation due to the lower occurrence of H-bonds. Experiments with *lacZ* fusions using varying lengths of the upstream region of *LsaA* have shown that transcription is maximal when a 50bp upstream region is employed. Expression analysis of the translational fusions also suggested that the LsaA promoter function was impaired in iron-depleted media, at a basic pH or at low temperatures. Interestingly, an acidic pH of 6 did not down-regulate expression suggesting *L. intracellularis* may be tolerant of acidic conditions, an important adaptation for a pathogen who must survive passage through the stomach to reach the site of infection (Alberdi *et al.*, manuscript in preparation).

A system for the production of recombinant LsaA has been previously established in the laboratory (McCluskey *et al.*, 2002). The Xpress™ (Invitrogen) system allows expression of recombinant proteins from a modified pUC-derived vector containing a phage T7 promoter that facilitates high levels of protein expression (Appendix I.01). *LsaA* has previously been cloned into the pRSET A vector and its insertion in the correct orientation and open reading frame verified. The insert is under control of the *lac* operon, therefore addition of IPTG (Isopropyl- β -D-Thiogalactopyranoside) to the growth media results in induction of expression rLsaA incorporating a six residue N-terminal His-tag. The (His)₆ tag is one of the most commonly used affinity tags due to its small size, strong metal ion binding capacity and ability to bind under denaturing, as well as native conditions (Gaberc-Porekar and Menart, 2001, Ueda, 2003). The tag enables selection of the fusion product using immobilised-metal affinity chromatography (IMAC). In this particular case, IMAC utilises a nickel ion chelated to nitrilotriacetic acid (Ni-NTA), NTA occupying four out of six nickel ion ligand binding sites, the two remaining sites being responsible for binding to the histidine tag. In binding proteins possessing a (His)₆ tag the IMAC system facilitates enrichment of the protein of interest and separation from extraneous molecules that have no affinity for nickel ions.

Although the function of relatively few members of the TlyA family have been studied in detail, TlyA from *H.pylori*, and now LsaA, have been shown to have roles in attachment/adherence to host cells (Martino *et al.*, 2001, McCluskey *et al.*, 2002). The cell surface location of most of the TlyA family is speculative, although it has been confirmed for LsaA via immunogold labelling experiments and immunohistochemistry (Figure 3.2) (McOrist *et al.*, 1989b, McOrist *et al.*, 1989a). Its cell surface location and immunodominant status would both support the existence of an important role for LsaA in the early stages of infection.

The aim of the work described in this chapter was to further elucidate the function of the LsaA protein theoretically, through bioinformatic analysis, and also functionally, by producing recombinant protein (rLsaA) for downstream use in the identification of a potential receptor molecule(s) on the host epithelial surface.



Figure 3.2: Immunogold labelling of LsaA with monoclonal antibody VPM53. Numerous 15-nm gold particles conjugated to VPM53 can be seen on the surface of the organism. Taken from McOrist *et al.*, (1989a).

3.2 Results

3.2.1 Analysis of *L.intracellularis* LsaA

Analysis of LsaA using a motif search algorithm found four possible functional motifs which indicate the potential for post-translational modification of fully mature LsaA protein (Bairoch *et al.*, 1997).

- 1 N-glycosylation site
- 4 Protein kinase C phosphorylation site
- 4 Casein kinase II phosphorylation site
- 1 N-myristoylation site

LsaA is predicted to be a soluble protein (Classification and Secondary Structure Prediction of Membrane Proteins, Mitaku Group, Department of Biotechnology, Tokyo University of Agriculture and Technology) and analysis with the dense alignment surface (DAS) algorithm found no predicted transmembrane regions. In this respect LsaA was found to differ from other members of the TlyA family which have a distinct hydrophobic stretch approximately in the middle of the linear amino acid sequence. Whereas other TlyA homologues were predicted to have one, or at most possibly two, membrane spanning regions the homologue from *Campylobacter jejuni* was found to possess several such stretches (Figure 3.3). Transmembrane helices in integral membrane proteins are composed of stretches of 15-30 predominantly hydrophobic residues separated by polar connecting loops. The DAS method identifies such stretches by comparing low-stringency dot-plots of the query sequence against a collection of non-homologous membrane proteins using a previously derived, special scoring matrix (Cserzo *et al.*, 1997).

PESTfind analysis software found only one poor putative PEST site in LsaA with a very low score of -24.37, well below the critical value of 10 (Rechsteiner M. and Rogers S.W., 1996). These sequences consist of short hydrophilic stretches of amino acids (12-50) that are rich in proline (P), glutamate (E), serine (S) and threonine (T) and flanked by positively charged residues. These sequences are recognised by 26S proteasomes and consequently degraded. The essential degradation of listeriolysin O

within the cytosol is dependent on the presence of PEST-like sequences (Rechsteiner M. and Rogers S.W., 1996). LsaA also had no signal sequences as detectable by the Signal IP programme (Bendtsen *et al.*, 2004).

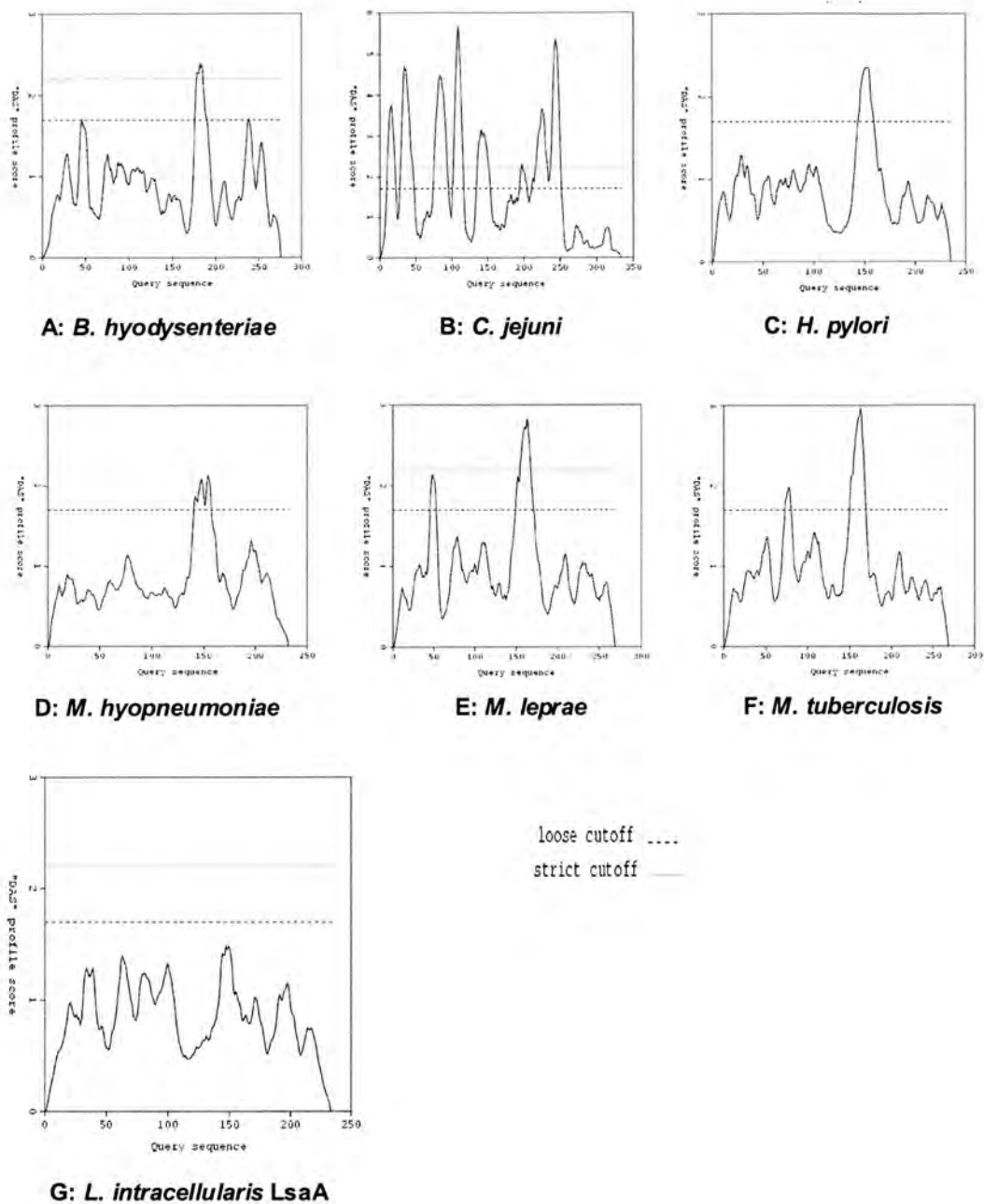


Figure 3.3: DAS (Dense alignment surface) method prediction of transmembrane helices. Analysis of TlyA family homologues from several organisms for presence of transmembrane alpha-helices (Cserzo *et al.*, 1997).

3.2.2 Expression and purification of rLsaA using IMAC

Expression of rLsaA has previously been optimised under denaturing conditions (McCluskey *et al.*, 2002). Cultures of *E. coli* BL21 (DE3)pLysS :: pRSETA/LsaA were grown to an OD₆₀₀ of 0.2 - 0.3 before expression was induced with IPTG. Following four hours of growth rLsaA was purified from the culture by Ni-NTA affinity and approximately ten 1ml fractions of eluate were collected following several washes to remove any contaminating proteins (Section 2.28.1). Figure 3.4 below shows the first six fractions eluted from the column blotted and probed with the monoclonal antibody against LsaA, VPM53. Since LsaA is a putative adhesin the aim is to use the rLsaA to detect protein: protein interactions between rLsaA and potential receptors it may bind to on the host cell surface.

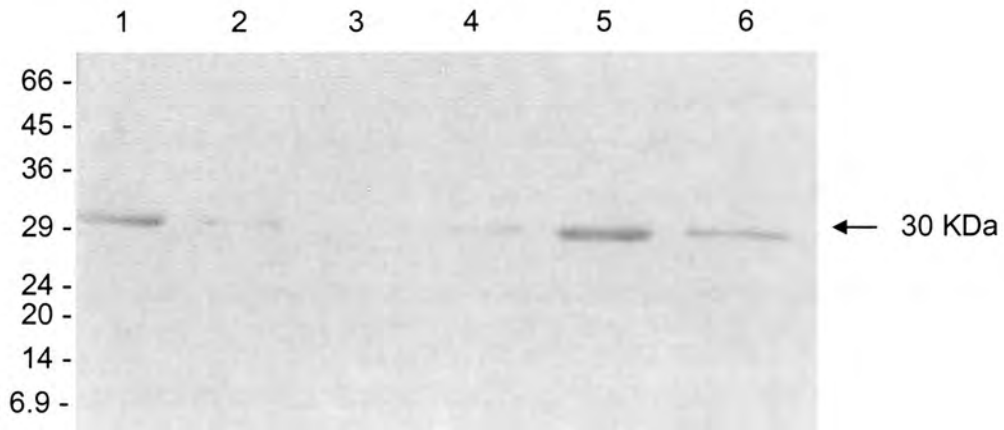


Figure 3.4: Fractions of rLsaA collected from Ni-NTA column. Western blot of first six fractions eluted from Ni-NTA purification column. Presence of rLsaA in the fractions was detected with VPM53 followed by a horseradish peroxidase conjugated secondary and developed using DAB. Due to the presence of the 3KDa His₍₆₎Tag the 27kDa LsaA is visible as a band of 30KDa.

3.2.3 Affinity Chromatography for detection of protein-protein interaction between immobilised rLsaA and host cell surface proteins

The concentration of protein eluted from the nickel affinity column was quantified by Lowry assay as 0.75mg/ml (Section 2.29) and was coupled to the sepharose in accordance with the manufacturer's instructions. CNBr activated sepharose is produced by the reaction of sepharose 4B with cyanogen bromide, an extremely toxic chemical whose use in the laboratory would be limited. Cyanogen bromide reacts with hydroxyl groups in the sepharose to produce active cyanate ester groups which in turn react with the primary amino groups of the ligand to form isourea linkages. The activation procedure also serves to crosslink the sepharose thus enhancing its chemical stability. CNBr-activated sepharose therefore provides a convenient and rapid way to immobilise ligands, which it achieves through attachment to amino groups or similar nucleophilic groups throughout the protein. DNA has also been coupled to Sepharose 4B and it has been used to purify enzymes which bind to DNA (Arndt-Jovin *et al.*, 1975).

Membrane enriched cell lysate was prepared from the semi-confluent intestinal epithelial cell line, INT-407 (Section 2.32), and passed through the column allowing for ligand binding (Section 2.33). Switching from washing to elution conditions yielded a single band of approximately 66kDa in fraction 9 (Figure 3.5).

A 'blank' column was also prepared, in which no rLsaA was bound to the CNBr activated sepharose but all other procedures carried out as before. Using this column it was also possible to obtain a band at the 66 kDa mark. The 66 kDa present in eluate from both the blank and rLsaA column was putatively identified as bovine serum albumin (BSA), presumably a contaminant of the mammalian cell extract. Fractions eluted from both the blank column (fractions 2 and 3) and the rLsaA column (fractions 4 and 5) containing a band at the 66 kDa mark were blotted with anti-bovine whole serum. All were positive, the results are shown in Figure 3.6.

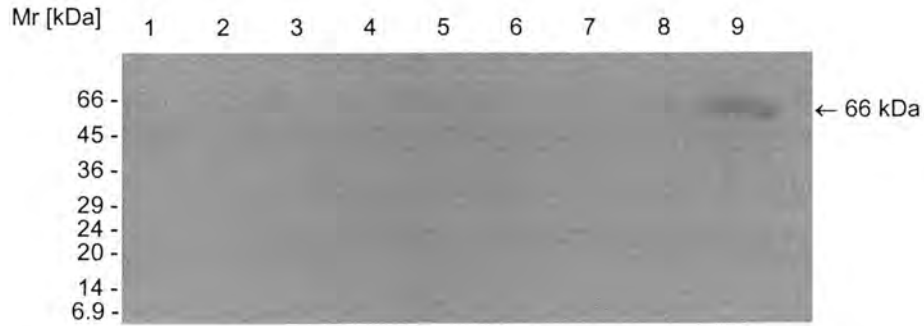


Figure 3.5: Fractions eluted from rLsaA column. Colloidal blue stained gel of the first nine 1ml fractions eluted from a rLsaA column. The column was treated with semi-confluent INT-407 cell lysate and, following washes to remove non-specifically bound proteins, treated with 1M NaCl to elute bound biomolecules. A 66kDa band is visible in fraction nine.



Figure 3.6: Western blot to confirm identity of 66kDa band as BSA. Western blot of fractions taken from the blank column (2 and 3) and the rLsaA column (4 and 5) which showed a band at 66kDa following colloidal blue staining. Lanes 1 and 6 are BSA 0.1mg/ml (10 μ l per lane) as controls. The blot was developed with rabbit anti-bovine whole serum and goat anti-rabbit horse radish peroxidase conjugate and visualised using DAB.

3.2.4 Affinity Chromatography incorporating biotin labelling of INT-407 cell surface proteins

With a view to increasing the sensitivity of detection of any potential binding partner eluted from the LsaA affinity matrix (Section 2.33), a strategy designed to biotin-label INT-407 cell surface proteins was implemented prior to cell disruption and application of the resulting lysate to the column (Section 2.34). As previously described, extraneous proteins were removed by multiple wash steps prior to switching to elution conditions. Analysis of the resulting fractions revealed bands purified in the presence of rLsaA but not from the blank column (Figure 3.6).

Since the bands of interest were not present in sufficient quantities to be detected via colloidal blue staining alone (data not shown) the 40 fractions collected from each column were pooled and concentrated by TCA precipitation (Section 2.35).

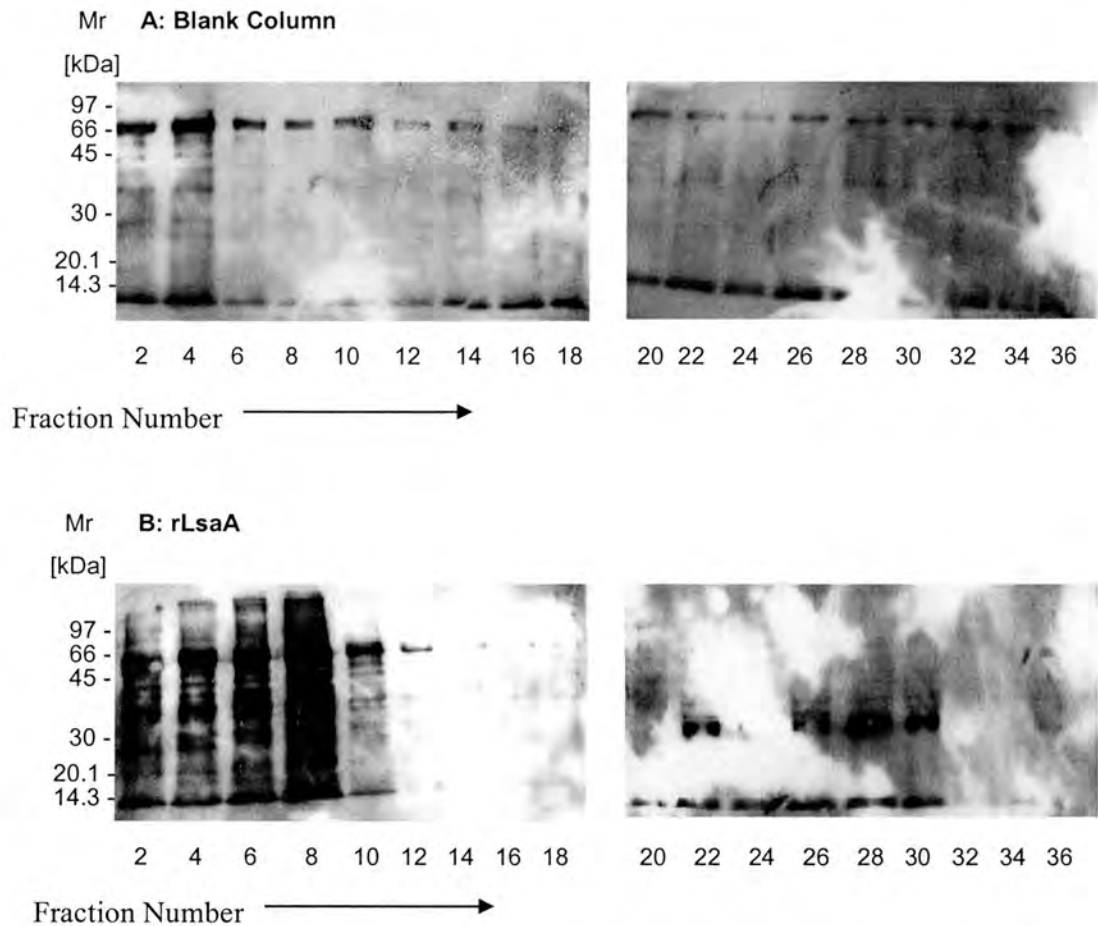


Figure 3.7: Western blot of fractions eluted from CnBr column either in the presence of (B) or without (A) rLsaA. The presence of biotin labelled INT-407 cell surface proteins was detected using avidin peroxidase conjugate. The blot was developed using ECL.

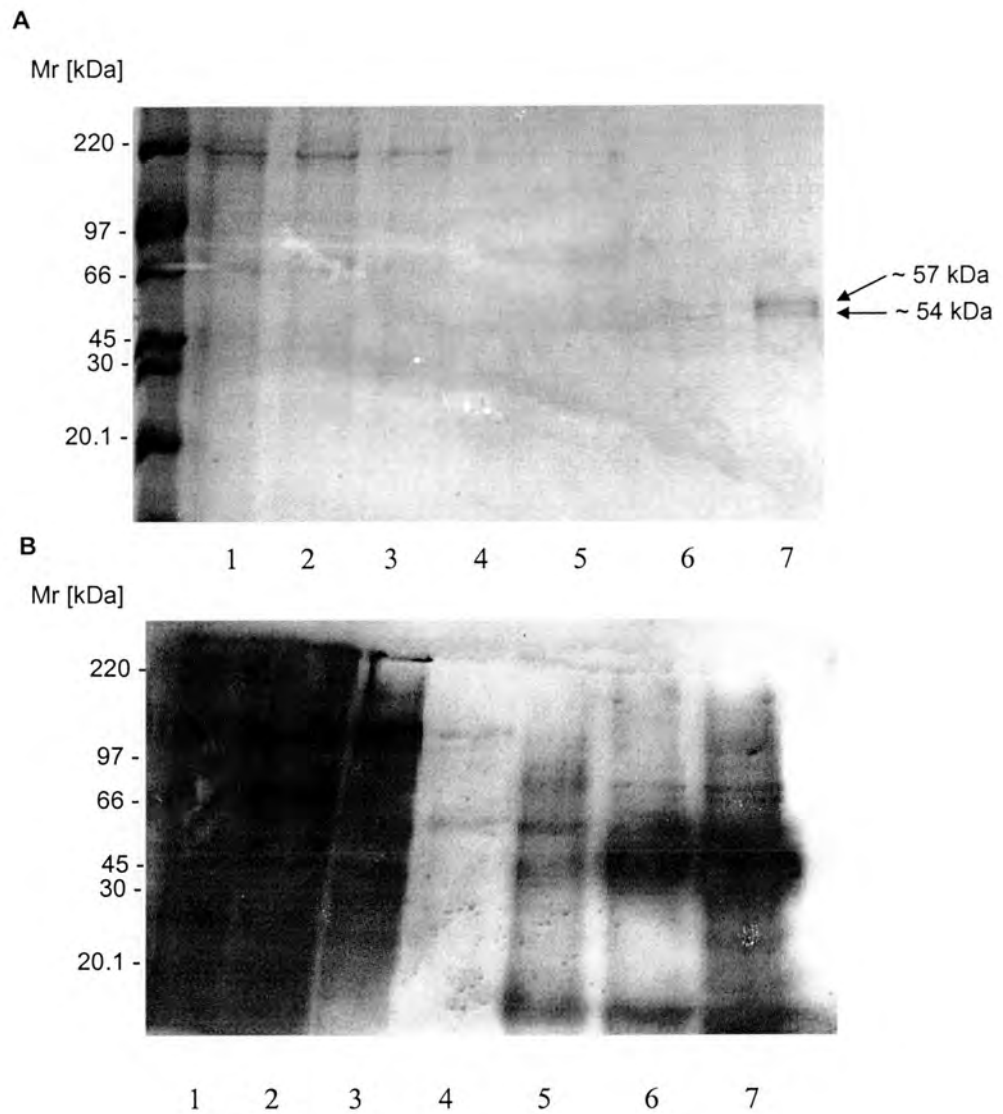


Figure 3.8: Pooled and TCA precipitated fractions from rLsaA column. Gels were either stained in colloidal blue (A) or transferred to nitrocellulose and blotted with avidin peroxidase conjugate (B) to detect biotin labelled INT-407 membrane proteins. The two indicated bands in lane 7 (A) were excised for analysis by MALDI-TOF mass spectrometry.

Isolated from the rLsaA positive column were two bands of approximately 54 and 57 kDa respectively eluted between fractions 31 and 39 which were not observed in the material collected from the blank 'column' (data not shown). The bands were excised and sent for analysis by MALDI-TOF mass spectrometry (Section 2.39).

Band	Hits (species)	MOWSE Score	Mass (kDa)
54kDa	Probable tautomerase (<i>Streptococcus mutans</i>)	23	7
57kDa	Preprotein translocase secG subunit (<i>Methanothermobacter thermautotrophicus</i>)	40	6
	Hypothetical protein TP0983 (<i>Treponema pallidum</i>)	39	27

Table 3.1: Table showing results of a Swissprot search for matches from the PMFs generated following MALDI-TOF mass spectrometry of 54 and 57kDa proteins. The proteins were isolated on a rLsaA column following incubation with a membrane enriched protein extract of semi-confluent INT-407 cells.

Unfortunately none of the hits detailed in table 3.1 were significant either in respect of MOWSE score or mass correlation therefore further investigation was not warranted. Therefore, a different line of approach was taken to continue the investigation of rLsaA binding to the cell membrane using Western blotting.

3.2.5 Far Western blotting to detect interaction of rLsaA with INT-407 membrane proteins

Far-Western blotting was originally developed to screen protein expression libraries with ^{32}P -labeled glutathione S-transferase (GST)-fusion protein. In recent years, Far-Western blotting has been used to determine receptor: ligand interactions and to screen expression libraries for protein-protein interactions. In a classical far-Western analysis, a labelled or antibody-detectable “bait” protein is used to probe and detect the target “prey” protein on the membrane. The sample (usually a lysate) containing the unknown prey protein is separated by SDS-PAGE or native PAGE and then transferred to a membrane. When attached to the surface of the membrane, the prey protein becomes accessible to probing. After transfer, the membrane is blocked and then probed with a known bait protein, which usually is applied in pure form. Following reaction of the bait protein with the prey protein, a detection system specific for the bait protein is used to identify the corresponding band (Prakobphol *et al.*, 1987).

In this case semi-confluent IEC-18 and INT-407 membrane-enriched cell lysate were separated on an SDS-PAGE gel, which was subsequently transferred to nitrocellulose, blocked and probed with rLsaA. Alternatively *L. intracellularis* (LR/189/S22, $10^7/\text{ml}$), at 37°C for several hours, were used in place of recombinant protein as a control measure. Membranes were then probed with VPM53 to detect bound rLsaA or whole *L. intracellularis* (Section 2.26). The results can be seen in Figure 3.9. IEC-18 cells have been used in the past for cultivation of *L. intracellularis*, however this cell line was superseded in the laboratory by the INT-407 line following a period of low infectivity of IEC-18 by *L. intracellularis*. Although bands did appear in similar locations on both the rLsaA and *L. intracellularis* blots, there was also an identical staining pattern evident in the control implying that this effect is due to non-specific binding of the primary or secondary antibodies and did not represent selective binding of rLsaA to proteins from the INT-407 membrane-enriched cell lysate.

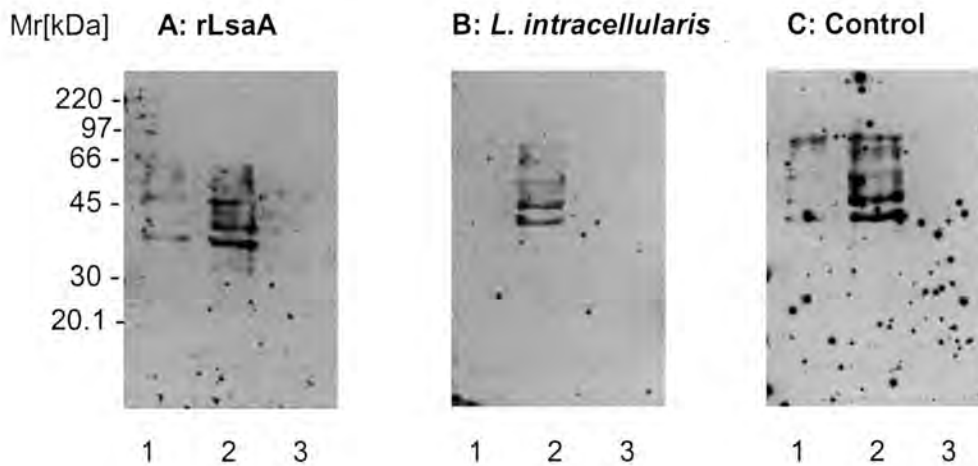


Figure 3.9: Far Western analysis of rLsaA to semi-confluent INT 407 membrane proteins. Binding of (A) rLsaA, (B) *L. intracellularis* (LR189) to solubilised semi-confluent intestinal epithelial proteins from two cell lines known to be permissive for *Lawsonia* infection was assessed. Membrane enriched solubilised lysates were initially separated by SDS-PAGE. Lane 1: solubilisation of 40-50% confluent INT-407's, Lane 2: solubilisation of 60-70% confluent INT-407 cells, Lane 3: solubilisation of 60-70% semi confluent IEC-18 cells. Bands were detected using VPM53 followed by goat anti-mouse horseradish peroxidase conjugate and developed using ECL. As a control (C) the blot was probed with VPM53 followed by secondary anti-mouse IgG horse radish peroxidase conjugate without prior incubation with either rLsaA or *L. intracellularis*.

3.2.6 *In Vitro* Adherence of rLsaA

To determine if the lack of success in receptor isolation was due to the failure of rLsaA to bind to the INT-407 cell monolayer the *in vitro* adherence of rLsaA to an INT-407 cell monolayer was established by immunohistochemistry.

Unfortunately the INT 407 monolayer could not withstand the denaturing conditions of the buffer in which rLsaA is eluted from the nickel column (8M urea, 20mM sodium phosphate, 500mM sodium chloride, pH 4). Attempts were therefore made to dialyse LsaA against elution buffer of a more neutral pH (8.3) and without urea (Section 2.30). The addition of 10% (w/v) glycerol and 5mM MgCl₂ was also attempted as recommended for His-tagged recombinant proteins. However, following overnight dialysis against such buffers the protein became insoluble and could not be resuspended, and hence could not be used in the direct binding assays.

3.2.7 Production of rLsaA under native purification conditions

The production of a recombinant protein in its naturally-folded tertiary conformation affords the preservation of the three dimensional structure of non-linear binding sites and maximises the potential for molecular interactions. By changing the buffers used in the purification procedure to exclude urea, a harsh denaturant, it was the intention to purify rLsaA in its natural conformation thereby maximising the chances of detecting an adhesion-receptor interaction.

3.2.7.1 Optimisation of soluble native rLsaA yield

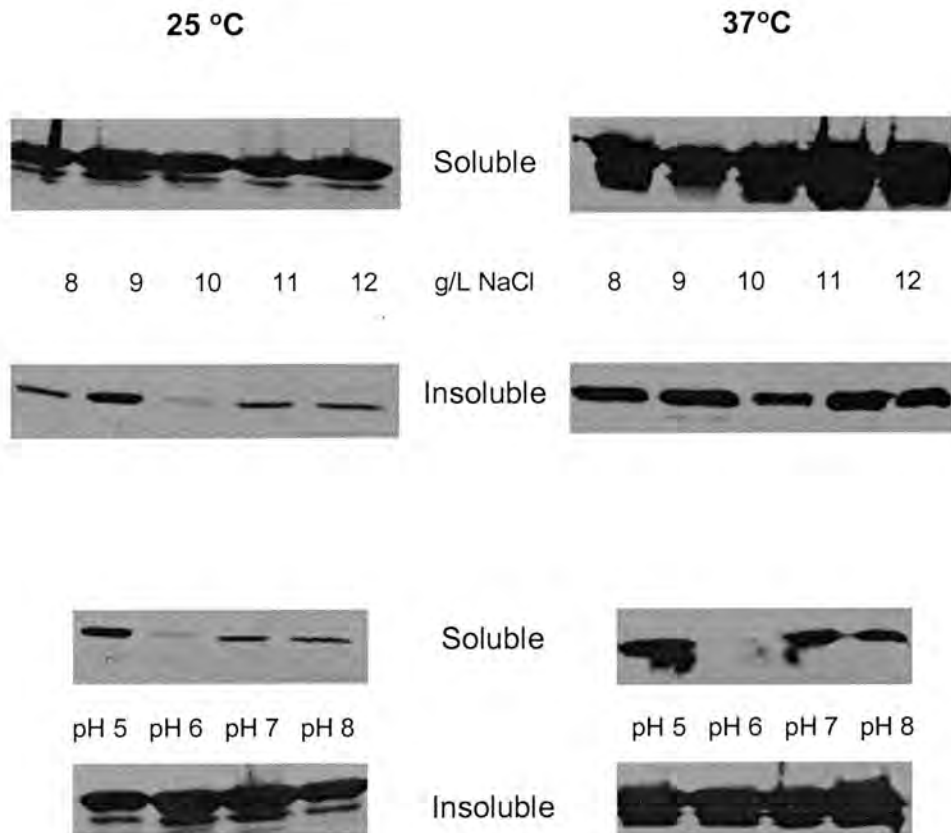
Expression of recombinant protein in *E.coli* results in the presence of over-expressed protein in either the host cell cytoplasm or its export to the periplasm. Production of rLsaA in the present system results in accumulation in the cytoplasm which often leads to the formation of insoluble aggregates known as 'inclusion bodies'. Inclusion bodies are the result of co-precipitation of recombinant protein in a denatured state alongside ribosomes, nucleic acids and other cytosolic proteins.

Alteration of growth conditions, such as temperature and osmolarity, is a long

established method of improving the expression of soluble native protein (Shenoy *et al.*, 1999, Van Wuytswinkel *et al.*, 1995). In some cases active proteins have been produced by cells induced at 25-30°C rather than the growth optimum of 37°C (Schien, 1989). High sodium chloride concentrations have been demonstrated to increase soluble protein yield (Chopra *et al.*, 1994). An increase in active recombinant protein and a decrease in inclusion body formation was observed by Blackwell and Horgan (1991), via the addition of sorbitol and betaine to the media when combined with a reduction in temperature (Blackwell and Horgan, 1991). When imposed on the cell such stresses encourage the slower, and hence more accurate, production of correctly folded recombinant protein.

3.2.7.1.1 Effect of pH, temperature and sodium chloride concentration on soluble protein expression

Standard LB broth is approximately pH 7, the pH of media used in this study was adjusted via the addition of hydrochloric acid or sodium hydroxide to give a range of pH5 to pH8. The modified media were inoculated with overnight cultures of *E. coli* BL21 (DE3)pLysS :: pRSETA/LsaA grown at 37°C. Similarly LB broth with concentrations of NaCl varying from the standard 0.171 mM down to 0.137 mM and up to 0.205 mM were also inoculated with overnight cultures. All cultures were then grown at either 37°C or 25°C and the growth rate recorded (A_{600}). Bacteria were then collected, lysed and split into soluble and insoluble protein fractions and analysed for rLsaA (Section 2.28.2). The results are shown in Figure 3.10. From this analysis it would appear that optimal growth conditions for the expression of soluble rLsaA are LB broth containing 0.154 mM NaCl, opposed to standard 0.171 mM, pH5 and 37°C for eight hours or more.



Concentration of NaCl	NaCl (g/L)
0.205 mM	12
0.188 mM	11
0.171 mM	10
0.154 mM	9
0.137 mM	8

Figure 3.10: Effect of varying NaCl concentration and pH on the production of soluble rLsaA at 25°C and 37°C. Overnight cultures of *E. coli* BL21 (DE3)pLysS :: pRSETA/LsaA were used to inoculate 100ml of LB broth modified in NaCl concentration or pH as indicated above. Following eight hours of growth at either 37°C or 25°C the culture was separated into soluble/insoluble protein fractions and rLsaA fusion protein detected using Anti-Xpress™ antibody conjugated to horse radish peroxidase.

3.2.7.1.2 Effect of betaine and sorbitol on expression of soluble protein

Blackwell and Horgan (1991) reported the increased production of soluble, active recombinant protein following the addition of betaine and sorbitol to the growth medium. The presence of higher internal concentrations of compatible solutes such as glycyl betaine minimises protein-solvent contact thereby reducing the hydration of the folding protein and stabilising protein structure. Uptake of betaine is encouraged by the presence of sorbitol, a non-digestible sugar. The same study also found induction of protein expression at lower temperatures substantially increased the yield of soluble protein (Blackwell and Horgan, 1991).

However, growth of *E. coli* seemed to be severely inhibited following the addition of sorbitol/betaine to the media even when the concentration of sorbitol was reduced. By process of elimination this effect was determined to be due to the low pH of the media. Therefore the effect of the addition of sorbitol/betaine was monitored in media at pH 7.0 containing 0.5M Sorbitol, 2.5mM Betaine, 0.154mM NaCl. Since the inclusion of sorbitol/betaine in the growth media did not afford any increase in the yield of soluble protein obtained (data not shown), both compounds were omitted from the formula finally selected for the production of rLsaA.

Following the optimisation of soluble rLsaA expression, Ni-NTA column selection of rLsaA was carried out under native conditions (Figure 3.11). In native conditions purification is carried out in a neutral pH buffer in the presence of imidazole. This compound is used for its high affinity for metal ions which is much greater than that of the His motifs in his-tagged recombinant proteins allowing the imidazole to successfully compete for binding space on the column. Initial binding to the column is performed in the presence of 10mM Imidazole to help prevent binding of non-specific proteins. Following this the column is washed several times with a slightly higher concentration of imidazole (20mM) to remove any loosely adhering proteins which may have been non-specifically absorbed onto the column. Finally the protein of interest is eluted using a high imidazole concentration of 250mM which should be sufficient to strip the column of all protein (Section 2.28.3).

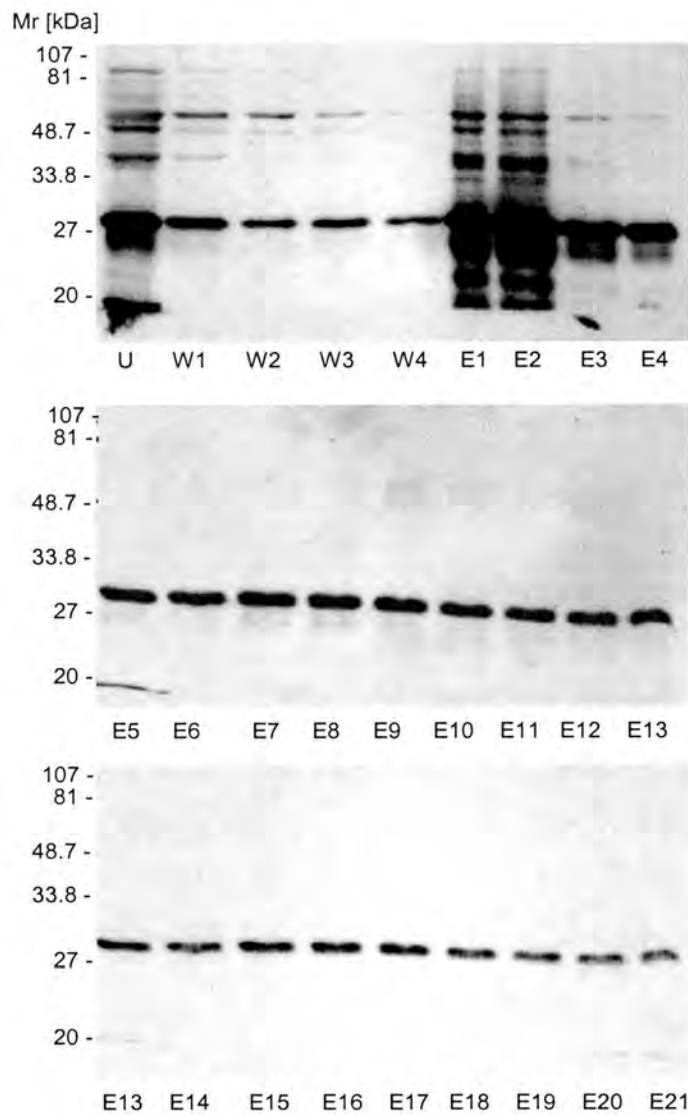


Figure 3.11: NiNTA column purification of rLsaA under non-denaturing conditions. Fractions collected from the NiNTA column during selection of His (6) tagged rLsaA detected using anti-LsaA followed by horseradish peroxidase-conjugated secondary. Initial fractions show unbound rLsaA (U), rLsaA lost through washes with native wash buffer containing 20mM Imidazole (W1-4) and fractions collected from column (E1-21) after addition of elution buffer containing 250mM imidazole. Presence of rLsaA in fractions was detected using anti-LsaA followed by a horse radish peroxidase conjugated antibody and the membranes developed using ECL.

Protein content of the collected fractions was quantified as before by Lowry assay (Section 2.29) and determined to be 2ng/ul. However, this cannot all be attributed to rLsaA. Fractions E1 and E2 show clearly the presence of additional contaminating proteins, visible through non-specific interaction of the primary or secondary antibodies, although subsequent fractions show only rLsaA. The level of which can be seen to decrease steadily as it is eluted from the column. To better define the extent of the contamination the eluted fragments were also stained with colloidal blue and silver stained (Figure 3.12).

Additionally, since the concentration of eluted protein from the column was known to be low, Centricon© centrifugal filter devices were also employed to concentrate the 25ml eluted from the column into smaller, more concentrated, volumes (Section 2.31). Results are shown in Figure 3.12.

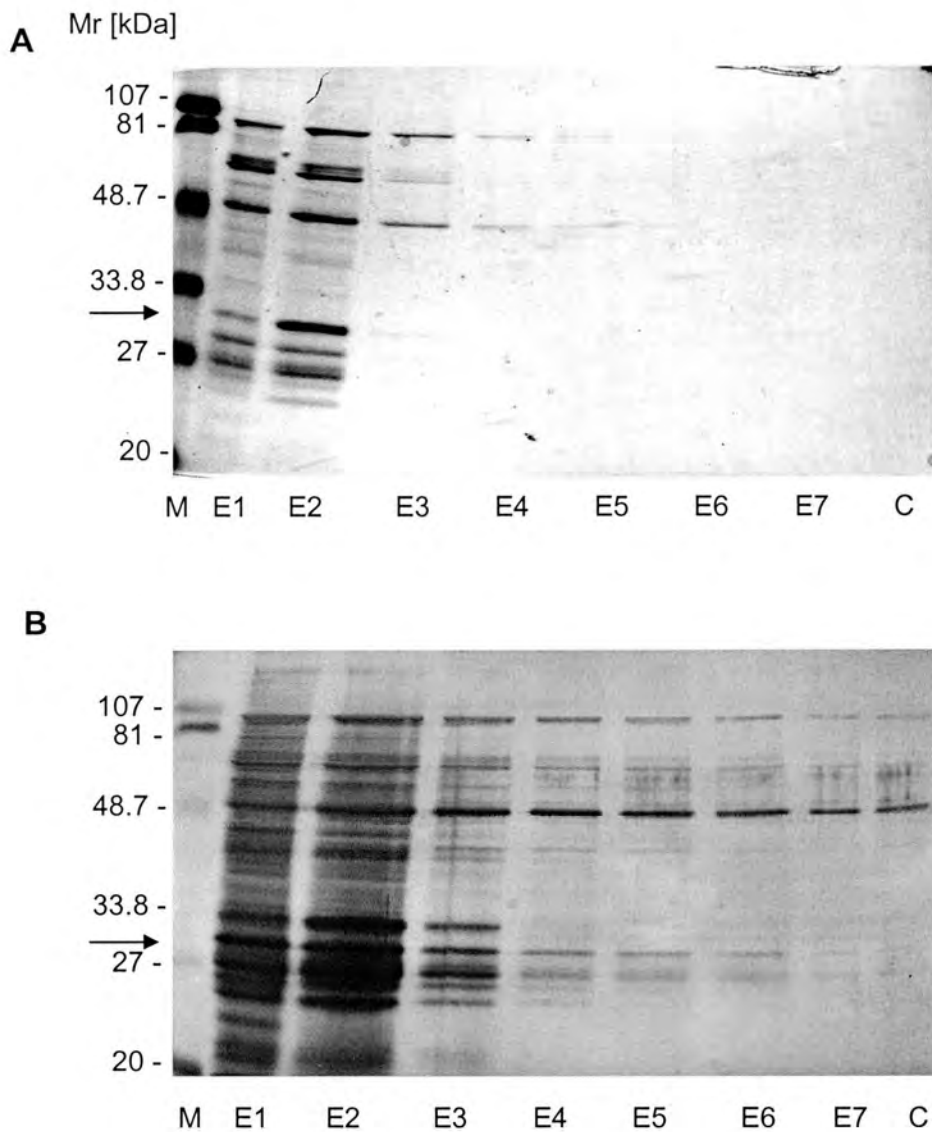


Figure 3.12: Ni-NTA column purification of rLsaA under non-denaturing conditions. Colloidal blue (A) and silver stained (B) gel of the first seven fractions (E1-7) eluted from Ni-NTA column following addition of 250mM imidazole and centricon column concentration of eluted fractions (C). Arrows indicate rLsaA.

3.2.7.2 Ion Exchange Chromatography

Since the fractions eluted from the Ni-NTA column were quite extensively contaminated with other proteins, as is evident in Figure 3.12, it was necessary to further purify rLsaA. In ion exchange chromatography, charged substances are separated via column materials that carry an opposite charge. LsaA is a basic protein of predicted pI 8.85 (positively charged at neutral pH). It was therefore attempted to use cation exchange chromatography to selectively purify, and thereby concentrate, rLsaA (Section 2.36). rLsaA (2ng/ul) was dialysed overnight against 20mM Na₂HPO₄ pH7.0 (2.30) in which buffer it was subsequently bound to the cation exchange column. Following absorption to the column, bound biomolecules can be subsequently displaced with the aid of an increasing salt gradient (0-0.5M NaCl over 30 mins was used in this instance). Such excessively high salt concentrations cause shielding of the charges on the protein surface and effective binding to an exchanger can no longer take place. The substances that have a higher charge density are bound correspondingly stronger to the column while the others elute rapidly thus enabling proteins varying in charge to be separated. The results of this experiment can be seen in Figure 3.13. Following collection of the flow-through (FT), containing molecules which failed to bind to the column, 1ml fractions were collected from the column every two minutes. When compared to the OD₂₈₀ absorbance data as a measure of protein yield it was decided to investigate fractions collected from the 16 - 48 mins giving a total of 17 x 1ml fractions. The results are shown in Figure 3.13.

In A the OD₂₈₀ absorbance levels are low showing that overall levels of protein eluted from the column are low. The fractions were run on an SDS PAGE gel, transferred to nitrocellulose and blotted with VPM53, monoclonal antibody against LsaA. Unfortunately none of the fractions showed a band of approximately 30kDa which could correspond to rLsaA, nor was rLsaA also not detectable in the flow through from the column. There was however a doublet in the region of 50-70 kDa which may represent a dimer of rLsaA (Figure 3.14).

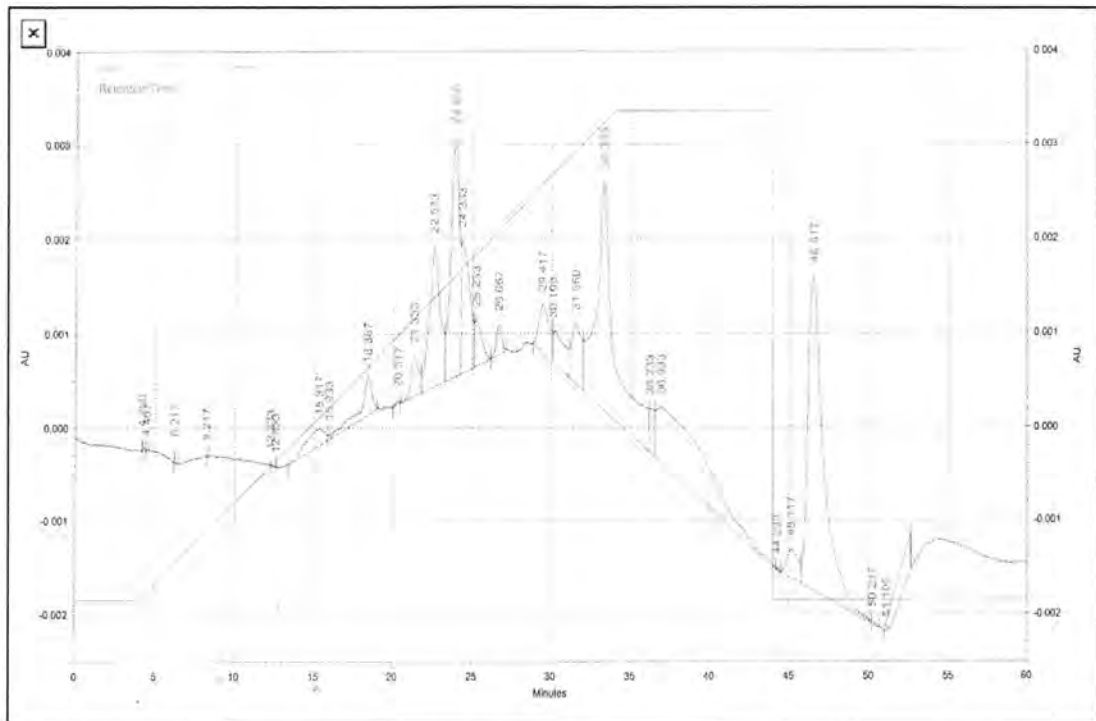


Figure 3.13: A Chromatogram resulting from the cation exchange HPLC of proteins contained in Ni-NTA purified rLsaA. Following dialysis of rLsaA against 20mM Na_2HPO_4 pH7.0 recombinant protein was bound to a cation exchange column. Absorbed proteins were eluted from the cation exchange matrix over a period of 60 min by the application of a steadily increasing NaCl gradient at a flow rate of 0.5ml/min. NaCl concentration was increased linearly from 0 – 0.5M between 4 min and 34 min, 0.5M for min. 1ml fractions were collected every 2 min from 4 min onwards and an A_{280} (AU) recorded to access their protein content.

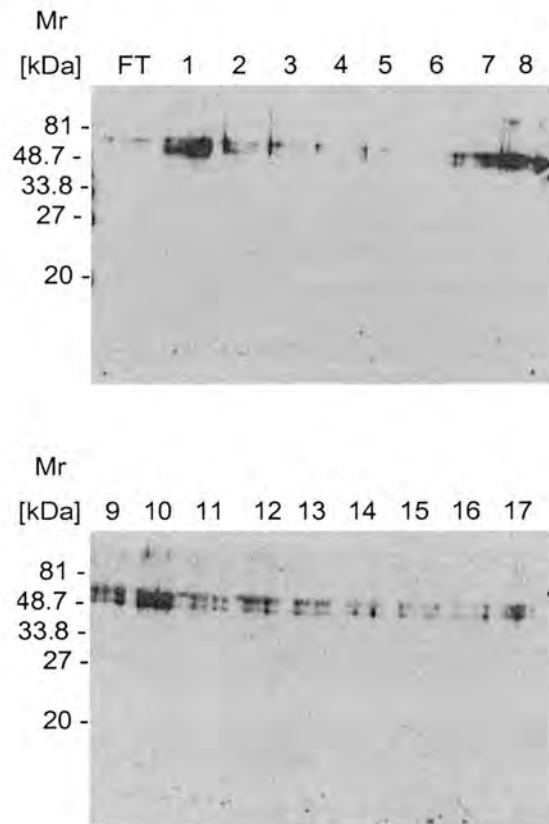


Figure 3.14: Western blotting analysis of selected fractions eluted from cation exchange column. Both the unbound molecules (FT) and each 1ml fraction collected in the period corresponding to the 16 - 48 min in Figure 3.13 (17 fractions in total) were analysed for the presence of rLsaA using monoclonal antibody VPM53. Since LsaA has a predicted pI of 8.85 it could be expected around the fourth fraction. However, this pI is theoretical and can change depending on the folding constraints imparted on the protein.

3.2.8 Far Western native gel

Despite the failure to purify rLsaA using cation exchange chromatography the early Ni-NTA column fractions (Figure 3.11: E1-E10), in which yield of rLsaA was greatest, were subsequently used in a Far Western assay on a native PAGE gel of semi-confluent membrane enriched INT-407 lysate (Section 2.32). Membrane-enriched semi-confluent INT-407 cell lysate was run on a native PAGE gel which was subsequently transferred to nitrocellulose (Section 2.25). rLsaA or *L. intracellularis* (DK15540C) were incubated with the membrane before washing to remove any unbound material. The procedure was unsuccessful however as attempts to detect bound recombinant proteins or intact bacteria using VPM53 gave a pattern of two bands (Figure 3.15) which could also be detected on the control, which had been incubated with PBS instead of bacteria or rLsaA. The reason for the lack of success even with whole bacteria is uncertain, however, it is possible that cell surface receptors recognised by *L. intracellularis* are non-proteinaceous in nature. A series of experiments were designed to test this theory as detailed below.

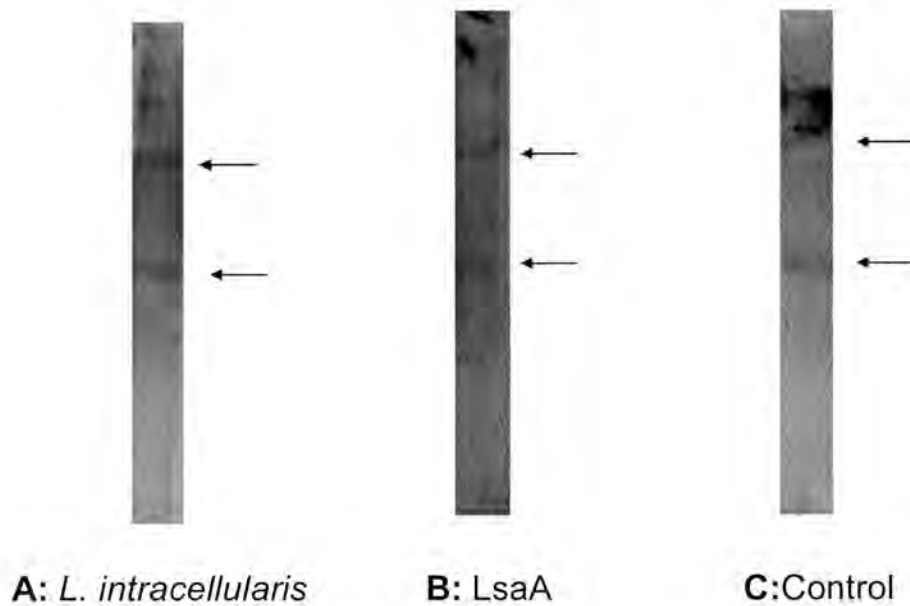


Figure 3.15: Far Western analysis of rLsaA to semi-confluent INT 407 membrane proteins. Binding of (A) *L. intracellularis* (LR189), (B) rLsaA to solubilised semi-confluent intestinal epithelial proteins from INT 407 cells known to be permissive for *Lawsonia* infection was assessed. Membrane enriched solubilised lysates were initially separated by SDS-PAGE. Following which the gel was transferred to nitrocellulose and incubated either with whole bacteria, recombinant protein or PBS alone. Following several washes with PBS to remove any unbound material, adherent bacteria or protein was detected using VPM53 followed by goat anti-mouse horseradish peroxidase conjugate and developed using ECL. As a control, (C) was probed with VPM53 followed by secondary anti-mouse IgG horse radish peroxidase conjugate without prior incubation with either rLsaA or *L. intracellularis*.

3.2.9 Adherence inhibition assays

The techniques described to date are limited to the detection of protein-protein interactions. It is possible however that *L.intracellularis* may interact with ligands which are non-proteinaceous in nature. The molecules involved in attachment/entry of *L. intracellularis* to the cell monolayer have only been briefly investigated previously (Section 1.7.3). In this section several compounds and their abilities to inhibit attachment and/or entry of *L.intracellularis* to an INT-407 cell monolayer were examined. Semi-confluent INT-407 cell monolayers were treated with trypsin (200µg/ml) or Proteinase K (200µg/ml) prior to infection with *L. intracellularis* (LR189/S22) to assess the contribution of glycoproteins to the infection process (Section 2.40). Unfortunately alongside low levels, or a complete failure of *L. intracellularis* to colonise the monolayer, there was also considerable difficulty with background staining making it impossible to accurately quantify the number of internalised organisms using fluorescent light microscopy. It is unlikely the low infectivity rate of *L. intracellularis* was due to the protease treatment since control organisms which had not been pre-treated with either trypsin or proteinase K had similarly low levels of infection.

3.2.10 Biotin labelling affinity assay

In an effort to identify which surface proteins from *L.intracellularis* are involved in binding to the INT-407 cell monolayer, *L.intracellularis* surface proteins were labelled with NHS-LC-biotin (BSP) at 4°C to minimise internalisation of the marker, before solubilisation in a detergent containing buffer (BDSP) (Section 2.37). Solubilised proteins were then added to a gluteraldehyde-fixed, semi-confluent, INT-407 monolayer which was then washed with PBS containing 0.5% Tween 20 to remove unbound material. Although several avidin reactive molecules were observed to remain bound to the monolayer following the washing process it was clear, using VPM53, that LsaA was not one of them although it was present in the both the BSP and BDSP fractions (Figure 3.16).

Adhering proteins were also probed with anti-sera from pigs inoculated with *L.intracellularis* (DK15540C), however, no sero-reactive antigens were identified (data not shown).

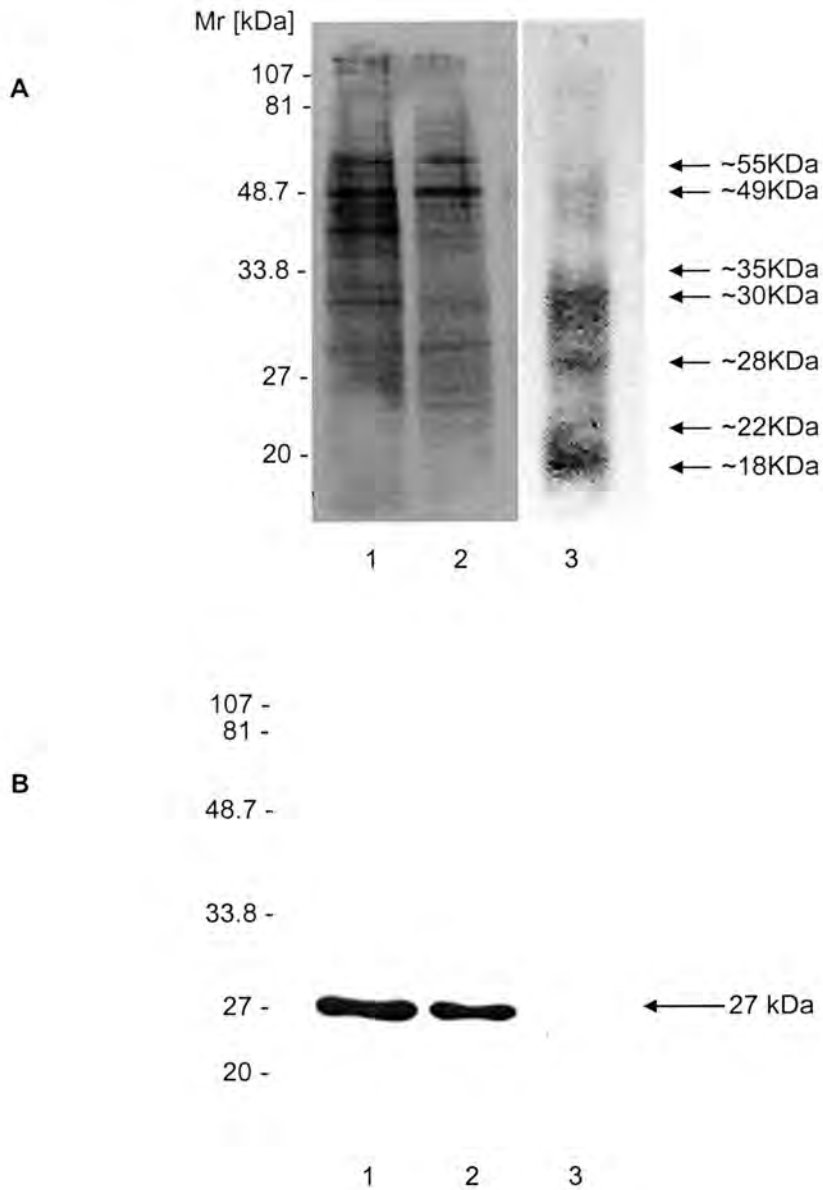


Figure 3.16: Binding of biotin-labelled *L. intracellularis* surface proteins to a semi-confluent, glutaraldehyde-fixed INT-407 cell monolayer. Lane 1: biotin-labelled surface proteins (BSP), Lane 2: biotin-labelled detergent-soluble membrane proteins (BDSP) and lane 3 biotin-labelled proteins which have bound to the INT-407 cell monolayer. In A the gel was transferred to nitrocellulose before blotting with a monoclonal antibody against biotin. In B, following transfer to nitrocellulose, the same fractions were blotted with VPM53, monoclonal antibody against LsaA.

3.3 Discussion

3.3.1 Sequence Analysis

Analysis of the LsaA ORF revealed several sites of potential post-translational modification including an N-glycosylation site, 4 Protein kinase C phosphorylation sites, 4 Casein kinase II phosphorylation sites and 1 N-myristoylation site. As LsaA is known to be surface-associated the validity of the predicted phosphorylation sites is uncertain. Indeed protein kinase C phosphorylation sites and casein kinase II phosphorylation sites are known to have a high probability of being predicted (Gattiker *et al.*, 2002) and may represent false positives.

However, myristate has been implicated in the attachment of RTX toxins to cell membranes (Lally *et al.*, 1999) but does not provide sufficient hydrophobicity alone to anchor the amino acid chain of the TlyA protein into the bacterial lipid bilayer. Since, LsaA is a surface associated protein this modification may be involved in anchoring the protein to the cell surface. Alternatively, although LsaA is reported to be non-haemolytic (McCluskey *et al.*, 2002) pore-forming activity of other members of the TlyA family (*H. pylori*, *C. jejuni* and *M. tuberculosis*) has been noted (Stabler, R.A. PhD. Thesis, LSHTM). RTX toxins also form pores and have been shown to require fatty acylation at one or two sites to acquire pore forming activity (Stanley and *et al.*, 1994, Issartel *et al.*, 1991). The fatty acid residue is the point of interaction between toxin and target cells and once acyl groups have established contact with the target cell, electrostatic forces and/or cell surface protein interactions are required for the RTX toxin to be inserted in the membrane (Lally *et al.*, 1999). Although forming membrane-spanning pores, not all RTX toxins are haemolytic. For instance, leukotoxin of *Mannheimia haemolytica* preferentially targets leukocytes thereby disrupting their normal function (Sun *et al.*, 1999, Czuprynski *et al.*, 1991). It remains a possibility that LsaA, and other members of the TlyA family, has cell-type specific activities that exclude haemolysis.

The involvement of glycosylated cell surface proteins in bacterial adherence is well documented (Benz and Schmidt, 2002). Most probably as a natural consequence of their sub-cellular location, cell surface molecules tend to feature prominently in the host humoral immune response to bacterial infections. However, there is currently

no documented evidence to suggest that LsaA is subject to any post-translational modification event. The molecular mass of natural LsaA, as predicted from a direct translation of the ORF, concurs with that observed in polyacrylamide gels. Although previously believed to be an exclusively eukaryotic modification, protein glycosylation in prokaryotes is now a well-documented phenomenon. Indeed most bacterial glycoproteins appear to be either associated with the surface of the organism, as in the case of pili or flagella, or to be secreted into the extracellular environment suggesting a role in interaction with the host (Benz and Schmidt, 2002). A direct function of bacterial glycoprotein carbohydrate moieties in adherence to host cells has been detected in several organisms. The 40 kDa major outer membrane protein (MOMP) of *C. trachomatis* requires glycosylation to take part in receptor binding. Removal of the carbohydrate residue leaves bacteria unable to attach to and infect HeLa cells (Kuo *et al.*, 1996). *C. jejuni* 81-176 contains a general protein glycosylation (*pgl*) system. The system affects many soluble and membrane associated proteins and most notably the flagella. Mutations in either the *pglB* or *pglE* genes, both involved in glycosylation pathway, resulted in a significant reduction in adherence to and invasion of INT407 cells *in vitro*, and a reduced ability to colonise the intestinal tract of mice (Szymanski *et al.*, 2002). More recently, the glycosylation of major surface protein 1a (MSP1a) from *Anaplasma marginale* has been shown to be involved in adherence to tick cells. Chemical de-glycosylation of MSP1a showed that, although the polypeptide backbone alone was capable of adhering to tick cells, the absence of glycans significantly decreased adherence (Garcia-Garcia *et al.*, 2004). Interestingly, glycosylation of prokaryotic surface proteins have also been implicated in interaction with the host immune response (Benz and Schmidt, 2002). The homologous immunodominant P140 and, surface exposed, P120 proteins of *Ehrlichia canis* and *Ehrlichia chaffinensis* are both known to be glycosylated (McBride *et al.*, 2000). By inference LsaA could be viewed as a comparable molecule; it is certainly highly immunogenic and, as the basis of a working hypothesis, involved in bacterial adherence (McCluskey *et al.*, 2002). Ultimately, the completion of the ongoing *L. intracellularis* genome project will reveal whether homologues of enzymes known to be involved in prokaryotic glycosylation events are present in this organism.

LsaA is set apart from the other members of the TlyA family in that it does not appear to incorporate a membrane-spanning region. All other members of the TlyA family so far examined have been predicted to incorporate at least one membrane-spanning region e.g. *M. tuberculosis* TlyA is predicted to have a membrane spanning helix incorporating residues 151-168. The preceding residues (1-150) are predicted to be intracellular and the subsequent residues (169-269) forming the extracellular portion of the molecule. The ability to span a membrane has been associated in the past with pore forming activities in other toxins, the insertion of this region into the host cell membrane forming the basis of the disruption and associated pore. There could be a functional correlation between the absence of such a region in LsaA and its lack of haemolytic activity. The TlyA homologue from *M. smegmatis* is also thought to be non-haemolytic, unfortunately the genome has not been sequenced as of yet to allow analysis of its predicted structure and comparison to LsaA.

Whether or not members of the TlyA family possess haemolytic activity, structural analysis predicts several possible functions for LsaA (and other TlyA proteins). In respect of LsaA functional assays have indicated a role for this surface protein in adherence and an objective of the current work was to examine this function further *in vitro*. To do so requires sufficient quantities of LsaA hence steps were taken to produce recombinant protein.

3.3.2 Recombinant LsaA Expression

There is an abundance of systems for the production of recombinant proteins. The ideal situation would be to purify the naturally-occurring protein directly from *L. intracellularis*. However, due to the fastidious growth requirements of *L. intracellularis*, it is not yet possible to readily cultivate sufficient amounts of material to enable such an undertaking. Hence the next best alternative is production of recombinant protein. The Xpress™ system has been used in the past for the production of biologically active TlyA proteins from *M. tuberculosis*, *M. leprae* and

H. pylori (Stabler, R.A., PhD. Thesis, LSHTM). Equally successful results would therefore be expected from production of rLsaA in the same system.

rLsaA was initially expressed and purified, by virtue of its N-terminal His₆ tag, under denaturing conditions. This method of production had previously been used in the laboratory and was capable of producing an acceptable quantity of recombinant protein, although the rLsaA was not in a pure form following this process. Using the rLsaA, several procedures were employed to attempt isolation of intestinal epithelial cell receptors. Firstly rLsaA was conjugated to sepharose beads as an immobile phase for affinity production of INT-407 cell surface components that selectively bind LsaA. However, only BSA, a component of the tissue culture medium, could be detected using this method. To improve sensitivity epithelial membrane proteins were labelled with biotin prior to their use in affinity assays. Two avidin reactive proteins, which selectively bound to a column containing rLsaA, were detected following affinity chromatography. MALDI-MS to identify these hits were unsuccessful and identified no convincing candidates on basis of either MOWSE score or correlation with expected mass from migration in PAGE gel.

Far Western analysis of rLsaA binding to semi-confluent IEC-18 and INT-407 membrane enriched extracts was also unsuccessful. No signal could be detected on blots that had been incubated with recombinant protein or intact bacteria that was not also detectable on a control which had been incubated in PBS alone before probing with a monoclonal antibody against LsaA. Separation of mammalian cell extract under denaturing conditions may have irreversibly destroyed the 3D structure of proteins and diminished the ability of receptor and adhesion to interact. This suggested that LsaA may not bind to membrane proteins removed from their normal membrane context therefore direct binding of rLsaA to epithelial cells in monolayer was carried out. However, the buffer in which rLsaA was eluted from the Ni-NTA column was too harsh for the cell monolayer and attempts to dialyse against a more physiologically tolerable buffer resulted in irreversible precipitation of rLsaA. Following this it was decided that production of native, soluble rLsaA, allowing

repetition of the above experiments under more physiological conditions, would increase the potential for detecting the *in vivo* interaction.

Although the natural product is found on *L. intracellularis* cell surface, rLsaA is found in the *E. coli* cytoplasm in both soluble and insoluble forms. It is possible that overexpression in *E. coli* may have affected normal cell processing pathways alternatively export of LsaA in *L. intracellularis* may utilise a pathway not found in *E. coli*. In order to optimise soluble protein expression growth conditions were altered and yield of soluble rLsaA was found to be greatest when protein expression was carried out in modified LB broth (pH5 and 9g/L NaCl). Interestingly inclusion of sorbitol and betaine in the growth medium, compounds which have been widely documented in the past to increase soluble protein yield, was found to hamper *E. coli* growth to an extreme extent. Such alterations in growth media are designed to put growing cells under stress and hence decrease the rate of recombinant protein expression in the hope of producing an increased amount of correctly folded, soluble protein. It is possible that the inclusion of sorbitol and betaine in the growth medium, in addition to changes in osmolarity and pH, was over the threshold of tolerable conditions. Recently the induction of protein expression during late log phase growth was found to increase soluble protein yield (Galloway *et al.*, 2003). However, since the affinity of the Ni-NTA column for the recombinant protein was the limiting step in the procedure the production of an increased proportion of soluble rLsaA was not investigated further.

Unfortunately, affinity of rLsaA purified under native conditions for the Ni-NTA column was extremely low compared to that of the denatured protein. It is possible this resulted from the His₍₆₎tag being partially 'hidden' in the core of the folded peptide. In such cases it may be possible to use a hybrid protocol in which protein is initially purified under denaturing conditions on the Ni-NTA column and subsequently refolded. However, since the function of LsaA as an adhesin or as a haemolysin is at present uncertain the absence of any assay for biological activity would make it impossible to test for a correctly folded and functional protein. Hence production of the protein in its native state is more desirable. Purification of rLsaA

using cation exchange chromatography was unsuccessful in that a band of 30kDa, corresponding to rLsaA, was not identified in a single fraction. Blotting of fraction eluted from the cation exchange column with VPM53 instead yielded a doublet band of approximately 50-70kDa 'smeared' across all fractions. Since this was recognised by a monoclonal antibody to LsaA it is possible that the doublet represents a dimer of rLsaA. This is a previously undocumented phenomenon and further analysis would be required to prove this hypothesis. Alternatively, the failure to purify rLsaA via cation exchange chromatography could be due to the low concentration of rLsaA initially available for this method. It would be hoped that elution of 8ml of rLsaA into a 1ml pure fraction would also serve to concentrate the protein. However, purification of recombinant proteins using this method can result in a 'smearing' effect of protein across several fractions. This phenomenon is likely to be due to the existence of a variety of folded conformations of the recombinant protein, each with their own associated pI and hence retention time on the column. In such cases the applied sample could potentially be diluted further which, in the case of rLsaA, could result in such a low concentration per aliquot such as to be undetectable (Dr N. Inglis, personal communication).

In the future a better approach to rLsaA purification may be to exclude the NiNTA step and apply crude *E. coli* BL21::pRESET A/LsaA lysate to the cation exchange column. Although this is unlikely to result in absolutely pure rLsaA it would be possible to use this method in conjunction with other biochemical techniques such as size exclusion and hydrophobic interaction chromatography until a pure sample is obtained. The omission of the NiNTA purification step would also hopefully serve to increase the available yield of rLsaA. Unfortunately it was not thought prudent to investigate these further during the course of this research project.

Identification of *L. intracellularis* proteins binding to epithelial cells was attempted using biotin labelling of *L. intracellularis* cell surface proteins and monitoring their adherence to a gluteraldehyde fixed semi-confluent epithelial cell monolayer. This approach would suggest that LsaA is not avidly involved in the attachment/adherence of *L. intracellularis* to host cells. LsaA was detectable in fractions of biotin labelled

L. intracellularis membrane proteins but was not amongst those which adhered to the glutaraldehyde fixed INT-407 cell monolayer. However, this technique could lead to the future identification of *L. intracellularis* surface proteins which may be involved in adherence to the host cell. Proteins of approximate molecular weight 55, 49, 35, 30, 28, 22 and 18 kDa were shown to remain bound to the INT-407 cell monolayer following several washes in PBS containing 0.5% Tween 20. These molecules could be selectively purified by means of an avidin affinity column and identified by mass spectrometry. Confirmation of their prokaryotic nature would also serve as a positive control for their *L. intracellularis* origin, since none of the putative surface proteins that remained on the INT-407 cell monolayer were seroreactive when probed with pig sera exposed to live *L. Intracellularis* (DK15540C). A similar technique has been used in the past with success for both *E. coli* (Bradburne *et al.*, 1993) and *H. pylori* (Sabarth *et al.*, 2002) to identify bacterial surface proteins which may mediate important host-pathogen interactions. However, in the absence of sufficient *L. intracellularis* sequences in public databases, analysis of potential *L. intracellularis* derived proteins by mass spectrometry may prove futile.

The failure to detect interaction of either rLsaA or whole organism (LR189/S22) with semi-confluent INT-407 membrane enriched extracts using the Far Western method could imply that *L. intracellularis* interacts with host cell surface molecules which are non-proteinaceous in nature. This was briefly investigated using immunohistochemistry to quantify the number of internalised *L. intracellularis* organisms in an INT-407 cell monolayer following treatment with either trypsin or proteinase K. Unfortunately it was not possible to suitably optimise this technique. A similar technique could have been employed to evaluate the adherence of native rLsaA to an epithelial cell monolayer. However, the possibility of similar problems could potentially have made this a fruitless procedure. In the future flow cytometry or confocal microscopy could be employed to give an accurate assessment of the effect of these compounds, and others, on the infective ability of *L. intracellularis*. Such experiments may give more insight into the nature of the compounds involved in *L. intracellularis* attachment and/or adherence to host cells and lead to more fortuitous lines of investigation in the future.

In conclusion, LsaA, although a surface protein associated with adherence does not appear to have any consistent binding activity. Since monoclonal antibodies to LsaA do inhibit infection *in vitro*, it is possible this occurred through steric hindrance rather than specifically occluding adhesion. Much further work is required until it will be possible to conclude whether LsaA contributes directly to adherence.

Chapter 4

MOLECULAR ANALYSIS OF RECOMBINANT LsaA INTERACTIONS

CHAPTER 4

Molecular analysis of recombinant LsaA interactions

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4.1 Introduction

The yeast two-hybrid system provides an alternative to the biochemical procedures that are commonly used to identify protein-protein interactions. The two-hybrid system exploits the ability of a pair of interacting proteins to bring a transcription activation domain into close proximity with a DNA binding site that regulates the expression of an adjacent promoter gene (Chien *et al.*, 1991). Its first use is attributed to Song and Fields (1989) who determined that the two separate and independent domains from the transcriptional regulatory protein GAL4, the DNA binding and the activation domain, could be expressed independently as chimeric proteins with each GAL4 domain fused to one partner of an interacting protein pair (Fields and Song, 1989).

In yeast the genes required for galactose metabolism are controlled by GAL4 and another regulatory protein, GAL80, as well as by the carbon source in the medium. When galactose is present the GAL4 protein binds to GAL4-responsive elements within the upstream activation sequence (UAS) of several genes involved in galactosidase metabolism and activates transcription. In the absence of galactose, GAL80 binds to GAL4 and blocks transcriptional activation. The tight regulation of GAL UASs by GAL4 makes it a valuable tool for manipulating expression of reporter genes in two hybrid systems that are dependent on the GAL4 DNA-BD. Thereby, the interaction of protein partners can be monitored by expression of a reporter gene under the control of the GAL4, or alternative, transcriptional activator. One commonly used reporter gene is *E. coli lacZ*, which causes blue colonies to be produced on plates or filters containing X-Gal (Fields and Sternglanz, 1994). Figure 4.1 depicts the normal function of a transcriptional activator protein and how this association may be used to screen for protein-protein interactions.

Since interactions between proteins mediate most biological processes the use of an *in vivo* system for their identification has several advantages. Firstly, there is no need to purify native protein, which can be time-consuming, all reactions occur *in vivo* under physiological conditions, and it is possible to screen a multitude of interactors.

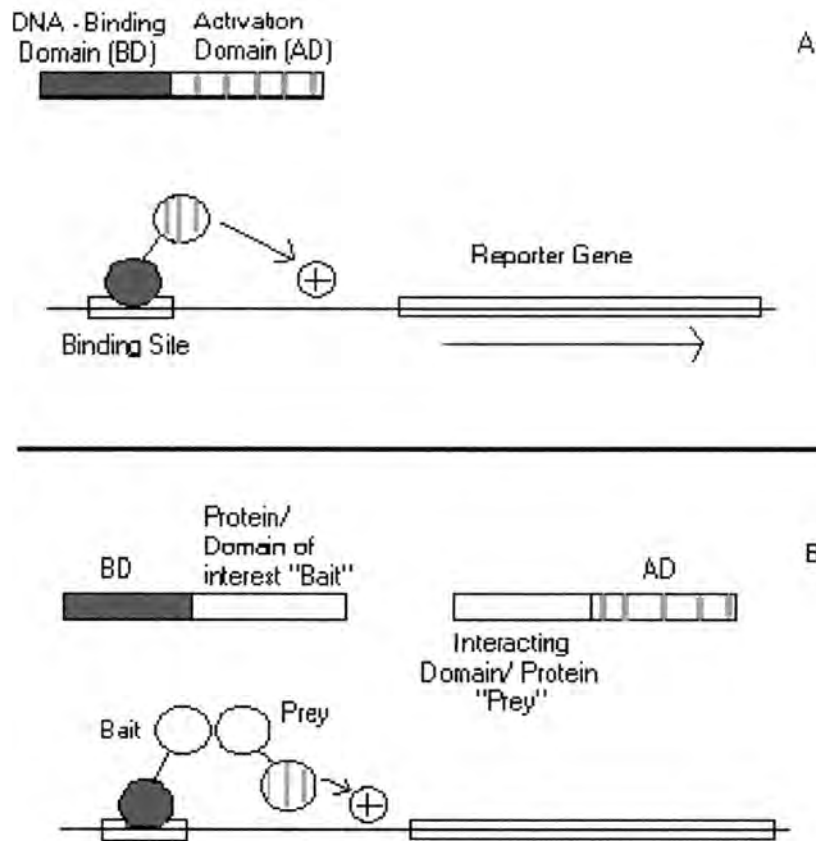


Figure 4.1 Molecular interactions involved in the yeast two-hybrid system. In (A) a schematic of the structure/ function properties of a typical transcription factor is depicted. The activation domain (AD) interacts with DNA polymerase whereas the binding domain (BD) interacts with the promoter. In (B) the exploitation of this interaction for use in the yeast-two hybrid system involving two distinct, but interacting, proteins forming fusions to the AD and BD is shown.

Unfortunately, however, in its strengths also lie its weaknesses as the *in vivo* reaction occurs in the yeast nucleus and possibly involves incorrectly folded proteins. It is therefore imperative that all potential interactions identified using this system must be verified in an independent manner. Nonetheless, the two-hybrid system is highly sensitive and can detect interactions not revealed by other methods. For example, interaction of the mammalian Ras protein with the protein kinase Raf was observed in the two hybrid system although this interaction had not been detected by immunoprecipitation (Fields and Sternglanz, 1994).

Screening of a HeLa cell cDNA library using the yeast two hybrid system had also been effectively used in the past to elucidate bacterial adhesion/effector with their target eukaryotic receptors (Viswantha *et al.*, 2004, Carlson *et al.*, 2000). The yeast two hybrid system showed host intermediate filament protein cytokeratin-18 (CK18) to be an interacting partner of both Enteropathogenic *Escherichia coli* type III secreted protein, EspF, (Viswantha *et al.*, 2004) and also of SipC, one of the major secreted invasion proteins essential for *Salmonella* entry to host cells (Carlson *et al.*, 2000). Similarly, screening of a human testis cDNA library with the EHEC EspB protein resulted in the isolation of several positive clones, including a fusion protein of the GAL4 activation domain that contained 295 amino acids of the C-terminal of α -catenin. The EspB protein is essential for attaching and effacing lesions formation presumably through the direct recruitment of α -catenin (Kodama *et al.*, 2002).

The aim of the work described in this chapter was to use a yeast two-hybrid system to screen a cDNA library derived from semi-confluent INT-407 cells with LsaA from *L.intracellularis* as 'bait' in a bid to identify possible interacting ligands not detected using the biochemical approach detailed in the previous chapter. The system of choice was the MATCHMAKER™ GAL4 Two-Hybrid system 3 from BD Biosciences. Plasmids provided for use with the system are described in Table 4.1. *E. coli lacZ* is the reporter gene in this system and monitoring of α -galactosidase and/or β -galactosidase activity forms the basis for detection of interactions in this system.

Vectors	Fusion	Yeast Selection	Function
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Cloning Vectors

pGBKT7	GAL4 DNA Binding Domain/bait	Tryptophan autotroph	GAL4 DNA Binding Domain/LsaA fusion protein
pGADT7	GAL4 Activation Domain/library	Leucine autotroph	GAL4 Activation Domain/ INT-407 cDNA library fusion

Positive Control Vectors

pCLI	Encodes the full length, wild-type GAL4 protein	Leucine autotroph	Provides a positive control for α -galactosidase and β -galactosidase assays
pGBKT7-53 and pGADT7-T	GAL4 DNA-Binding Domain and murine p53 GAL4 Activation Domain and large T-antigen	Tryptophan autotroph Leucine autotroph	P53 and large T-antigen interact in a yeast two-hybrid assay and thus provide a positive control for the system.

Negative Control Vectors

pGBKT7-Lam	GAL4 DNA-Binding Domain/ lamin C	Tryptophan autotroph	Lamin C neither forms complexes nor interacts with most other proteins (Ye and Worman, 1995, Bartel <i>et al.</i> , 1995) and provides a control for a fortuitous interaction between an unrelated protein and either the pGADT7-T control or the AD/library plasmid
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Table 4.1: Plasmids used in MATCHMAKER™ GAL4 Two-Hybrid system

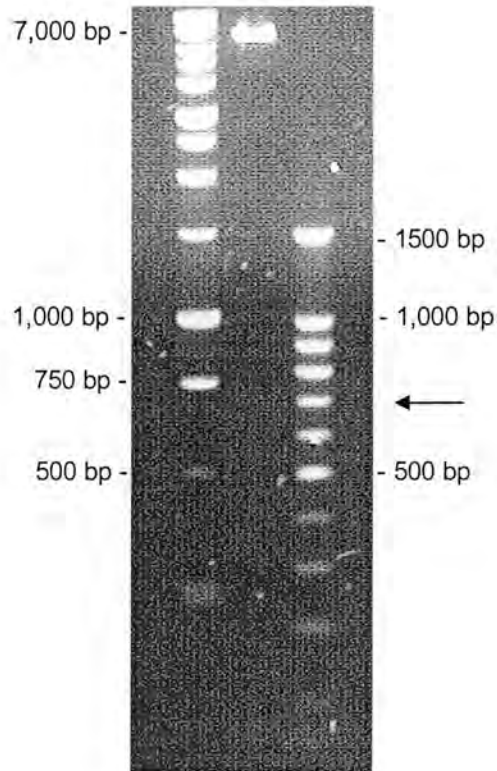
3

False positives usually due to non-specific binding of prey or, more frequently, the ability to induce transcription without direct interaction with the bait are the major problem of the yeast two hybrid system. The MATCHMAKER GAL4 system has been optimised to avoid the detection of false positives by using three reporters- ADE2, HIS3 and MEL1 (*or lacZ*) - under the control of distinct GAL4 upstream activating sequences and TATA boxes. These promoters yield strong and specific responses to GAL4 and thereby virtually eliminating the possibility of false positives due to direct interaction with sequences flanking the GAL4 binding site and interaction with transcription factors bound to specific TATA boxes.

4.2 Results

4.2.1 Construction of pGBKT7/LsaA

The first step in a yeast two hybrid screen is the construction of the bait, in this case LsaA/GAL4 DNA BD fusion protein. Since LsaA has no detected signal sequences (see Section 3.2.1) the fusion protein should be directed to the yeast nucleus due to the nuclear localisation sequence of GAL4, precluding the necessity of removing any signal sequence from the LsaA ORF. Therefore the complete 702 bp *LsaA* reading frame was amplified using the primer pair LsaA_LibraryFor and LsaA_BamHIRev (Table 2.2) using standard PCR conditions. The product was then digested with *EcoRI* and *BamHI* (section 2.21.1) and ligated into similarly digested expression vector pGBKT7 (Appendix I.04). Successful cloning was confirmed by restriction digest analysis. Figure 4.2 shows pGBKT7 with LsaA insert excised via digestion with *EcoRI* and *BamHI*. The plasmid was purified from TOP 10 competent cells via maxi-prep in order to give plasmid DNA of sufficient concentration for subsequent transformation into yeast competent cells.



Restriction Enzymes	p GBKT7 only	pGBKT7/ LsaA
<i>BamHI</i> and <i>EcoRI</i>	7.3 kb	7.3 kb + 702 bp

Figure 4.2: Restriction analysis of pGBKT7/ LsaA. *BamHI/Kpn1* restriction digests of pGBKT7/LsaA to confirm insert size. Lane 1: 1Kb DNA Ladder, Lane 2: *BamHI/Kpn1* restriction digests of pGBKT7/LsaA, Lane 3: 100bp DNA Ladder. Insert can be seen in the centre lane migrating at the expected size (arrow).

4.2.2 Confirmation of LsaA expression

To ascertain if LsaA was being expressed in the environment of the yeast nucleus, pGBKT7/LsaA and control vector pGBKT7-p53 were transformed into yeast strain AH109 using the polyethylene glycol/lithium acetate (PEG/LiAc) mediated method of yeast transformation (Section 2.8.2). As a positive control the pGBKT7-p53 vector, which contains a fusion of the GAL4 DNA-BD and murine p53 protein, was also assayed for expression of p53 fusion proteins. Untransformed yeast strain AH109 serves as the negative control for this experiment. Figure 4.3 shows yeast protein extracts from AH109 transformed with pGBKT7p53 and pGBKT7/LsaA probed with a monoclonal antibody against the GAL4 DNA-BD and shows LsaA to be expressed in the yeast cells.

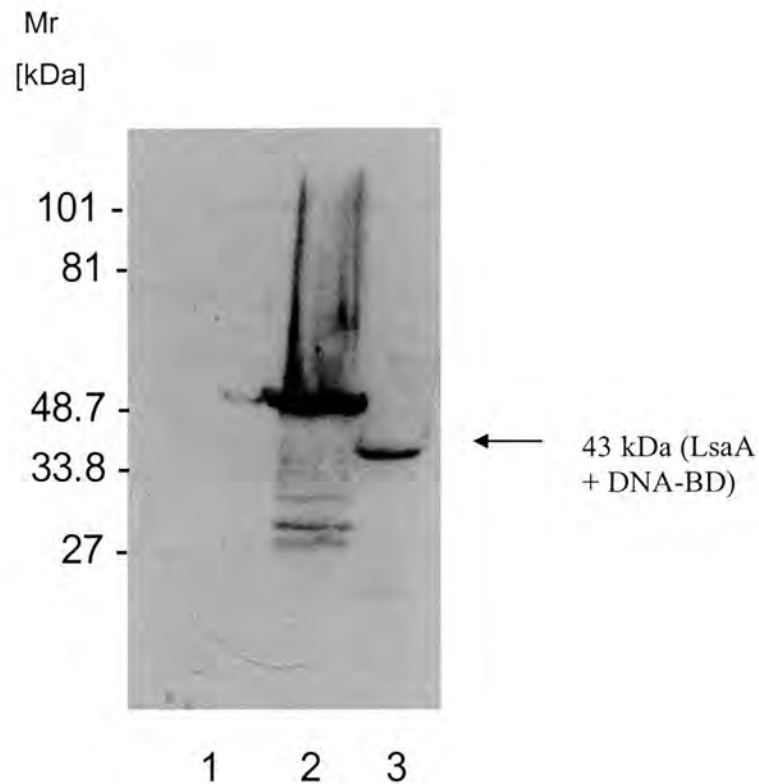


Figure 4.3: Western blot in detect expression of LsaA DNA-BD fusion protein. Whole cell protein extract of Yeast strain AH109 untransfected (Lane 1) or transfected with pGBKT7p53 (Lane 2) or pGBKT7/LsaA (Lane 3) was probed with GAL4 DNA-BD Monoclonal antibody and rabbit anti-mouse horse radish peroxidase conjugate. Since the Gal4 DNA-BD is 16 kDa, an LsaA fusion should appear at the 43 kDa mark, indicated by arrow. The murine p53 DNA BD fusion is around 51 kDa (35 kDa + 16 kDa).

4.3.3 Construction of cDNA library from semi-confluent INT-407 cells

Approximately 1.3 mg was extracted from semi-confluent INT-407 cells using the Qiagen RNeasy maxi prep kit (Section 2.14.2.1). From this, poly A⁺ mRNA was purified and quantified using an Oligotex-dT™ kit (Qiagen) following the manufacturers instructions (Section 2.14.2.2) (Figure 4.4). The cDNA was synthesised (Section 2.44) from the polyA⁺ RNA template using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and DNA polymerase I. The cDNA was size fractionated to exclude DNA fragments of less than 500 bp and ligated into the pGBKT7-T vector then used to transform competent *E.coli* XL10-Gold™ cells (Section 2.2.1.3 + 2.8.1). Despite multiple attempts, using different RNA or cDNA preps, no clones could be detected therefore further application of the system and progression to yeast-two hybrid analysis was not possible in the absence of a library containing 'prey' clones.

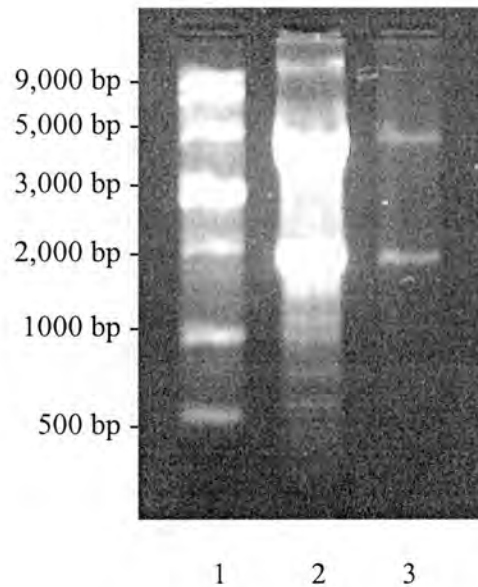


Figure 4.4: Extraction of RNA from semi-confluent INT-407 cells. Lane 1: RNA Ladder (Promega); Lane 2: Total RNA was extracted from semi-confluent INT-407 cells; poly A⁺ mRNA. Total RNA profile exhibits the typical 28S (5 kb) band that is approximately 2 times more intense than the 18S (1.9 kb) ribosomal band. The 28S and 18S bands are also apparent in the polyA⁺ mRNA (Lane 3) indicating some degree of contamination with ribosomal RNA.

4.3 Discussion

Yeast-two hybrid analysis is a powerful technique to define protein-protein interactions. Its application to the identification of ligands for bacterial proteins has been described previously. Due to the difficulties associated with the biochemical approaches used to detect a partner for LsaA a molecular approach, the yeast-two hybrid system, was attempted. However, it was decided not to invest in a full screen. This was due to technical difficulties encountered in cDNA library construction, which was initially attempted without the use of a pre-optimised commercial kit and resulting time constraints on the project. Moreover, it is probable that a portion of membrane proteins will not be effectively expressed within the nuclear milieu hence limiting the possibility of detecting an interacting partner for LsaA. Failure of system in this respect could be due to a number of reasons. Complex protein folding motifs, often involving intricate cysteine bond formation, may not pair adequately to attain functional shapes when expressed intracellularly in yeast cells. It is also possible that since membrane proteins possess a signal sequence different from the nuclear localisation sequence this may result in a fusion protein that never reaches the yeast nucleus to interact with the bait protein (Williams *et al.*, 1998). In the past, membrane proteins have been portioned into fragments and relocalised to the nucleus to ensure their successful expression (Fetchko and Stagljar, 2004). However, the problem is at least partially overcome by screening partial cDNAs that do not include the appropriate membrane-targeting sequences. These partial sequences are created randomly in the creation of any cDNA library. Furthermore, if a fusion protein did possess two different functional localisation sequences, the net result would probably be an equilibration between the two targeted environments. However, if the bait protein interacts with a glycoprotein, and this interaction is dependent on sugar residues, it will not be detected in this system as fusion proteins are targeted to the yeast nucleus and not through the secretory pathway of the yeast cells where the appropriate glycosylation would perhaps occur (Williams *et al.*, 1998). LsaA itself would appear to be expressed in the yeast cells, however, its intracellular localisation may also be called into question. Although its homology to a methyltransferase, FtsJ, might suggest a nucleolar localisation, studies described in Chapter 6 would suggest that LsaA may have a perinuclear localisation *in vitro*.

Williams *et al.*, (1998) successfully used the yeast two hybrid system with the opacity associated protein (Opa), an outer membrane protein from the intracellular pathogen *Neisseria gonorrhoeae*, to screen a HeLa cell DNA library for Opa-interacting proteins. The system was used to successfully identify the interaction of Opa and pyruvate kinase isoenzyme M2, a novel mechanism for acquisition of an essential intracellular carbon source and growth substrate. However, Opa is also known to be involved in adherence of bacteria to host cells. Opa proteins have been shown to interact with either soluble or cell associated glycosaminoglycans and the CD66a family of surface glycoproteins (Jerse and Rest, 1997). The latter interaction is known to occur via protein-protein interactions (Bos *et al.*, 1998) and should have theoretically been detectable using the yeast two-hybrid screen in question (Gray-Owen *et al.*, 1997, Bos *et al.*, 1997). However, an interaction between OpaP of *N. gonorrhoeae* strain F62SF and a member of the CD66 family was not detected (Williams *et al.*, 1996).

A variety of interactions which normally occur extracellularly have been established using two-hybrid methods, thereby demonstrating that they are not entirely precluded from investigation using this amenable and powerful method (Young, 1998). Many membrane-associated proteins have been expressed and properly folded using yeast systems (Eckart and Bussineau, 1996, Sudbery, 1996) and in addition several successes have been reported with transmembrane receptors. Appropriate extracellular receptor-ligand interactions were demonstrated for the growth hormone, prolactin and growth hormone releasing receptors (Kajkowski *et al.*, 1997, Young and Ozenberger, 1995). The interaction of insulin-like growth factor 1 (IGF-1) and its receptor has also been investigated using the yeast two hybrid system. This study also incorporated the use of random mutagenesis to study the structural requirements of the IGF-1 ligand required for receptor interaction (Zhu and Kahn, 1997). Furthermore interactions of antigen-antibody complexes have been reported using standard two-hybrid methods (Hoeffler *et al.*, 1994).

A yeast two hybrid system has also recently been developed which is more suited to the detection of membrane protein interactions. The split-ubiquitin membrane yeast

two-hybrid (MbYTH) assay utilizes complementation between separable domains of the cytosolic protein ubiquitin. It is based on the ability of the N- and C-terminal halves of ubiquitin, Nub and Cub, to reassemble into a quasi-native ubiquitin. Ubiquitin-specific proteases (UBPs), present in the cytosol and nucleus of all eukaryotic cells, recognise such reconstituted ubiquitin but not its halves and cleave off a reporter protein that is linked to the C-terminal of Cub (Fetchko and Stagljar, 2004). The system has been used with success to isolate interacting partners of human proteins BAP31 and ErbB3 from human cDNA libraries (Thaminy *et al.*, 2003, Wang *et al.*, 2003). The bait proteins described above are both known to be integral membrane proteins; however, there is no reason why the system should not be suitable for a cell surface protein such as LsaA. Although it is not thought to be an integral membrane protein, LsaA is known to be exposed to the eukaryotic cytosol. The advantage of the split ubiquitin method would be in the expression of potential interactors for LsaA which, if indeed involved in entry of *L. intracellularis* to host cells, may well be integral membrane proteins.

Alternatively other molecular and genetic based mechanisms for investigation of protein-protein interactions include the use of phage display libraries. Phage display libraries are a well documented method used for the identification of high-affinity ligands for target molecules. The strategy involves the display of a cDNA expression library on a filamentous phage by fusing the cDNA product to the minor coat protein pVI. This allows the selection and enrichment of those cDNA products that interact with a chosen immobilised or labelled ligand. Unbound phage can be washed away whilst the remainder are amplified to enrich for cDNA products displayed on the phage surface conferring adherence to the target molecule (Smith and Petrenko, 1997).

Bacterial surface display systems have also been developed. When used in concert with a gentamicin protection assay this system has the advantage of being able to differentiate between peptides mediating entry and those mediating adherence to host cells. Libraries of short peptides are inserted in the surface-exposed loops of *E. coli* OMPs, e.g. LamB and FhuA, and used to screened for interactive peptides (Taschner *et al.*, 2002). It was this system which led to the discovery of the interaction of

invasion of *Y. pseudotuberculosis* and the β 2 integrins (Isberg *et al.*, 1987). The latter technique could be employed to non-specifically isolate *L. intracellularis* proteins conferring adhesion/invasion to host cells.

However, the present system may prove a useful tool in the future if a receptor is identified for LsaA. Providing LsaA, or the reciprocal molecule, did not prove to be a transcriptional activator appropriate domains of the receptor molecule could be cloned and used to study the interaction more specifically. Such an approach has been widely used previously as discussed above and also used to localize the intimin-binding region of EPEC translocated intimin receptor (Tir) (Liu *et al.*, 2002, Grado *et al.*, 1999, Hartland *et al.*, 1999). The system may also have broader applications in the field of *L. intracellularis* research since it is foreseeable from the pathology of PE that *Lawsonia* has untold effects on cellular proliferation. Generally this involves interaction with host cell proteins involved in the control of such processes and if factors are identified in the future for the *L. intracellularis* genome screening of the INT-407 cDNA library may be used to identify specifically the host cell proteins involved.

Chapter 5

INVESTIGATION OF THE FUNCTION OF TlyA AND LsaA IN ADHERENCE

CHAPTER 5

Investigation of the function of TlyA and LsaA in adherence

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5.1 Introduction

Adherence is an important process for mucosal pathogens, particularly for obligately intracellular bacteria such as *L. intracellularis*. So far, investigation of *L. intracellularis* with intestinal epithelium has focused on invasion although a putative adhesin has been identified.

A similar role in attachment has been implicated for LsaA due to the reduced *in vitro* colonisation ability (>50%) of the NCTC 12657 and LR189/5/83 strains following treatment with a monoclonal antibody, VPM53, against LsaA (McOrist *et al.*, 1997). As with *H. pylori*, the standard procedure in such instances would be to produce a knockout of the gene of interest and clarify the phenotype. However, molecular techniques do not yet exist for this to be routinely feasible in obligate intracellular bacteria such as *L. intracellularis*. Therefore, the ability to complement the function of the *H. pylori* TlyA protein would allow insight into the function and mechanisms of action of LsaA and the family of TlyA proteins.

H. pylori colonises the human gastric mucosa by adhering to the mucous epithelial cells and the mucous layer lining the gastric epithelium (Blaser, 1993). In 95% of cases infection remains asymptomatic, however, in a small proportion of individuals, infection with *H. pylori* results in peptic ulceration, gastric adenocarcinoma and gastric lymphoma (Williams and Pounder, 1999). Since the gastric epithelium and mucous are in continual turnover, and peristalsis ensures constant movement of food and cell debris, it has been vital for *H. pylori* to evolve mechanisms to keep them stationed specifically in this environment (Blaser, 1993, Kirschner and Blaser, 1995). As a result *H. pylori* has developed the ability to physically interact with various types of host cells, host mucins and extracellular matrix proteins using a number of different adhesins. *H. pylori*'s adaptability in this aspect is evidenced by its proven ability to exist in a single host for decades (Evans Jr. and Evans, 2000). Adherence of *H. pylori* to sulphated oligosaccharides expressed on glycoproteins (Piotrowski *et al.*, 1991, Tsouvelekis *et al.*, 1991) and glycolipids (Kamisago *et al.*, 1996, Saitoh *et al.*, 1989, Slomiany *et al.*, 1989), to phosphatidylethanolamine (Lingwood *et al.*, 1992), and to the fucosylated blood group oligosaccharide Lewis^b (Borén *et al.*,

1993, Falk *et al.*, 1993) has also been reported. In addition, binding to basement membrane constituents such as lamin and collagen (Trust *et al.*, 1991), to heparin sulphate (Chmiela *et al.*, 1995, Ascencio *et al.*, 1993), to sialic-acid containing oligosaccharides, and to unspecified neuraminidase-insensitive ligands (Fauchère and Blaser, 1990) has been described.

Reciprocally adhesion of *H. pylori* to human gastric epithelial cells is multifactorial. There are over 30 members of the *H. pylori* outer-membrane protein (Hop) family, three of which have been identified as adhesins although several more may also play a role in adherence (Odenbreit *et al.*, 1999a, Odenbreit *et al.*, 1999b, Borén *et al.*, 1993, Tomb *et al.*, 1997). Attachment to the blood group antigen Lewis^b (Le^b) is mediated by the BabA adhesin (Ilver *et al.*, 1998) and *H. pylori* lipopolysaccharide-linked Lewis antigens may also play a role in adherence (Edwards *et al.*, 2000, Taylor *et al.*, 1998). Neutrophil activating protein (*H. pylori* NAP), a 16kDa surface protein, has the capacity to adhere to oligosaccharide ligands such as sulphated Lewis^a (Le^a) antigens present on mucin glycoproteins (Navamar *et al.*, 1998) whilst neuraminyl-lactose-binding haemagglutinin (NLBH) binds to sialic acid residues (Chaturvedi G. *et al.*, 2001). It has also been reported that broth grown *H. pylori* produces a second, non-NLBH, sialic acid-specific adhesion referred to as polyglycosylceramide-binding adhesion (Chaturvedi G. *et al.*, 2001).

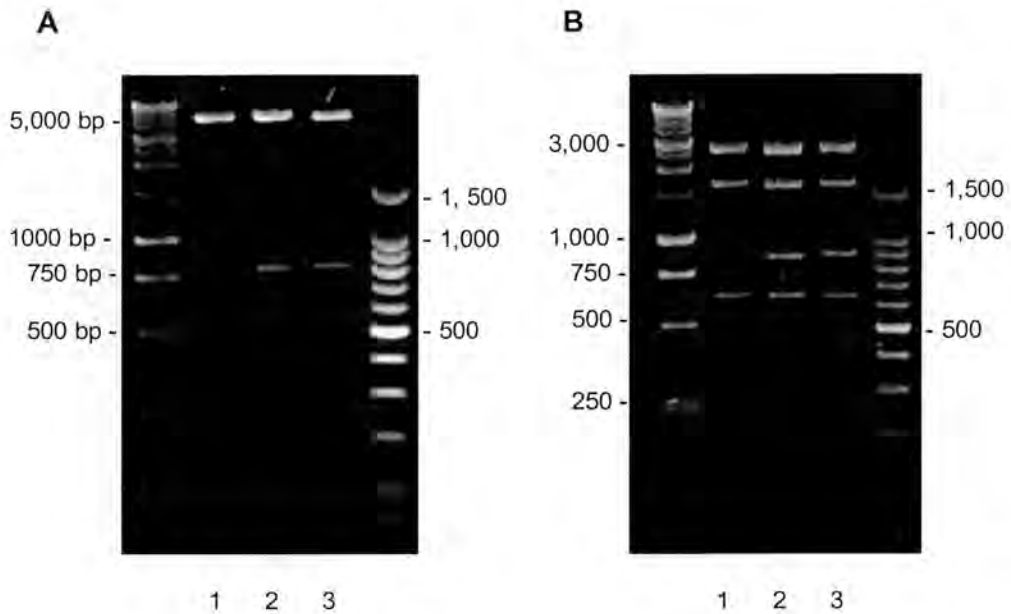
The *H. pylori* TlyA homologue is one of the factors implicated in attachment of these bacteria to the host cell surface. As discussed previously, a knockout of the *H. pylori* *TlyA* gene has been made in the SS1 strain of *H. pylori* by Martino *et al.* (2001). The mutation was produced via the insertion of a kanamycin resistance cassette into the *TlyA* open reading frame. The resulting knockout (RS7) had a phenotype showing reduced ability to adhere to an human gastric epithelial cell monolayer and ability to colonise the mouse model of *H. pylori* infection (2.9% of RS7 colonised mice compared to 72.4% wild-type SS1) (Martino *et al.*, 2001) indicating that TlyA is an important determinant in adherence of *H. pylori* to the gastric epithelium.

The aim of the work in this chapter was complementation of the *tlyA* knockout in *H. pylori* with LsaA from *L. intracellularis* and with *H. pylori* TlyA. To achieve this aim, a *H. pylori*/*E. coli* shuttle vector, pHel2, was utilised to express the LsaA protein in *H. pylori* RS7. The vector pHel2 is a derivative of the cryptic 2.93Kb plasmid pHel1. The small plasmid belongs to a family of plasmids carrying a series of direct repeats, the iterons, and a replication initiation protein (RepA) (Heuermann and Haas, 1995). This plasmid has been modified and successfully used as a shuttle vector in *H. pylori* and *E. coli* via the addition of a ColE1 origin of replication, a chloramphenicol resistance cassette, an *oriT* sequence and a multiple cloning site (Heuermann and Haas, 1998) (Appendix I.03). The effect of this complementation on the phenotypic characteristics of the organism such as haemolytic activity and adherence to host cells was monitored to assess the function of LsaA.

5.2 Results

5.2.1 Construction and confirmation of expression of *LsaA* and *H.pylori* *TlyA* inserts from pHel2 constructs.

Genomic DNA from *L.intracellularis* (LR189) and *H.pylori* SS1 was used to amplify the *lsaA* (702bp) and *tlyA* (705bp) genes respectively (Section 2.16). Although a gradient PCR was used initially for amplification subsequent PCR was carried out at 58°C. The forward primer incorporates a 100bp upstream region into the product which has been shown to contain the promoter region for *LsaA* (Dr. P. Alberdi, manuscript in preparation). Primers used incorporated a 5' *BamHI* site and a 3' *KpnI* into the amplified product. The fragments were 'blunt end' cloned into PCR4 TOPO (Appendix I.06) and subsequently excised by virtue of their incorporated *BamHI* and *KpnI* restriction sites for ligation into a similarly digested pHel2 vector prior to transformation into the *E.coli* TOP 10 competent cells (Figure 5.1).



Restriction Enzyme (s)	pHel2 only	pHel2/ <i>H. pylori</i> TlyA	pHel2/ LsaA
<i>KpnI</i> and <i>BamHI</i>	5.1 kb	5.1 kb + 805 bp	5.1 kb + 802 bp
<i>EcoRI</i>	2704, 1673, 636 and 88 bp	2704, 1673, 636 and 884 bp	2704, 1673, 636 and 879 bp

Figure 5.1: Restriction analysis of pHel 2 constructs. Lane 1 = pHel2, Lane 2= pHel2/ LsaA, Lane 3= pHel2/ *H. pylori* TlyA. In (A) all plasmids were digested with *KpnI* and *BamHI* to excise cloned insert. In (B) all constructs were digested with *EcoRI* which should result in four fragments one of which corresponding to the entire MCS and insert. Following restriction digest analysis the products were electrophoresed on a 1% agarose gel. The insert size is calculated as 100bp (upstream region) + 702 bp (LsaA) or 705bp (TlyA). A 1Kb (250bp-10,000bp) and 100bp (100bp – 1,000bp) DNA ladder were included as DNA markers (Promega).

5.2.2. Conformation of LsaA and TlyA_{HP} expression in RS7

In order to determine if the recombinant proteins were being expressed in RS7, polyvalent anti-sera against each protein was produced by inoculating rabbits with His-tagged recombinant TlyA_{HP} and recombinant LsaA. The denatured TlyA_{HP} protein has been expressed previously in the same system employed for expression of rLsaA (R.A. Stabler, LSHTM, PhD Thesis) as described in Section 2.28.1.

Unfortunately polyclonal anti-sera obtained against the recombinant antigens gave consistently high background when used to probe whole cell *H. pylori* lysate (data not shown). The high background was not lowered by adsorption of the anti-sera with the RS7 strain and, although improved by three rounds of precipitation with saturated ammonium sulphate (pH 7.8) (Figure 5.2), not eradicated to enable a definitive result to be distinguished. Probing the blot using secondary antibody alone showed no non-specific interactions which could potentially be producing an interfering signal.

Repeated attempts were also made to demonstrate expression of the inserts via RTPCR. RNA was extracted from *H. pylori* RS7 transformed with pHel2/ LsaA or pHel2/LsaA as described in section 2.15. The RNA was converted to cDNA and amplification attempted with primers specific for *lsaA* or *tlyA_{HP}* via RTPCR. However, negative controls which had not undergone a reverse transcription step consistently gave positive bands due to DNA contamination of the sample. Despite treatment with DNaseI to remove contaminating genomic DNA prior to RTPCR it was not possible to rid the RNA sample of contamination. Therefore it was not possible to conclusively determine if the *lsaA* and *tlyA* genes were being transcribed in RS7.

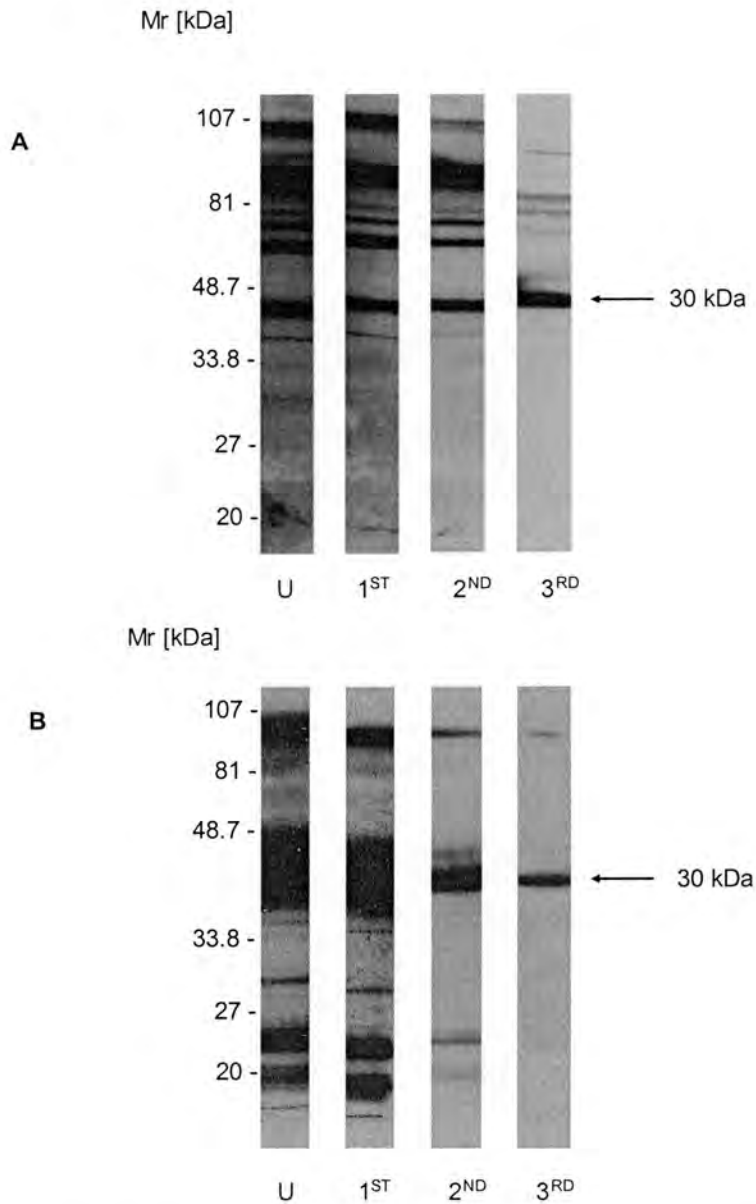


Figure 5.2: Ammonium sulphate precipitation of rabbit derived polyvalent antiserum. Precipitation of anti-sera increased specificity to Ni-NTA purified TlyA_{HP} (A) and rLsaA (B). Antiserum was treated to three rounds of precipitation with saturated ammonium sulphate (pH 7.8). U = pre-precipitated serum (1/200), 1st = 1st precipitate, 2nd = 2nd precipitate (1/200), 3rd = 3rd precipitate (1/200).

5.2.2 Adherence of *H. pylori* transformants to an AGS cell monolayer.

pHel2 plasmids containing the *lsaA* and *TlyA_{HP}* inserts were isolated from *E. coli* following overnight growth using a maxiprep kit and transformed into *H. pylori* RS7 via electroporation (Section 2.8.2.1) Following growth on selective agar, transformants were resuspended at the desired concentration, as determined by a viability assay (Section 2.47), in PBS. Following resuspension, bacteria were added to a monolayer of AGS cells, which had previously been counted via haemocytometer and seeded at the correct density, to give ratio of 100:1 bacteria: cells. Monolayers were incubated with bacteria for 1 hour at 37°C before washing with PBS to remove any non-adherent bacteria (Section 2.45). The extent of *H. pylori* binding was then determined by virtue of a urease assay as described in section 2.45.1 (Figure 5.4). Production of the virulence determinant urease by *H.pylori* is essential for successful colonisation of the host and accordingly is one of the most abundant proteins produced by *H.pylori*. To date, no regulatory signals have been found for *H.pylori* urease and it is constitutively expressed even in a neutral environment (Grosdent *et al.*, 2002, Akada *et al.*, 2000, Marais *et al.*, 1999). Urease catalyses the reaction depicted in Figure 5.3 below. Therefore the addition of a urea solution containing the pH indicator phenol red enables a quantitative measurement of alkaline end product, ammonia, and hence of adherent bacteria.

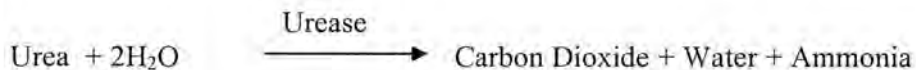


Figure 5.3: Reaction catalysed by Urease.

Although it would have been preferable to grow the bacteria in liquid culture to the desired density this was not possible. Only after 24 hour periods of incubation in pre-reduced BHI broth (10% FBS) did even minimal growth occur. Both Mueller-Hinton and Brucella broth were tested for their ability to propagate either SS1 or RS7, both without success, and incubation in a VAIN incubator (Courtesy of Dr P. Everest, University of Glasgow) had no positive influence on growth rate either.

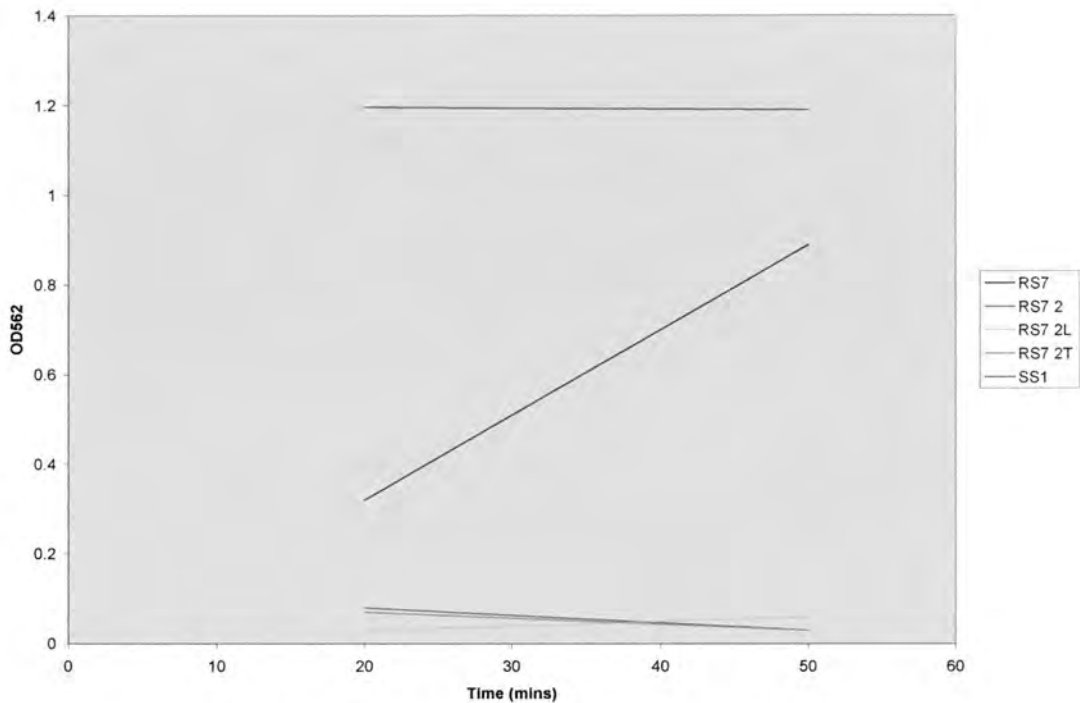


Figure 5.4: Adherence of *H. pylori* to an AGS cell monolayer quantified by means of a urease assay. The graph shows relative adherence of *H. pylori* strains SS1 and *tlyA* mutant RS7 to an AGS cell monolayer as quantified by urease assay. The OD₅₆₂ of a solution of PBS containing 300mM urea, 0.5% (w/v) phenol red was added to wells of AGS adhered bacteria and used to quantify the extent of *H. pylori* adherence to the monolayer. Adherence of RS7 is reduced to around 33% compared to that of the wild-type. However, the viability of RS7 strains transformed with the *H. pylori* vector pHel2 seem to be severely compromised (RS7 2: RS7 pHel2, RS7 2L: RS7 pHel2/LsaA, RS7 2T: RS7 pHel2/ TlyA_{HP}).

Adherence of RS7 to AGS cells has previously been measured at 71.4% +/- 7.5% compared to 92.8% +/- 2.6% observed with SS1 (Martino *et al.*, 2001)

By 60 min, relative levels of adherence detected using the urease assay broadly corresponded with those obtained by Martino *et al.* 2001. However, transformation of *H. pylori* with pHel2 constructs appeared to present a substantial fitness burden reflected in adherence of ~ 5% relative to SS1 and RS7 (at 50 min).

It is known that different restriction/modification systems can severely inhibit the transformation of bacteria with heterologous DNA (Miller *et al.*, 1988). *H. pylori* strains demonstrate considerable variability in the efficiency of transformation by plasmids from *E. coli*, and many strains are completely resistant to transformation. Numerous strain-specific restriction-modification systems are known to be high among the barriers to transformation. Donahue *et al.* (2000) have developed a method to protect plasmid DNA from restriction by *in vitro* site-specific methylation using cell-free extracts of *H. pylori* before transformation which they successfully used to transform SS1 with the pHel3 shuttle vector (Donahue *et al.*, 2000). The technique was applied to the pHel2 constructs generated in this chapter from hereon. A comparison of natural transformation and electroporation of RS7 was also carried out. Based on the growth of transformants, once transferred to selective media, electroporation of RS7 using CFE-treated plasmids seemed to be the most efficient and reliable method.

It was also thought necessary to implement an alternative method of adherence quantitation other than the urease assay due to the possibility of differences in levels of urease activity between the RS7 and SS1 strains as discussed in the following chapter. Therefore, labelling of adherent bacteria with an anti-*H. pylori* antibody (Section 2.45.2.1) and quantification using flow cytometry was investigated (2.45.2). This technique was initially used by Martino *et al.* (2001) to characterise the reduced adherence phenotype of RS7 (Martino *et al.*, 2001). Unfortunately significant background readings were associated with the primary antibody. Also, despite repeated attempts propidium iodide staining indicated that a large proportion of the cells had become membrane-permeable during the procedure and this cell population

appeared to have an increased affinity for the primary antibody (Figure 5.5).

To circumvent this problem bacterial nucleic acid was pre-labelled with the nucleic acid stain SYTO 9 (Section 2.45.2) and the adherence and washing steps carried out as documented previously (Section 2.45.2.2.1). Using this method 100% of the cells were positive for SS1. However, 100% of cells were also found to be positive for RS7 and DH5 α , a non-adherent *E. coli* laboratory strain known included as a negative control (Figure 5.6).

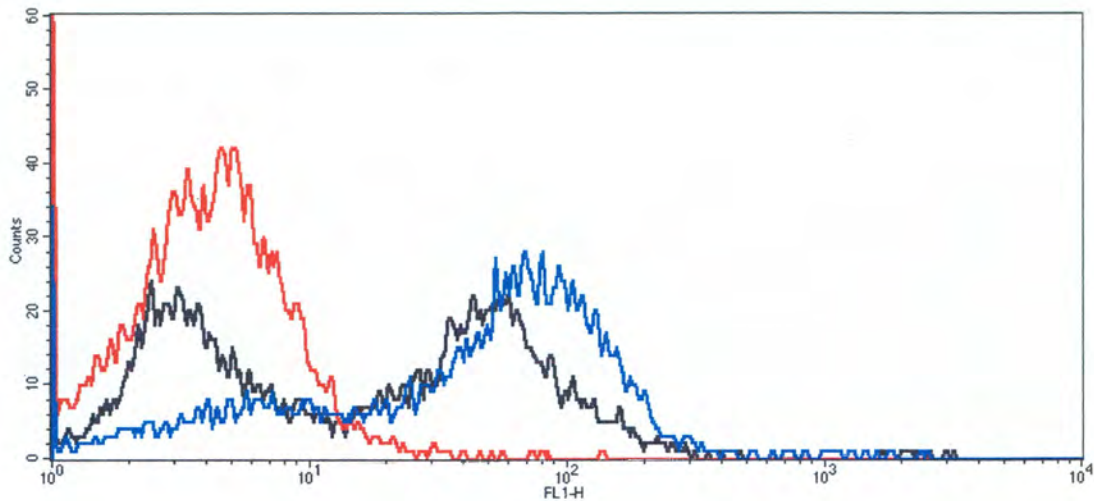


Figure 5.5: Histogram (fluorescence vs. no. of events) showing non-specific adherence of anti- *H. pylori* antibody to AGS cells. SS1 bound to AGS cells and labelled with anti- *H. pylori* primary and Alexa Fluor® 488 goat anti-rabbit secondary (blue line). A single peak representing the cells positive for SS1 is observed. AGS cells alone treated with anti- *H. pylori* primary and Alexa Fluor® 488 goat anti-rabbit secondary (black line). The first peak (left), as expected, represents the unlabelled cells, however, a second peak (similar to that observed for SS1) is also visible indicating that some cells have become non-specifically labelled with antibodies despite never being exposed to SS1. AGS cells incubated with SS1 and probed with the secondary alone (red line). It is apparent that non-specific binding is due to the primary antibody since incubation of AGS incubated with SS1 and probed with secondary alone shows only a peak in a similar position to that of the unlabelled cells.

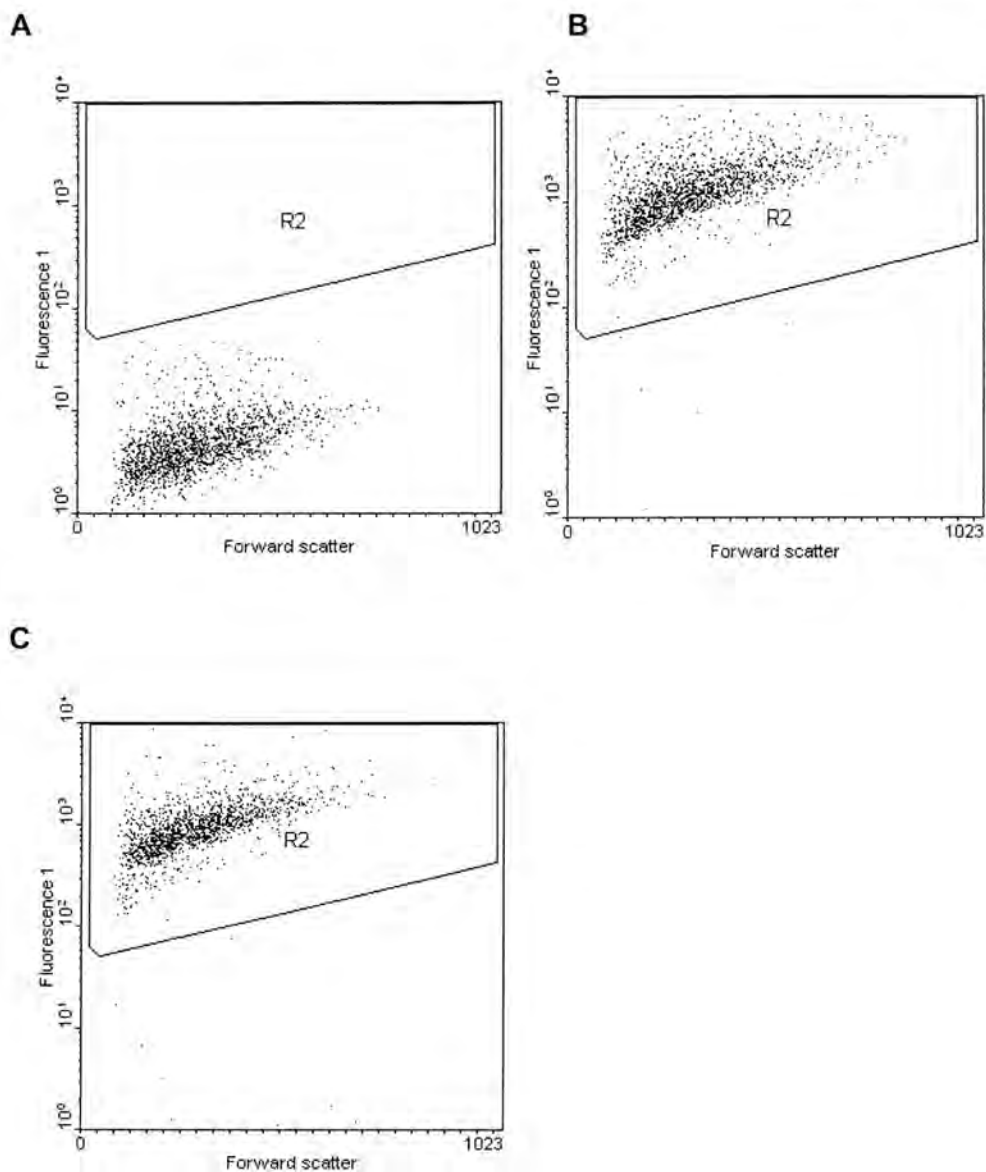
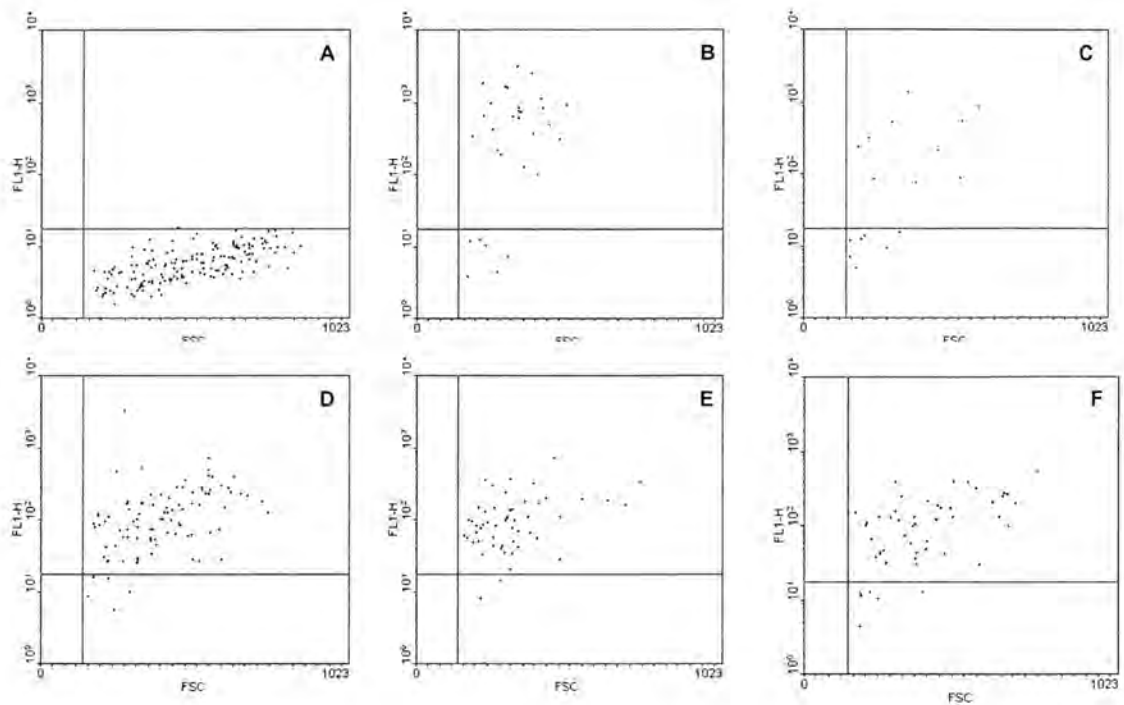


Figure 5.6: Flow cytometric profile (forward scatter vs. fluorescence) of bacterial adherence to AGS cells. The flow cytometric profile of (B) SYTO 9-labelled *H. pylori* SS1 and (C) SYTO 9-labelled *E. coli* DH5 α adhering to AGS cells. There is a clear difference in fluorescence compared to that of (A) AGS cells alone. However, there is no difference in the number of AGS cells positive for SS1 and *E. coli* DH5 α (R2).

The addition of bacteria to AGS cells in suspension appears to lead to an increased 'affinity' of the cells for bacteria which may be due to their entry into apoptosis. AGS cells examined immediately following their removal from the monolayer showed negligible signs of membrane permeability, as detectable via trypan blue staining (Section 2.46). To circumvent this problem a method whereby the AGS cells were not removed from the monolayer until immediately prior to flow cytometric analysis was employed (Section 2.45.2.2.2). SYTO 9-labelled bacteria were co-incubated with a monolayer of AGS cells (100:1) at 37°C for 1 hour. After washing to remove non-adherent bacteria, epithelial cells were detached with EDTA (2mM) and analysed by flow cytometry, (Figure 5.7) a method that has also been used successfully in the past to quantify adherence of *H. pylori* to gastric epithelial cells (Logan *et al.*, 1998).



Profile	Bacterial Strain	Y Mean
A	AGS cells alone	6.61
B	SS1	714.04
C	RS7	421.75
D	RS7/ pHel2	177.50
E	RS7/ pHel2 LsaA	134.67
F	RS7/ pHel2 <i>H. pylori</i> TlyA	153.05

Figure 5.7: Flow cytometric profile (forward scatter vs. fluorescence) of SYTO 9 labelled bacteria adhering to an AGS cell suspension. SYTO 9 labelled SS1 (B) and RS7 (C) transformed with CFE treated plasmids pHel2 (D), pHel2/LsaA (E), pHel2/ TlyA_{HP} (F) were allowed to adhere to a monolayer of AGS cells for an hour at 37°C. Following which non-adhered bacteria were removed by washing with PBS and the cells detached with 2mM EDTA for analysis by flow cytometry. In (A) AGS cells alone are shown. The Table above shows the mean fluorescence (Y mean) of flow cytometric profiles A-F.

Although the low abundance of cells in Figure 5.7 allows the results to be only indicative, there is a very similar pattern to that previously observed. Adherence of the RS7 strain appears to be reduced compared to wild-type SS1. However, as was found previously, the 'fitness' of RS7 carrying the pHel2 plasmid with or without insert seems to be severely compromised despite treatment of the plasmids with *H. pylori* CFE. It is therefore not possible to detect any effect of heterologous or homologous complementation of the *H. pylori* TlyA protein on the reduced adherence phenotype exhibited by the RS7 strain.

5.3 Discussion

Difficulties were encountered when trying to confirm the expression of TlyA_{HP} or LsaA in RS7. Since the chloramphenicol acetyltransferase gene as well as the cloned genes have their own promoters growth of a colony alone was not sufficient to show expression of functional protein. Initially a western blotting approach was used with anti-sera derived from rabbits exposed to Ni-NTA purified rLsaA or rTlyA_{HP}. Unfortunately probing whole cell lysate of RS7 transformed with the pHel2/LsaA/TlyA constructs gave considerable background. This made it impossible to detect the presence or absence of a 30kDa band corresponding to TlyA_{HP} or LsaA. The blot was probed with the secondary antibody which, alone, gave no background demonstrating that the observed background could be attributed to non-specific binding of the primary antibodies. Presumably the background is arising as a result of prior exposure of immunised rabbits to *E. coli* and possibly also *L. intracellularis*. Ammonium sulphate precipitation of the rabbit anti-sera was performed with the aim of selectively precipitating the IgG fraction. Pre-absorption with RS7 was also carried out to remove any antibodies present which reacted with *H. pylori* cells surface components and may lead to a confusing signal. Using the RS7 strain, as opposed to SS1, removed the possibility of loss of antibodies specific to TlyA. Perhaps disruption of RS7 using a mechanical method, such as a French press, may have reduced background further. Since pre-absorption with intact bacteria will have removed only cell surface reactive components which is unlikely to be the extent of an immune response against the pathogen.

Since analysis of protein expression proved unsuccessful in detecting expression of TlyA or LsaA it was decided to look for expression at the RNA level. Detection of mRNA corresponding to *tlyA_{HP}* or *lsaA* could help to confirm expression initially. Therefore total RNA was extracted from RS7 transformed with pHel2 TlyA/LsaA and subjected to RTPCR with primers specific to *tlyA* or *lsaA*. However following RTPCR bands could consistently be detected in the sample and negative control (no RT step). This indicated that DNA contamination was present and precluded the identification of a definitive result. Removal of the DNA was attempted by treatment of the RNA sample with DNaseI, however, this did not fully eliminate the

contamination.

Queries over expression of the recombinant proteins could be allayed in the future by placing the recombinant proteins under the control of a promoter known to be constitutively expressed in *H. pylori* such as the *UreB* promoter (Marais *et al.*, 1999).

Assessment of *H. pylori* binding to AGS cells by monitoring urease levels was broadly in line with that observed by Martino *et al.* (2001) for RS7 and SS1. Due to the fitness burden exerted by the pHel2 plasmid it was not possible to assess the presence or absence of complementation of RS7 by either *TlyA_{HP}* or *lsaA*. It was decided however to opt for another means of quantifying bacterial adherence due to differences in the levels of UreA and UreB expression detected in RS7 and SS1, as discussed in section 6.31.

Therefore, following CFE treatment of plasmids, in an attempt to alleviate the fitness burden, adherence of *H. pylori* to AGS was monitored by flow cytometry. However, following removal of AGS cells from the monolayer and incubation with *H. pylori* followed by antibody labelling of bound bacteria a certain population of the AGS cells were observed to be membrane permeable, and possibly in the initial stages of apoptosis. The background was determined to be associated with non-specific binding of the primary antibody. Therefore, as an alternative to the use of antibodies, the bacteria were pre-labelled with SYTO-9, a fluorescent nucleic acid stain. However, 100% of AGS cells could still be seen to be positive for bacterial adherence, whether it was from SS1 or *E. coli* DH5 α , a non-adherent laboratory strain. It is possible that membrane permeability is an early sign of apoptosis in this cell population. It is unclear the reason for the increased affinity since it is not specific to *H. pylori*.

Since the increased membrane permeability, and hence non-specific adherence of bacteria, seemed to be increase in accordance with the length of time AGS cells were removed from the monolayer a new technique minimising this period was implemented. SYTO-9 labelled bacteria were incubated with the AGS monolayer for 1 hour at 37°C before washing to remove unattached bacteria and only then were

AGS cells detached and analysed by flow cytometry. This technique did diminish the non-specific adherence of *H. pylori* and gave results for SS1 and RS7 that were broadly in line with those documented by Martino *et al.* (2001). Although the small number of cells meant the results could not be quantified statistically there was clear evidence of the 'fitness burden' imposed by the pHel2 plasmid in transformed strains. As a result, the role of LsaA in adherence remains unconfirmed as a result of the failure to complement the *tlyA* knockout, RS7.

Although it was not possible to confirm expression of LsaA or TlyA in RS7 transformation of *H. pylori* with the shuttle vector pHel 2 alone severely compromised the viability of the bacteria. Natural transformation of *H. pylori* with the pHel2 shuttle vector has been shown to result in stable autonomous replication in *H. pylori*. Although the shuttle vectors are known to be targeted by the restriction/modification system of *H. pylori* it was hoped that treatment with cell free extract would alleviate this inhibition (Heuermann and Haas, 1998). Furthermore electroporation of *H. pylori* with the pHel2 shuttle vector containing the cloned genes *ureA* and *ureB* and their specific promoter sequence has been used, with success, to genetically complement a *H. pylori* urease mutant N6*ureB*::TnKm (Rokita, E and Makristathis, A. 2001). The authors did report a rate of around 5% homologous recombination, a phenomenon which has been observed previously (Suerbaum, S *et al.*, 1998).

In the future it may be advisable to try conjugative transfer of the shuttle vector and inserts. This method has been shown to circumvent the restriction barrier between *E. coli* DH5 α and *H. pylori* P1 observed in transformations (Heuermann and Haas 1998) and has also been used in the past to successfully complement *H. pylori* knockout strains with homologous genes, for example, in the characterisation of genes involved in *H. pylori* transformation. Natural competency and electroporation abilities of a *comE3* homologue knockout in *H. pylori* were restored when the strain was complemented with a pHel2 vector carrying the deleted ORF via conjugative transfer (Yu-Chin, Tzu-Lung 2003).

Chapter 6

THE PUTATIVE REGULATORY ROLE OF THE TLYA FAMILY

CHAPTER 6

The Putative Regulatory Role of the TlyA family

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6.1 Introduction

Although a relationship between haemolytic activity and the *tlyA* locus has been demonstrated in *Brachyspira hyodysenteriae* (TerHuurme *et al.*, 1992) and *Helicobacter pylori* (Martino *et al.*, 2001) the direct demonstration of the *tlyA* locus encoding a beta-haemolytic protein has not yet been confirmed by protein sequencing. It has been suggested that the product of the *tlyA* locus may in fact cause up regulation of separate haemolysins from the bacterial genome, resulting in the haemolytic phenotype and that the TlyA family of cytolysins may in fact function as regulatory proteins (Hsu *et al.*, 2001). Evidence from the study of the *S. hyodysenteriae* haemolysin is particularly compelling. Several investigators have successfully isolated and purified the *S. hyodysenteriae* haemolysin from culture supernatant. However, the reported size of the isolated haemolysin has varied from 19KDa (Kent *et al.*, 1998) to 68KDa (Knoop, 1981) and 74KDa (Saheb *et al.*, 1980). Since the open reading frame of the *tlyA* ORF encodes a protein of 26.9KDa, unless attributed to post-translational modification, it seems an unlikely correlation between TlyA and haemolysin (Muir *et al.*, 1992). Subsequently, Hsu *et al.*, (2001) reported the purification of a beta-haemolytic toxin, of between 19 and 21KDa, from log phase cultures of spirochetes, and the N-terminal sequence was determined. The subsequent unexpected lack of N-terminal homology between *tlyA*, or two other ORFs (*tlyB* and *tlyC*) encoding putative haemolytic proteins, and the purified beta-haemolysin, HlyA, led to the suggestion that the haemolytic phenotype observed was due to the up-regulation of this separate and distinct haemolysin from the *B. hyodysenteriae* genome. Similarly the haemolytic activity observed in *E. coli* transformed with *tlyA*-containing plasmids may be attributable to the upregulation of a cryptic haemolysin, a phenomenon recently identified in the *E. coli* genome by del Castillo *et al.*, (1997) which was expressed when a plasmid encoding a transcriptional regulator, *slyA*, of *Salmonella* was introduced. Although purified recombinant TlyA protein from *M. tuberculosis*, *M. leprae*, *H. pylori* and *C. jejuni* has been tested and found positive for haemolytic activity (R.A. Stabler, LSHTM, PhD. thesis) the physiological relevance of this is unclear. Two domains present in the LsaA protein, and in all other TlyA proteins studied so far (Table 1.1), have been identified which may account for the regulatory function.

These domains, as found in LsaA, are shown in Figure 6.1 below: an S4 RNA-binding domain at residues 3-63 of the protein found in conjunction with a methyltransferase domain, FtsJ (residues 3-63) and documented in Table 1.1.



Figure 6.1: Two functional motifs identified in LsaA. An S4 RNA binding domain at residues 3-63 of the protein and a methyltransferase, FtsJ, at the C-terminus. This homology is well conserved throughout the TlyA family (Table 1.1).

About 2/3 of the mass of a ribosome consists of RNA and 1/3 of the mass is protein. The proteins are named in accordance with the subunit of the ribosome which they belong to - the small (S1 to S31) and the large (L1 to L44). S4 is a small protein, consisting of 60-65 amino acid residues, and is one of several that make up the small ribosome subunit S4 RNA-binding domains which usually decorate the rRNA cores of the subunits (Davies *et al.*, 1998). RNA binding proteins are a diverse group of proteins whose structures vary widely from each other and have clearly evolved multiple strategies to interact with RNA. However, several RNA-binding proteins are known to use independent globular domains of 60-90 residues to interact with RNA (Staker *et al.*, 2005). The S4 RNA-binding domain has been detected in the bacterial ribosomal protein S4 and eukaryotic ribosomal protein S9. However, the S4 domain can also be found as a single copy at various positions in over 500 proteins, from at least seven different protein families (Aravind and Koonin, 1999, Korber *et al.*, 1999). In six of these families it is found in association with other domains such as the N-terminal alpha helix rich globular domain found in S4 proteins, the pseudouridine synthase catalytic domain, the methylase domain, the tyrosyl-tRNA synthetases domain or the deaminase domain (Korber *et al.*, 1999). It can also be found alone as in several small bacterial proteins, e.g. YabO from *B. subtilis* and YrfH from *E. coli* (Aravind and Koonin, 1999). The seventh family is the heat shock protein Hp15 (Korber *et al.*, 1999).

It has been proposed that the S4 domain is capable of recognizing complex, unique 3D features in highly folded RNA molecules such as rRNAs, tRNAs, and untranslated regions of mRNAs (Korber *et al.*, 1999). S4 is known to be one of the proteins that autogeneously regulates the expression of other ribosomal proteins by binding to a polycistronic mRNA (Thomas *et al.*, 1987, Dean and Nomura, 1980). The RNA binding ability of the S4 domain is thought to be attributable to its homology to the α L motif initially identified in heat shock protein Hsp15 (Staker *et al.*, 2005) (Figure 6.2).

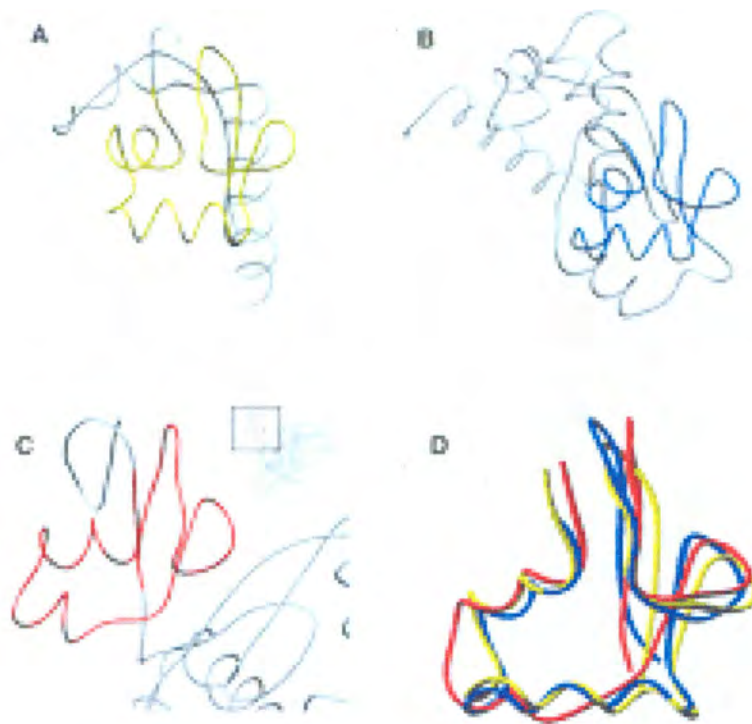


Figure 6.2: Proposed α L RNA-binding motif (taken from Staker *et al.*, 2000). The L motif in three different protein structures. The peptide backbones of three structures, ribosomal protein S4, Hsp15 and threonyl-tRNA, are compared. The region highlighted in color is the L motif that is shared by all three proteins. (A) Hsp15 with its L motif highlighted in yellow. (B) Ribosomal protein S4 with its L motif highlighted in blue. (C) Threonyl-tRNA synthetase with its L motif highlighted in red. (D) Overlay of residues 9-57 of Hsp15 (yellow), 92-141 of ribosomal protein S4 (blue) and 18-59 of threonyl-tRNA synthetase (red).

The α L motif was also found to be present and similarly folded in the S4 protein with a comparable patch of surface exposed residues. S4 conserved residues that are likely to be solvent accessible include R93, T106, R111, H118, D122 and G123 and these are the likely candidates for forming an RNA binding surface. Of these R93 and R111 in S4 (R10 and R28 in Hsp15) are absolutely conserved in all members of the Hsp15 and S4 families (Staker *et al.*, 2005).

In the TlyA family the S4 domain is found in conjunction with a putative RNA methyltransferase, FtsJ (Ching *et al.*, 2002). The methyltransferases represent a large family of enzymes catalyzing the transfer of a methyl group from the ubiquitous *S*-adenosylmethionine (SAM) to nucleophilic atoms of nucleic acids, phospholipids, proteins, and small molecules (Chiang, 1996, Niewmierzycka and Clarke, 1999). The two most frequent types of RNA modification are *S*-adenosylmethionine-dependent methylation of the 2'-O position of ribose and conversion of uridine into pseudouridine. The function of such modifications is still elusive but it is known they can alter the three-dimensional structure of RNA as well as its interaction with ligands (Feder *et al.*, 2003). The *E. coli* FtsJ/ RrmJ protein has been shown to be a SAM-dependant Um₂₅₅₂ methyltransferase of the A loop of 23S rRNA within 50S ribosomal subunits *in vitro* and *in vivo* (Bügl *et al.*, 2000, Caldas *et al.*, 2000). While deletions in most of the known 23S rRNA methyltransferases has been shown not to affect *E. coli* growth or ribosome assembly, strains with null mutations in *ftsJ/rrmJ* show severe growth retardation and an increase in the proportion of free 30S and 50S subunits at the expense of functional 70S ribosomes (Caldas, 2000, Caldas *et al.*, 2000).

While *E. coli* cells have only one FtsJ homologue, eukaryotic cells usually have several. Yeast cells, for instance, have been found to harbour three FtsJ homologues: a cytosolic (Pintard *et al.*, 2002b), a mitochondrial (Pintard *et al.*, 2002a) and a nuclear homologue (Pintard *et al.*, 2000). Whilst the ribosomal and the nuclear homologues have been shown to function as rRNA methyltransferases, the cytosolic homologue, Trm7p, has been shown to be responsible for two 2'-O-ribose

methylations at position 32 and 34 in the anticodon loop of certain yeast tRNAs. *E.coli* FtsJ has also been shown to methylate tRNAs *in vitro* in addition to 23S rRNA (Bügl *et al.*, 2000). Intracellular localisation of human FTSJ2 has been examined in cultured mammalian cells transfected with a plasmid expressing the GFP-FTSJ2 protein. Using confocal microscopy the GFP-FTSJ2 construct was found in the nucleus of the transfected cells with enrichment in the nucleolus (Ching *et al.*, 2002), the main site of ribosome production (Carmo-Fonseca *et al.*, 2000). The expression of FTSJ2 in human tissues and cells has also been examined using Northern blotting. Of the tissues examined, expression was most abundant in muscle, placenta and heart tissues and notably in all cancer cell lines. Comparing band intensities of the FTSJ2 band normalised to the β -actin signal, the FTSJ2 transcripts were found to be 8.9-fold more abundant in A549 lung carcinoma cells than in normal lung tissue (Ching *et al.*, 2002).

Comparison of FtsJ with several known methyltransferases has led to the identification of a common putative catalytic tetrad K-D-K-E in the binding pocket of these proteins (Bujnicki and Rychlewski, 2001). The residues K-D-K were later found to be essential for the MTase activity, while the fourth residue, E, appears to play only a minor role in catalysis (Hager *et al.*, 2002). This putative catalytic tetrad would also appear to be conserved throughout the TlyA family (Feder *et al.*, 2003).

Juxtaposition of S4 and FtsJ domains support the deduction that TlyA/LsaA could possess nucleic acid binding activity and potentially regulate expression. The aim of this chapter was to further elucidate the role of the TlyA family as regulatory proteins. Since systems for the genetic manipulation and mutagenesis of *L. intracellularis* have not been sufficiently developed, examination of the putative role of TlyA was carried out in another bacterial species, *Helicobacter pylori*. Two dimensional electrophoresis of whole cell proteins from RS7, the *tlyA* knockout strain of *H.pylori* SS1, and wild-type SS1 (Martino *et al.*, 2001), will allow the effect of the loss of TlyA on protein expression profiles between the two strains. LsaA is known to be a surface associated protein and this may preclude it functioning as a regulatory factor in *L. intracellularis*, although, its cell surface location would put it

in an excellent position for a role in the regulation of host cell protein expression. *L. intracellularis* is often observed *in vivo* and *in vitro* in a peri-nuclear position, an ideal location in which to interact with mRNA translation on the ribosome (McOrist *et al.*, 1995, Rowland and Lawson, 1974). The production of translational fusions of *LsaA* and the *TlyA* from *H. pylori* and to the EGFP protein will allow the intracellular localisation of these proteins to be observed in a similar experiment to that performed with FTSJ2 by Ching *et al.*, 2002.

6.2 Results

6.2.1 2D PAGE of whole cell proteins from *H.pylori* strain RS7 and SS1

Plate grown *H. pylori* protein extracts were prepared for 2-D gel electrophoresis as described in section 2.38. Initially 7cm strips were used to optimise the technique and protein profiles using these strips were suggestive of differences between RS7 and SS1. Full scale analysis of protein expression using 24cm strips was then attempted and, as expected, gave much clearer resolution of differences in protein expression between strains. Although the experiment was not intended as a comprehensive analysis of protein expression the profile was sufficiently similar to existing 2D PAGE *H. pylori* protein profiles (Jungblut *et al.*, 2000).

Several differences in protein expression were observed on larger gels between RS7 and SS1 (Figure 6.4). Proteins observed as being expressed differently between strains were selected for analysis by MALDI-MS. Some proteins whose expression remained consistent between strains were also selected for reference purposes. Selected proteins are highlighted in Figure 6.3 and their identities listed in Table 6.1. Several identified proteins were observed to be of integral membrane origin.

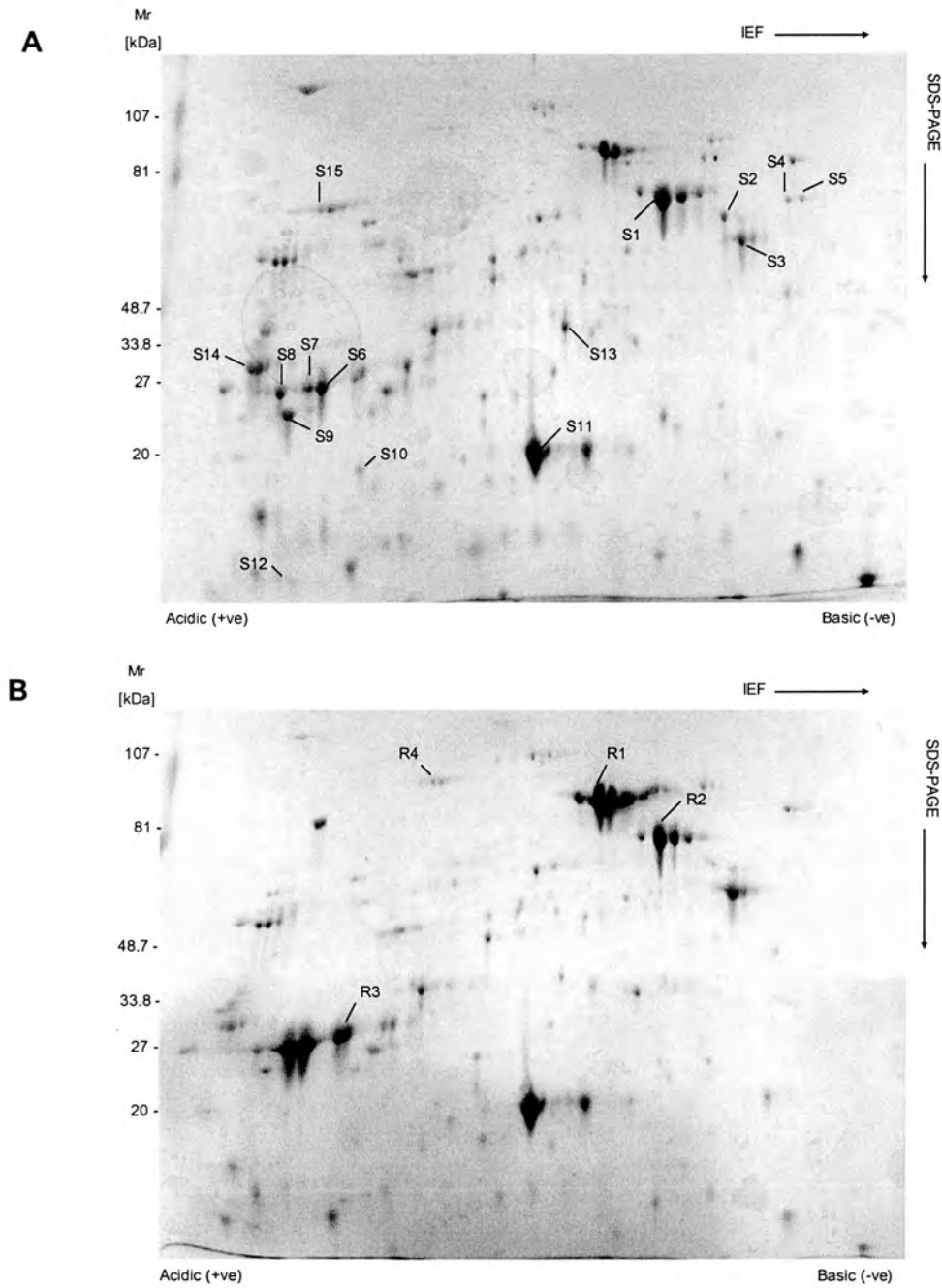


Figure 6.3: 2-D Gel Electrophoresis of SS1 (A) and RS7 (B) whole cell lysate. Spots marked with numbers were excised for identification by peptide mass fingerprinting MALDI-ms are indicated.

Spot ID	Hit	Expression levels	Score	Expected pI	Expected Mass (kDa)
R1	Urease β subunit	Up regulated in RS7	181 (1.4×10^{-13})	5.64	61.7
S1	GroEL Hsp60	-	70 (0.016)	5.55	58.3
R2	GroEL Hsp60	-	108 (2.7×10^{-6})	5.55	58.3
S2	Trigger Factor	-	54 (0.69)	5.33	52
S3	Elongation Factor (EFTu)	-	80 (0.0016)	5.17	43.5
S4	Flagellin B	Up regulated in SS1	97 (3.7×10^{-5})	5.95	53.8
S5	Flagellin B	Up regulated in SS1	103 (8.6×10^{-6})	5.95	53.8
S6	Urease α subunit	Up regulated in RS7	76 (0.0039)	8.47	26.6
S7	Urease α subunit	Up regulated in RS7	76 (0.0039)	8.47	26.6
S8	Urease α subunit	Up regulated in RS7	143 (8.6×10^{-10})	8.46	26.6
S9	Urease α subunit	Up regulated in RS7	108 (2.7×10^{-6})	8.46	26.6
S10	Ribosome recycling factor	-	71 (0.015)	7.79	20.9
S11	Probable peroxiredoxin	-	123 (8.6×10^{-8})	5.88	22.2

S12	50S ribosomal protein	-	39 22	8.81	16.5
S13	Fructose bisphosphate aldolase	-	106 (4.3×10^{-6})	5.90	33.8
S14	Hypothetical protein JHP0161 precursor	-	123 (8.6×10^{-8})	9.29	34.0
S15	Catalase	Up regulated in SS1	55 (0.58)	8.70	58.6
R3	Urease α subunit	Up regulated in RS7	99 (2×10^{-5})	8.46	26.5
R4	Fumarate reductase	-	54 (0.27)	6.87	80.1

Table 6.1: Spots excised from 2D-PAGE of SS1 and RS7 and identified by peptide mass fingerprinting MALDI-MS. The presence of multiple spots with the same identity is most likely due to the existence of differences in post-translational modification of the same protein. Significant MASCOT scores are shown in bold with probability that the match is a random event given in brackets below.

6.2.2 Intracellular localisation of LsaA and TlyA_{HP} within epithelial cells

The differences between protein expression profiles in RS7 and SS1 would indicate that the TlyA_{HP} appears to have a role in affecting protein expression in bacteria (*H. pylori*). However, S4 and FtsJ domains are also present in eukaryotic regulatory proteins. Therefore LsaA and TlyA_{HP} were examined to determine whether they localised within epithelial cells to particular organelles, specifically the nucleus or nucleolus.

As mentioned previously, *L. intracellularis* within enterocytes has been observed to lie closely associated with mitochondria and rough endoplasmic reticulum in a perinuclear pattern (McOrist *et al.*, 1995, Rowland and Lawson, 1974). In order to confirm this finding in this study a semi-confluent INT-407 cell monolayer was infected with *L. intracellularis* (LR189/S22) (Section 2.4.3) and stained as described in section 2.42.2. The result was identical to that described above showing that this pattern is consistent with *L. intracellularis* infection (Figure 6.4).

LsaA is predominantly a surface protein, however the conserved domains in TlyA family members suggests possible functions within the nucleus/ nucleolus. Therefore an investigation of the intracellular localisation of LsaA and TlyA_{HP} was also undertaken. To do so, EGFP fusions to LsaA and TlyA_{HP} were constructed.

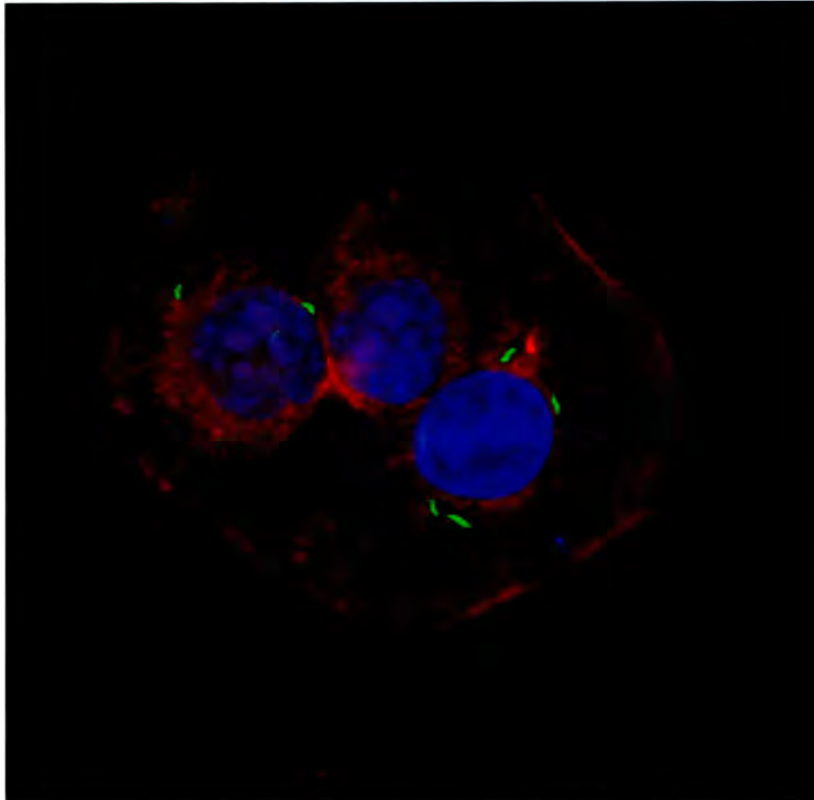
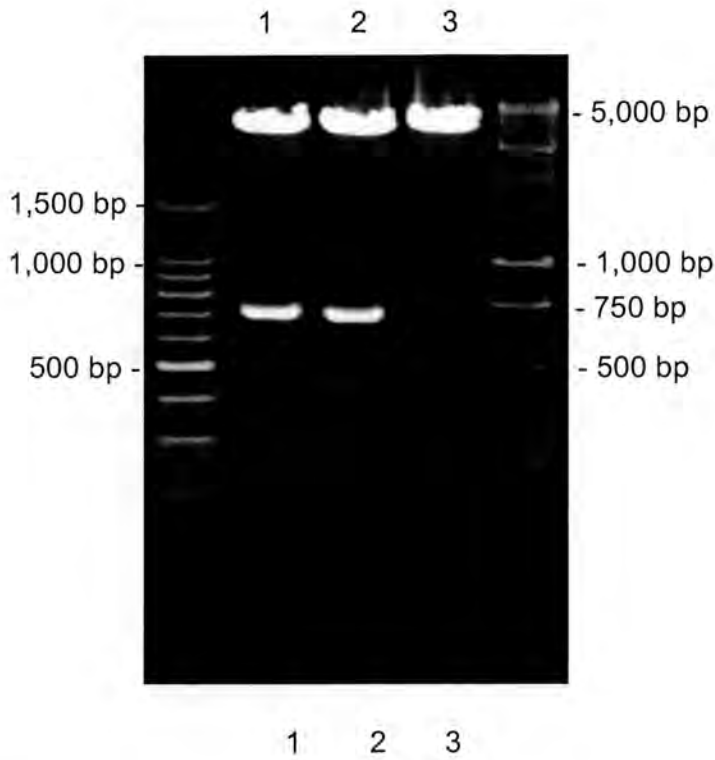


Figure 6.4: *L. Intracellularis* infected INT-407 cells showing peri-nuclear localisation of the organism. INT-407 cells were infected with *L.intracellularis* (LR189) (green), fixed and stained with VPM53 followed by an anti-mouse FITC conjugated antibody 24 hrs p.i. INT-407 cells were counterstained with phalloidin-conjugated TRITC (red) and the nucleus stained with TOPRO-2 (blue).

6.2.2.1 Expression plasmid construction

Genes were amplified via PCR using primers designed to incorporate a *BglIII* site and a *KpnI* site into the 5' and 3' ends respectively of the *lsaA* and *tlyA* gene. The products were then cloned directly into the vector PCR-4-TOPO (Appendix I.06) from which they were excised using the newly incorporated restriction sites, *BglIII* and *KpnI*, and directionally ligated into a similarly digested pEGFP-C1 vector (Appendix I.02) to create translational fusions to green fluorescent protein (GFP) (Figure 6.5). The MCS site in pEGFPC1 is between the EGFP coding sequences and the SV40 poly A. Genes cloned into the MCS will be expressed as fusions to the C-terminus of EGFP if they are in the same reading frame as EGFP and there are no intervening stop codons. The pEGFPC1 plasmid incorporates the cytomegalovirus immediate early promoter which fires transcription of the GFP protein and of any in-frame insertions in the C-terminal MCS therefore no promoter regions were included in the genes of interest.



Restriction Enzymes	pEGFPC1	pEGFPC1/ LsaA	pEGFPC1/ <i>H. pylori</i> TlyA
<i>Bgl</i> III and <i>Kpn</i> I	4.7 kb	4.7 kb + 702 bp	4.7 kb + 705 bp

Figure 6.5: Restriction analysis of GFP fusion constructs. All plasmids were *Bam*HI/*Kpn*I digested. Lane 1= pEGFPC1/*H. pylori* TlyA, Lane 2= pEGFPC1/ LsaA, Lane 3= pEGFPC1.

6.2.2.2 Transfection and expression of GFP fusion proteins in semi-confluent INT-407 cell monolayer.

Plasmids were transfected into the intestinal epithelial cell line INT-407, known to be permissive for *L. intracellularis* infection, which had previously been seeded to give a 30-40% semi-confluent monolayer following overnight growth. DOTAP liposomal transfection reagent was used to transfect cells and medium containing the DOTAP-DNA complexes was added to the cell monolayer for 6 hours before being changed for fresh media (Section 2.41). Cells were then incubated for either 12 or 24 hrs post transfection before being fixed and stained (Section 2.42.1).

Following optimisation of lipofection conditions, 5% of the cells could be seen to be fluorescing. It therefore remained to ascertain if the GFP was being expressed as a translational fusion. Initially whole cell protein extracts were blotted with an anti-GFP antibody and also VPM53. However, it was not possible to detect fusion protein expression using this method, possibly due to the low transfection efficiency and hence low yield. Initial attempts at RTPCR were also unsuccessful due to DNA contamination. Samples were DNase I-treated to remove contamination (Section 2.14.1.3.2), however this was also unsuccessful. Removal of template DNA contamination from RNA samples has been shown to be most efficient when RNA is treated to DNase I digestion in parallel with oligo (dT) affinity column purification of mRNA (Section 2.14.2.2) (Stocher and Berg, 2004). Using this method it was possible to confirm expression of the fusion protein using primers designed to amplify a 600bp fragment incorporating the final 300bp in the GFP ORF and the initial 300bp from *LsaA* or *TlyA* (Figure 6.6).

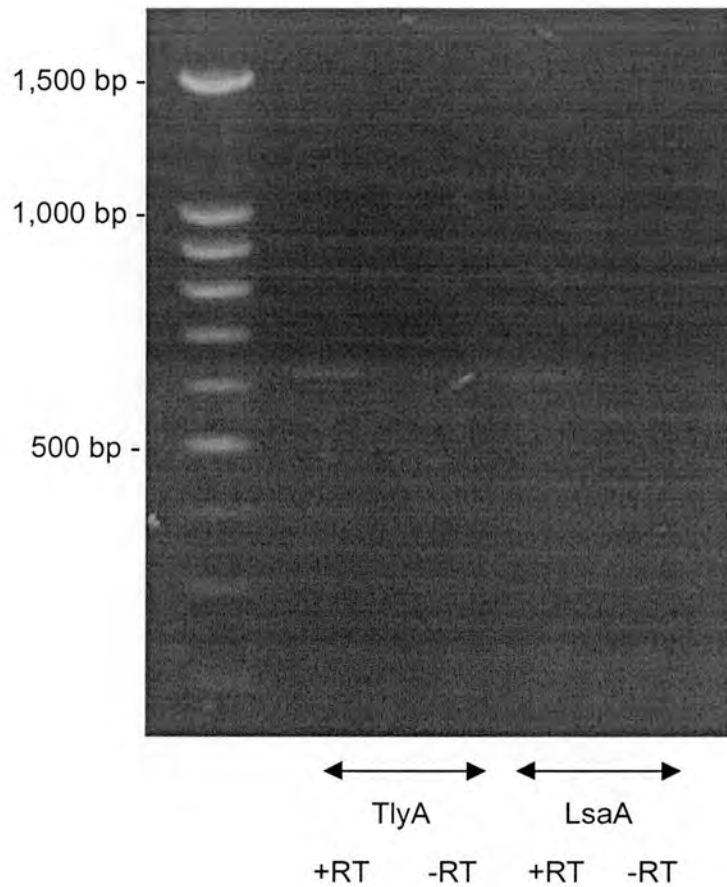


Figure 6.6: Conformation of GFP-LsaA and GFP- TlyA_{HP} fusion protein expression. RTPCR on RNA extracted from INT407 monolayer transformed with pEGFPC1/LsaA or pEGFPC1/TlyA_{HP} respectively, using primers designed to amplify a 600bp fragment incorporating the last 300bp of the gene of interest and the first 300bp from the GFP protein.

Following confirmation of fusion protein expression cells were fixed and stained for confocal microscopy (Section 2.42.1).

Figure 6.7: Confocal microscopy of GFP-LsaA and GFP-TlyA_{HP} transfected semi-confluent INT 407 monolayer. Following transfection of semi-confluent INT-407 cells with LsaA or TlyA_{HP}-EGFP fusion construct, cells were cultured for either a 12 or 24 hr period before being fixed and stained with phalloidin-conjugated TRITC (red) and TOPRO-2 (blue) to highlight the nuclear material. Following 24hrs GFP (A), GFP-LsaA (B) and GFP-TlyA (C) were frequently observed in a punctuate staining pattern within cells. However, GFP alone could also be observed as diffuse staining throughout the cytoplasm and nucleus at both 24hrs (D) and 12hrs (E) post-transfection. Whereas, GFP-LsaA was observed at 24hrs post-infection throughout and surrounding the host cell nucleus (F, G and H). A similar distribution of GFP-TlyA was also visible 12 hrs post-transfection (I, J and K). Scale bars are all equivalent to 10 μm .

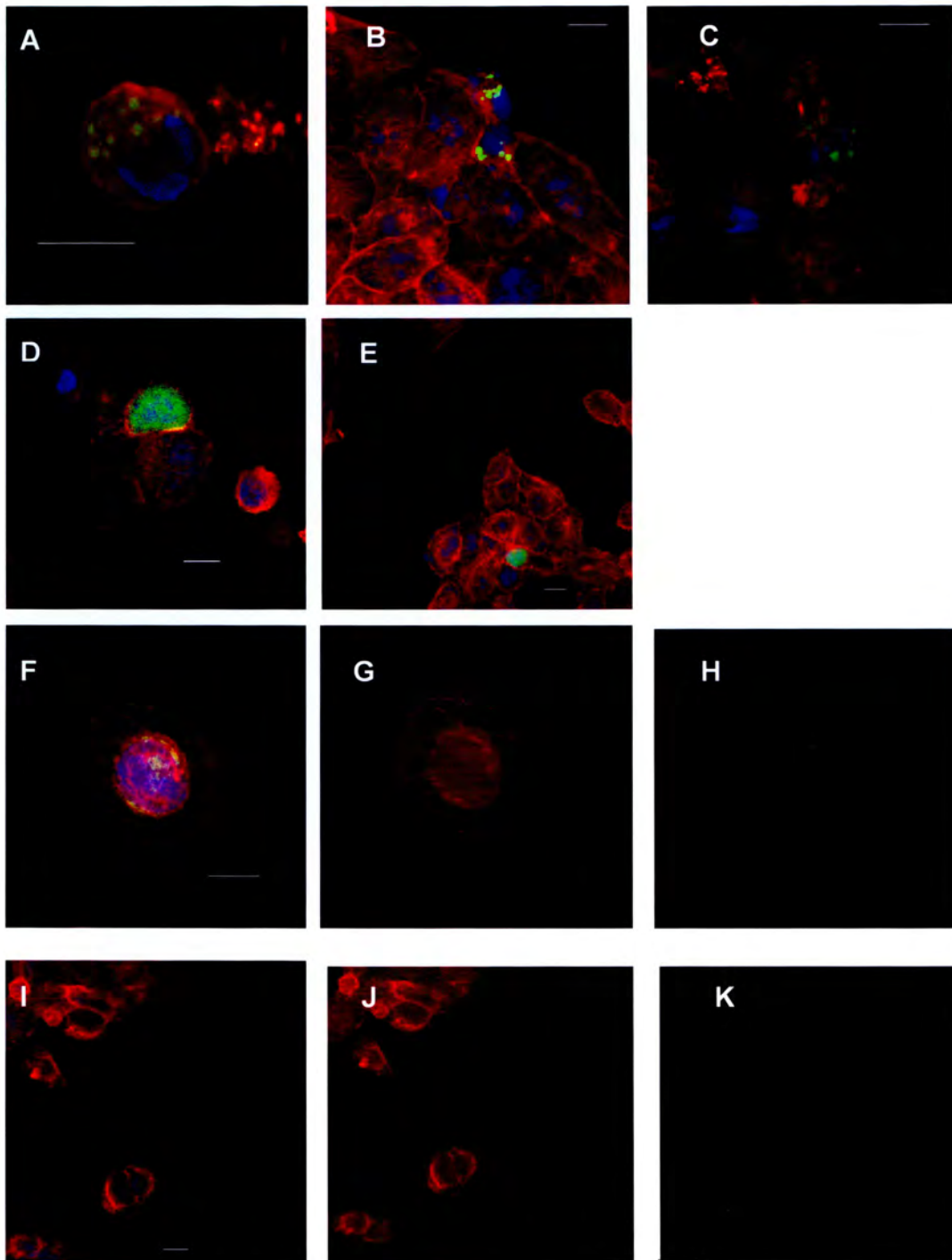


Figure 6.7

6.3 Discussion

6.3.1 Whole cell protein analysis of RS7 and SS1

2-D electrophoresis of whole cell protein extracts from RS7 and SS1 showed some major variations in protein expression between the wild-type and the knockout strains. Although the levels of several proteins, including GroEL Hsp60, were equivalent between strains there was a noticeable absence of flagellin B and catalase in RS7 compared to SS1. Flagellin B is one of the two flagellins, FlaA and FlaB, which comprise each flagellar filament. *H. pylori* carries a tuft of about five sheathed flagella located at one pole. The minor flagellin species, FlaB, localizes to the base of the flagellum, while the more abundant one, FlaA, lies in the outer region (Kostrzynska *et al.*, 1991). *H. pylori* has been shown to require flagella for infection of the stomach. Flagellated but non-motile mutants have been constructed in *H. pylori* showing that it is motility, not merely the presence of flagella that is important for initial colonisation (Ottemann and Lowenthal, 2002). Elimination of *flaB* (FlaB-) yields normal-looking flagella that retain some function and propel the bacterium about 60% as well as normal (Josenhans *et al.*, 1995); (Suerbaum *et al.*, 1993). Elimination of *flaA* (FlaA-) yields truncated flagella that confer only slight bacterial motility. Elimination of both flagellins (FlaA- FlaB-) results in a flagellated bacteria that are nonmotile (Josenhans *et al.*, 1995). The phenotypes of these mutants in the piglet colonisation model roughly parallel their motility: mutants missing either *flaB* or *flaA* were able to transiently colonise piglets (for four days) but at levels about 10^4 fold lower than those of their wild-type parents (Eaton *et al.*, 1996). FlaA⁻FlaB⁻ double mutants were able to infect animals to levels similar to those of the single flagellin mutants only until the earliest time-point, day 2. These results suggest that partial motility can support some colonisation but wild-type motility is needed for the bacterium to reach and maintain high levels of infection in the piglet. The reduced level of FlaB in the *tlyA* mutant may therefore be predicted to compromise motility and lead to attenuation.

Similarly catalase has been implicated in assisting *H. pylori* colonisation of the gastric mucosa. Catalase is a ubiquitous enzyme that is responsible for the enzymatic dismutation of hydrogen peroxidase into water and molecular oxygen and can protect organisms against the potentially damaging effects of hydrogen peroxide

(Farr *et al.*, 1988, Cadenas, 1989). Catalase has been proposed as a *H. pylori* adhesin in the past and postulated to bind to a glycolipid receptor (Lingwood *et al.*, 1993). However, this finding has been disputed by Odenbreit *et al.*, (1996) who constructed transposon insertion mutants of the catalase gene, *KatA*, in three independent *H. pylori* strains and found no significant difference in the ability of wild type and mutant strains to bind to epithelial cells (Odenbreit *et al.*, 1996). Nevertheless, catalase can clearly contribute to virulence by neutralising antibacterial activities of hydrogen peroxide.

These results are interesting since a phenotype of RS7, the *tlyA* knockout, is a reduced ability to adhere to AGS cells and to colonise the mouse model (Martino *et al.*, 2001, Chapter 3). It had previously been assumed that this must be due to a direct role of the TlyA protein in adherence/attachment to host cells, however, these results would suggest that the TlyA protein in fact plays an indirect role in reduction in the virulence of the RS7 strain. Although the PMF data from catalase did fall slightly short of the significance threshold, it would appear that down-regulation of virulence genes such as flagellin B, and possibly catalase, in the absence of TlyA, may be responsible for the observed phenotype.

However urease, another well documented virulence factor of *H. pylori*, was also observed to have different expression levels in the two strains. Expression of both α and β subunits of *H. pylori* urease would appear to be upregulated in RS7 compared to SS1, perhaps reflecting wider dysregulation in the *tlyA* mutant. Urease (urea amidohydrolase), accounting for 5-10% of the total cellular protein of *H. pylori*, is central to the pathogenesis of *H. pylori* infection and disease. The protein, comprised of six copies each of two structural subunits, UreA and UreB, is a nickel-requiring metalloenzyme that hydrolyses urea to ammonia and carbon dioxide (Mobley, 1997); (Mobley *et al.*, 1995). Urease-generated ammonia neutralises gastric acid (Goodwin *et al.*, 1986), causes damage to gastric epithelial cells (Smoot *et al.*, 1990), and is assimilated into proteins by synthesis of glutamine from ammonia and glutamate catalyzed by glutamine synthetase (Garner *et al.*, 1998) or by synthesis of glutamate from ammonia and α -ketoglutarate catalysed by glutamate

dehydrogenase (ferrero *et al.*, 1988). The role of urease in colonisation has been assessed by testing the virulence of a urease-negative mutant of an *H. pylori* strain, generated by mutagenesis with nitrosoguanidine, in the gnotobiotic piglet model of gastritis (Eaton *et al.*, 1991). The mutant, which retained only 0.4% of the urease activity of the parent strain, was unable to colonise any of ten orally challenged piglets as assessed at 3 or 21 days after challenge and no pathology was observed in these piglets. In contrast, the parent strain successfully colonised all seven piglets and elicited gastritis. It was later confirmed by Eaton and Krakowka (1994) that it is urease enzymatic activity and not simply the inactive apo-urease protein is essential for colonisation (Eaton and Krakowka, 1994).

It could be postulated that the apparent up-regulation of these factors is an attempt by the organism to compensate for the reduced expression of other factors involved in colonisation of the gastric mucosa. However, this is purely speculative.

6.3.2 Intracellular localisation of LsaA and TlyA_{HP}

As well as affecting expression of genes in prokaryotes it is possible that LsaA/TlyA family members could affect expression in eukaryotic cells also. The bacterial cell surface location of LsaA would put it in an ideal position to do so. If so, S4/FtsJ containing proteins may localise to the nucleus or to sites of protein expression in the endoplasmic reticulum (ER). Therefore, the *in vitro* localisation patterns of GFP-LsaA and GFP-TlyA_{HP} fusion proteins were investigated using confocal microscopy. Transfection of semi-confluent INT-407 cells with the LsaA and TlyA_{HP} GFP fusion proteins resulted in punctuate staining following a 24 hour incubation periods with the constructs. This effect was also seen in cells transfected with the plasmid expressing only GFP, however, GFP alone could also be observed as diffuse staining throughout the cytoplasm and nucleus at both 24hrs and 12hrs post-transfection. In contrast, GFP-LsaA was observed at 24hrs post-infection in a distinctly peri-nuclear position and also distributed within the host cell nucleus. A similar distribution of GFP-TlyA_{HP} (i.e. peri-nuclear) was also visible 12 hrs post-transfection. Targeting to this intracellular site suggests that LsaA/TlyA_{HP} possess localisation signals or, alternatively, interacts with ligands which preferentially localise to this site.

However, a search for known signal sequences failed to find any homologues in LsaA (Emanuelsson *et al.*, 2000, Section 2.3.1). There is an intriguing correspondence in localisation of LsaA (and TlyA_{HP}) and *L. intracellularis* itself and it is tempting to speculate that LsaA could confer intracellular tropism, since LsaA appears to be a major surface protein.

Whether or not LsaA is involved in the regulation of host cell protein expression *in vivo* and functions as a virulence determinant in this respect is yet to be determined. However, this work supports the previously implied role of the TlyA family in regulation of bacterial protein expression. In the future selective cultivation of resistant INT-407 cells carrying the pEGFPC1/LsaA construct could be cultivated until sufficient material was available to perform 2-D electrophoresis and analyse protein expression against an untransfected control.

Together, the findings herein suggest that LsaA/TlyA may have pleotropic roles and confirms their importance as a determinant of virulence.

Chapter 7

IDENTIFICATION OF FURTHER IMMUNODOMINANT SURFACE ANTIGENS FROM *L. INTRACELLULARIS*

CHAPTER 7

Identification of further immunodominant surface antigens from *L. intracellularis*

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7.1 Introduction

When considering the properties of an efficacious vaccine, it is important to identify the *L. intracellularis* proteins which elicit a strong immune response in the host in order to analyze their capability to confer protective immunity. Furthermore, the identification and characterization of immunodominant proteins can contribute to the improvement of serological tests for detecting and monitoring *Lawsonia* infections.

At present, only a few immunogenic proteins from *L. intracellularis* have been identified (Section 1.5). These include LsaA (27kDa) and LsaB (25kDa), the main proteins identified in immunoblots analysed with hyperimmune polyclonal antibody (4F5) raised in rabbits against bacteria in adjuvant (Lawson *et al.*, 1979, McOrist *et al.*, 1989b).

The aim of the work described in this chapter was the identification of further immunogenic antigens. One experimental approach designed for the discovery of immunorelevant proteins from bacteria consists of 1D or 2D gel separation of bacterial proteins followed by immunoblotting and detection of antigens with host sera. The gastric pathogen *H. pylori* has been the subject of several such studies. Nilsson *et al.* compared the expression and immunogenicity of cell surface proteins from a strain of *H. pylori* in two different states - spiral-shaped and coccoid (Nilsson *et al.*, 2000). Another group has identified immunogenic proteins in *H. pylori* strains Z-170 (McAtee *et al.*, 1998a) and ATCC 43504 (McAtee *et al.*, 1998b). The above studies were performed by immunodetection with pooled convalescent sera from several patients. In addition, the technique has been extended to identify common patterns of *H. pylori* antigens which are recognized by sera from patients showing various gastroduodenal pathologies. The technique successfully identified several immunogenic proteins of *H. pylori*; however no clear correlation between antigenic patterns and gastroduodenal pathologies was evident. The authors suggested several reasons for this including the possibility that specific virulence factors were produced in amounts below detection limits of this technique. Alternatively, they also suggest that the strain in question may have lost virulence traits due to prolonged *in vitro*

passages or that factors contributing to virulence may not be expressed under *in vitro* conditions precluding their detection using this method (Kimmel *et al.*, 2000).

The strategy for the identification of immunogenic *L. intracellularis* proteins and potential vaccine candidates is based on a comparative analysis of the humoral immune response of pigs inoculated with a live attenuated vaccine strain of *L. intracellularis* (Boehringer Ingelheim) and experimentally infected with live, virulent organisms of the same strain (DK-1790). To this end, *L. intracellularis* (DK-1790) proteins resolved by 2D gel electrophoresis were blotted onto nitrocellulose and screened using immune sera from vaccinated and experimentally-infected pigs.

This strategy was adopted on the rationale that immune reagents raised in the natural host species could conceivably identify *L. intracellularis* immunogens that were not detected in previous studies where laboratory rabbits immunised with inactivated organisms were used to generate immune sera.

Furthermore, an additional aim was to use the polyclonal antisera, 4F5, to select and further characterise the *Lawsonia* surface antigen B (LsaB) and other possible immunogenic components.

7.2 Results

Normal or membrane enriched extracts of *L. intracellularis* (DK-1790) proteins were separated by 2D gel electrophoresis (Section 2.38). Gels were either stained in colloidal blue or transferred to a nitrocellulose membrane and immunoscreened using anti-sera from challenged animals (84 days p.i.) or, as a control, with sera from 3-4 week old unchallenged, unvaccinated animals. The results can be seen in Figure 7.1.

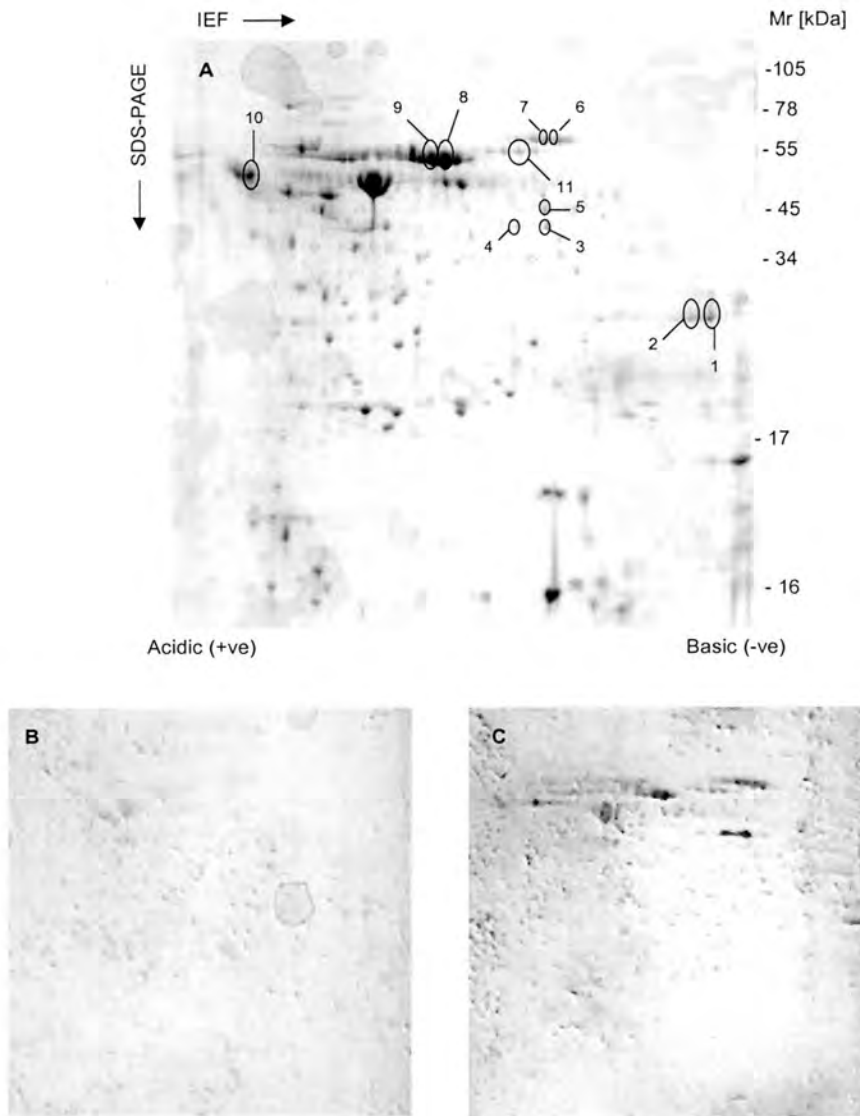


Figure 7.1: Immunoscreening of *L. intracellularis* proteins separated by 2D electrophoresis with pig sera. Two dimensional blot of *L. intracellularis* (DK-1790) membrane enriched proteins stained with colloidal blue (A) or transferred to nitrocellulose and blotted with anti sera from challenged (84 days p.i.), unvaccinated (C) and unchallenged, 3-4 week old pigs (B). Proteins picked for identification by MALDI-MS are indicated (No.s 1-10).

It was found that there was no difference in the detectable proteins by the preparation of bacterial extracts in buffer enriching for membrane proteins or without. Spots excised from the gel were analysed by MALDI-TOF mass spectrometry (Section 2.39). Peptide mass fingerprints (PMF) generated from tryptic peptide digests produced from each protein were screened using the Swissprot database for matches of either a eukaryotic or prokaryotic nature. The best matches were mainly against eukaryotic proteins. Unfortunately the obligate intracellular nature of *L. intracellularis* requires it to be maintained in mammalian tissue culture making such contamination an unavoidable problem.

Therefore the unmatched peptides were searched against a prokaryotic database in order to identify any prokaryotic homologues masked by a predominantly overwhelming eukaryotic protein presence. The results are shown in Table 7.1 with scores of proteins determined to be significant hits highlighted in bold.

A similar approach was taken to identify surface antigen LsaB with polyclonal antibody 4F5; the result can be seen in Figure 7.2 and Table 7.2.

Table 7.1: Proteins highlighted in Figure 7.1 and identified by peptide mass fingerprinting MALDI-MS. PMF were initially screened against a eukaryotic database following which unmatched peptides were then screened against proteins from a prokaryotic database. *Mr* was estimated from the 2-DE position. Scores calculated to be significant are shown in bold.

Spot No.	All entries	Score	Mass (kDa)	Prokaryotic Hits	Mowse Score	Mass (kDa)	Mr (kDa)
1	Voltage-dependent anion-selective channel protein 1 Rabbit	133	30	N utilization substance protein B homolog (NusB protein) <i>Borrelia burgoderferi</i> 50S ribosomal protein L23 <i>Bacillus subtilis</i>	44 44	17 11	30
2	Voltage-dependent anion-selective channel protein 1 Rabbit	155	30	Hypothetical protein RP448 <i>Rickettsia prowazekei</i> ATP synthase alpha chain <i>Haemophilus influenzae</i>	52 51	51 56	30
3	Alpha enolase Mouse	83	47	Cysteinylyl tRNA synthase <i>Helicobacter pylori</i> J99	45	53	40
4	Alpha enolase Mouse	58	47	30S ribosomal protein <i>Sinorhizobium meloti</i>	32	9	40
5	Aldehyde dehydrogenas, mitochondrial precursor Mouse	77	66	ATP-dependent RNA helicase <i>Vibrio cholerae</i>	57	49	45
6	Serotransferrin precursor Bovine	185	77	Hypothetical protein BU358 <i>Buchnera aphidicola</i>	45	20	60
	Extracellular Lipase Precursor <i>Aeromonas hydrophila</i>	68	77	ATP phosphoribosyl transferase <i>Prochlorococcus marinus</i>	55	23	

	Tyrosine-protein phosphatase YopH <i>Yersinia enterocolitica</i>	58	51				
7	Serotransferrin precursor Bovine	199	77	ATP phosphoribosyl transferase <i>Prochlorococcus marinus</i>	48	23	60
	Tyrosine-protein phosphatase YopH <i>Yersinia enterocolitica</i>	68					
8	Serum albumin precursor	367	69	Hypothetical UPF0247 protein WS0156 <i>Wolinella succinogenes</i>	42	17	55
	Propionyl-CoA carboxylase beta chain, mitochondrial precursor	82	58				
9	Serum albumin precursor	303	69	16S rRNA processing protein rimM <i>Buchnera aphidicola</i>	40	21	55
	Propionyl-CoA carboxylase beta chain, mitochondrial precursor	80	58				
10	Alpha-2-HS-glycoprotein precursor Bovine	110	38	30S ribosomal protein S11 <i>Chlamydia muridarum</i>	52	14	50
11	Serotransferrin precursor Bovine	123	77	50S ribosomal protein L29 <i>Rickettsia conorii</i>	30	8	40

Table 7.1

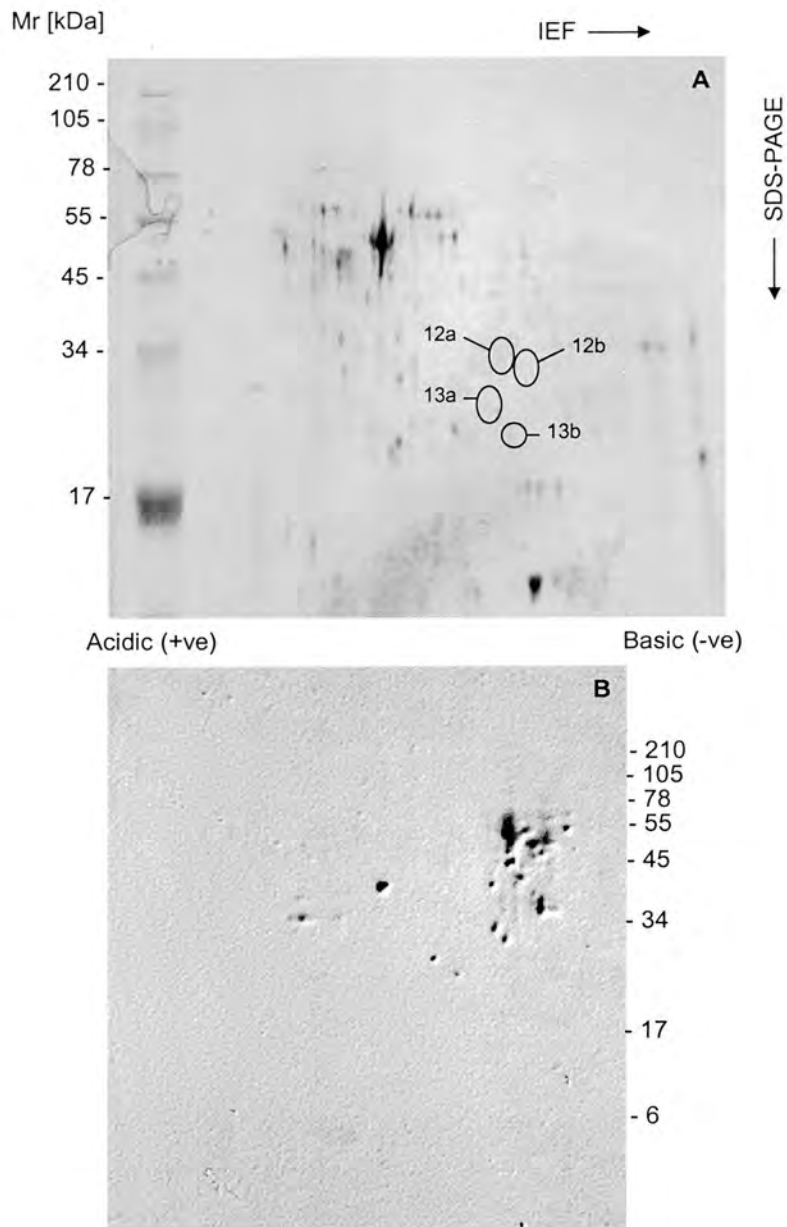


Figure 7.2: Immunoscreening of *L. intracellularis* proteins separated by 2D electrophoresis with polyclonal antibody 4F5. (A) Membrane enriched *L. intracellularis* (DK-1790) whole cell lysate separated by 2-D gel electrophoresis and stained with colloidal blue. (B) Membrane enriched *L. intracellularis* whole cell lysate separated by 2-D electrophoresis, transferred to nitrocellulose and immunoscreened with polyclonal antibody, 4F5, against LsaA and LsaB. Proteins picked for identification by MALDI-MS are indicated (No.s 12a-13b).

Although there is a significant amount of background the known mass (27 kDa) and pI (8.85) of LsaA and the known mass of LsaB (25 kDa) can be used to predict the approximate positions of the corresponding spots.

Spot no.	All Entries	Score	Mass (kDa)	Prokaryotic Hits	Mowse Score	Mass (KDa)	Mr (kDa)
12a	Keratin Human	64	-	Glutamyl-tRNA reductase <i>Aquifex aeolicus</i>	42	47	~30
12b	Peroxiredoxin 4 Mouse	92	30/31	DNA-directed RNA polymerase gamma chain <i>Prochlorococcus marinus</i>	54	72	~30
13a	T-complex protein 1, theta subunit Mouse	49	59	Transcriptional regulator slyA (Salmolysin) (Cytolysin slyA) synthase <i>Salmonella typhinurium</i>	16	43	~25
13b	Nuclear autoantigen Sp-100 Chimpanzee	54	25	Cop protein <i>Staphylococcus aureus</i>	15	45	~25
	Retrovirus-related Gag polyprotein	50	29				

Table 7.2: Spots highlighted in Figure 7.2 and identified by peptide mass fingerprinting MALDI-MS. PMF were initially screened against a eukaryotic database following which unmatched peptides were then screened for homologous proteins of a prokaryotic nature. *Mr* was estimated from the 2-DE position. Scores calculated to be significant are shown in bold.

7.3 Discussion

In immunoproteomics, immunogenic bacterial proteins can be detected using antibodies from infected animals or humans. Those immunogenic proteins can then be identified using mass spectrometry or alternative methods. This approach has previously been applied successfully to the identification of antigens of importance in *H. pylori* (Nilsson *et al.*, 2000, McAtee *et al.*, 1998a, McAtee *et al.*, 1998b), *C. trachomatis* (Vandahl *et al.*, 2003) and *Borrelia garinii* (Jungblut *et al.*, 1999), and its application to *L. intracellularis* also shows promise. Although there were significant problems arising from contamination with eukaryotic proteins, an unavoidable problem due to the conditions required for *L. intracellularis* culture, it was possible to obtain significant hits of a prokaryotic nature. Screening of *L. intracellularis* membrane enriched extract with sera from a pig exposed to live replicating *L. intracellularis* identified a YopH homologue (*Yersinia enterocolitica*) and an extracellular lipase precursor homologue (*Aeromonas hydrophila*) as two of the immunogenic proteins produced by *L. intracellularis* during infection *in vivo*. Both were significant hits in terms of MOWSE score and MW, and although the primary hit was against eukaryotic serotransferrin it is entirely possible that due to the low resolving power available with 7cm strips more than one species of protein was present. It has recently been estimated that only around 30% of the spots from a 2D gel are truly attributable to a single protein species and overlapping of this nature is common (Campostrini *et al.*, 2005). Theoretically, it should be possible to increase resolution in the future, if more starting material were available, by using longer 1st dimension strips or by focusing the separation across a narrower pI range.

7.3.1. YopH homologue

The putative identification of a YopH as an immunogenic protein from *L. intracellularis* is an intriguing finding. YopH is one of the *Yersinia* outer proteins (Yops) encoded on a 70kb plasmid, pYV, found in all pathogenic *Yersinia* species (Gemski *et al.*, 1980). Direct cell contact, in combination with a type III secretion apparatus, allows *Yersinia* to translocate the Yops from the cytoplasm of the bacteria directly into the cytoplasm of a host cell to modify host cell function. In general *Yersinia* Yops are involved in modulating the host immune defences to allow

bacteria to replicate extracellularly in lymph tissues and organs (Simonet *et al.*, 1990, Cornelis *et al.*, 1998). Studies have shown that YopH plays an essential role in the *Yersinia* pathogenesis since a *Yersinia* mutant expressing a catalytically inactive form of YopH is avirulent in the mouse model (Andersson *et al.*, 1996, Persson *et al.*, 1997).

As a powerful tyrosine phosphatase, YopH targets many proteins leading to a variety of different effects in cultured cells. A characteristic of *Yersinia* infection is invasion of lymphatic tissue and extracellular proliferation in the lymph nodes in the absence of an immune response (Brubaker, 1991, Simonet *et al.*, 1990). YopH is now known to be involved in resistance to engulfment by professional phagocytes and is essential for pathogen survival and replication in lymphoid tissue (Rosqvist *et al.*, 1988).

Through dephosphorylation of P130^{Cas} and focal adhesion kinase (FAK) (Black and Bliska, 1997, Persson *et al.*, 1997), YopH has been found to cause the disassembly of the focal adhesion complex (Black and Bliska, 1997, Persson *et al.*, 1997, Black *et al.*, 2000, Hamid *et al.*, 1999), an action which is ultimately responsible for prevention of *Yersinia* phagocytosis. Additionally, YopH has been shown to inhibit Fc receptor-mediated phagocytosis in macrophage-like cell line J774 (Fällman *et al.*, 1995). Since *L. intracellularis* does, and must, enter epithelial cells of the gut to survive, and has been observed to accumulate inside macrophages in large numbers (MacIntyre *et al.*, 2003), (MacIntyre *et al.*, 2003) there must be differences in functionality or expression of YopH between the bacteria. However, *Y. pseudotuberculosis* is known to enter M cells before invading the underlying lymphoid follicles of the small intestine (Marra and Isberg, 1997, Autenreith and Firsching, 1996) and so it must be possible to circumvent the anti-phagocytic activity of YopH. Possibly via invasion of M cells prior to Yop expression or perhaps a different complement of cell surface molecules on M cells may prevent delivery of the Yops (Logsdon and Mecsas, 2003).

YopH also has major effects on the innate and adaptive immune responses in several ways, a function which may be highly relevant to *L. intracellularis* pathogenesis. As mentioned previously, YopH is considered as the key factor in the down-regulation

of the host inflammatory response during *Yersinia* infection (Sauvonnet *et al.*, 2002). Incidentally a key feature of *L. intracellularis* infection is the marked absence of inflammation or immune response in the infected tissue (McOrist *et al.*, 1992). YopH has been shown to inactivate the PI 3-kinase/Akt signalling pathway in host cells via its tyrosine phosphatase activity. This pathway is involved in a number of processes in the host cell including endocytosis, cell survival and proliferation (Sauvonnet *et al.*, 2002) and in macrophages, PI 3-kinase/Akt inactivation by YopH was linked to the downregulation of the gene coding for the chemokine MCP-1, responsible for directing the migration of macrophages into inflammatory sites (Palframan *et al.*, 2001). YopH can also prevent T-cell proliferation and, in infected cells, this is also mediated via the PI 3-kinase/Akt signalling pathway (Sauvonnet *et al.*, 2002). Inactivation of this pathway prevents IL-2 secretion by stimulated T lymphocytes (Sauvonnet *et al.*, 2002, Yao *et al.*, 1999), thereby preventing their proliferation (Sauvonnet *et al.*, 2002). It is tempting to speculate that down regulation of villus epithelial-associated CD3⁺ T-cells observed in *L. intracellularis* infection (MacIntyre *et al.*, 2003) could also be mediated by a YopH homologue. Similarly, complete T-cell activation requires a signal delivered through TCR-peptide/MHC complex and the engagement of costimulatory molecules CD28/B7.2, expression of which is inhibited by YopH (Yao *et al.*, 1999). A failure to induce normal levels of B7.2 on B cells has previously been associated with the induction of nonresponsiveness in T cells (Greenfield *et al.*, 1998).

However, the idea that YopH may be involved in the aspects of *L. intracellularis* pathogenesis discussed above is purely speculative and further studies would have to be carried out to confirm any link between expression of YopH by *L. intracellularis* and the immunosuppressive features of PE. Initially expression of YopH would have to be reconfirmed and, in the absence of *L. intracellularis* genome sequence, tandem mass spectrometry carried out to confirm the protein sequence.

7.3.2 Extracellular Lipase Precursor Homologue

Extracellular lipases are produced by a variety of microorganisms: fungi, yeasts, and bacteria, including actinomycetes (Sztajer H. and Maliszewska I, 1994). Lipases are glycerol ester hydrolases that catalyze the hydrolysis of triacylglycerols to free fatty acids and glycerol. They resemble esterases in terms of catalytic activity, but differ in that their substrates are water-insoluble fats containing medium to long fatty acid chains (Brockerhoffer and Jensen, 1974). An extracellular lipase, LipA, from *Actinobacter* sp. RAG-1 has been cloned and sequenced and found to contain several conserved regions common to bacterial lipases (Sullivan *et al.*, 1999).

Interest in lipases has increased recently due to their recognition as important virulence factors. Lipases of *Propionibacterium acnes* and *Staphylococcus epidermidis* may be involved in colonization and persistence of these bacteria on the human skin. Lipases of *S. aureus* and *P. aeruginosa* are produced during the bacterial infection process and, at least *in vitro*; considerably impair the function of different cell types involved in the human immune response like macrophages or platelets. The present state of knowledge suggests to classify the lipases as important bacterial virulence factors which exert their harmful effects in combination with other bacterial enzymes, in particular the phospholipases C (Jaeger K.E. *et al.*, 1994).

Lipase activity has been detected in *L. pneumophila* supernatants but is dependant on the presence of a functional type II secretion system, also required for intracellular infection (Rossier and Cianciotto, 2001, Flieger *et al.*, 2001, Aragon *et al.*, 2001, Aragon *et al.*, 2000). Screening of the *L. pneumophila* genome with the consensus sequence [LIV]-X-[LIVFY]-[LIVMST]-G-[HYMV]-S-X-G-[GSTAC] identified two unlinked ORFs, designated *lipA* and *lipB*, containing both the lipase consensus sequence and a signal sequence. Mutations in *lipA* in particular reduced supernatant activity against mono- and triacylglycerols. However, loss of *lipA* and/or *lipB* did not affect the ability of *L. pneumophila* to infect *Hartmannella amoebae* or U937 cell macrophages (Aragon *et al.*, 2002).

The role of phospholipase C in escape of *L. monocytogenes* from the host cell vacuole has been well documented (Cossart and Lecuit, 1998). However, disruption

of the phospholipase C from *Legionella pneumophila* had no such distinct effect on the intracellular infection (Aragon *et al.*, 2002). It is plausible to assume that lipases, and related phospholipases, indeed play an important role in virulence. In the case of intracellular bacteria, such as *L. pneumophila* and *L. intracellularis*, it is tempting to speculate that these factors have a role to play in the establishment of intracellular infection as in *L. monocytogenes*. However, further studies are clearly required to determine their exact function in virulence of these pathogens. It will be interesting to screen the *L. intracellularis* genome, once sequenced, for lipase and phospholipase homologues.

7.3.3 Immunoblotting of whole cell *L. intracellularis* membrane enriched cell lysate with polyvalent antibody, 4F5

Screening of *L. intracellularis* membrane enriched extract with polyclonal antibody, 4F5, failed to identify the 25kDa surface antigen LsaB, or LsaA. Again, due to the way in which *L. intracellularis* is cultivated, contamination with mammalian proteins is always a possibility and this was indeed found to be a major problem in this case. The difficulties associated with cultivating *L. intracellularis* also limits the amount of material available for analysis and it is possible that virulence factors, such as LsaA, may be produced in amounts below the detection limits of the applied method. The success of the technique may therefore have been improved if enough material had been available to use a larger 1st dimension separation, for example an 18 or 24 cm strip opposed to the 7cm strip employed.

Although 2D-PAGE is a powerful technique in terms of its sensitivity and resolving power it does have limitations. Certain classes of protein are under represented in 2D-PAGE gels, including hydrophobic proteins, those with low copy number and highly basic proteins (Wilkins *et al.*, 1998). Since any cell surface antigens are at least likely to be membrane associated it is possible that this technique may have been biased against such proteins. Although bacterial extracts were prepared in chaotropic conditions which should have enriched for membrane proteins, it is

possible that the relatively low level of material and resolution may have compromised any potential advantage of such a measure.

In recent years several new methods have been developed which are designed to eliminate discrimination against membrane proteins or those present in low abundance. For example, two-dimensional semi-preparative electrophoresis follows a similar protocol to 2D-PAGE, however, in the first dimensional separation proteins are enriched up to 500 times in liquid fractions at their respective isoelectric points. IEF enriched proteins are then separated by SDS-PAGE, eluted from the gel into liquid fractions, digested and analyzed by mass spectrometry. Alternatively, two-dimensional liquid chromatography-tandem mass spectrometry (2D-LC-MS/MS) entirely circumvents the use of gels which can be time consuming. Proteins from an entire proteome or cellular compartment are reduced to peptides then loaded onto a 2D chromatographic system which is coupled to an electrospray ionisation source. The peptides are subjected to MS/MS analysis, and the uninterpreted spectra used to search protein databases in order to retrieve identities (Nilsson, 2002). This technique is referred to as MudPIT (Multidimensional Protein Identification Technology). MudPIT analysis has recently been used to investigate the proteome of the pathogen *Plasmodium falciparum* with the aim of identifying factors expressed throughout different stages of the pathogens lifecycle (Florens *et al.*, 2002). MudPIT technology was also used in later analysis of immunogenicity of several of the ORFs identified to assess their potential as anti-malarial vaccine candidates (Doolan *et al.*, 2003). However, the MudPIT approach precludes the collection of visual data which can be useful for analytical and quantitative purposes. Additionally, MudPIT is incapable of detecting post-translational modification or cleavage events, all of which maybe visible with 2-DGE (Boyce *et al.*, 2004).

Methods for analysis of obligate intracellular bacterial pathogens are limited due to the lack of molecular methods for manipulating these bacteria. However, analysis of protein expression has shown promise for such pathogens including *Lawsonia*. Although problems with cultivating sufficient quantities of material for a more extensive 2-DGE examination of *L. intracellularis* in the future still remain, the

combined resolving power and sensitivity of 2D-LC-MS/MS represents a way forward. Furthermore, once the fully sequenced and annotated *L. intracellularis* genome becomes available the MudPIT approach could also be implemented.

Chapter 8

DISCUSSION AND FURTHER STUDIES

CHAPTER 8

Discussion and Further Studies

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8.1 Further studies and discussion

8.1.1 Future Work

8.1.1.1 Function of LsaA as an adhesin

The findings in this thesis were inconclusive regarding the role of LsaA in adherence of *L. intracellularis* to host cells. Several attempts, using both biochemical and molecular methods, were tried to establish a role for LsaA in adherence to host cells. Due to technical difficulties associated with the production of native protein it was not possible to bring the work to a definitive conclusion. Although the findings in this thesis would point towards an alternative role in pathogenesis for the TlyA family of proteins, an alternative/additional role in adhesion cannot be entirely ruled out at the present for many of the TlyA family.

In the case of *L. intracellularis*, understanding of the molecular interactions occurring between pathogen and host cell are rudimentary at best. The use of proteases such as trypsin and proteinase K were implemented to try and establish a definite role of host cell surface proteins in bacterial adherence. These were also inconclusive due to problems with *L. intracellularis* infectivity, also observed in the control. It would be suggested that the wider characterisation of attachment/adherence to host cells was continued. Further understanding of the nature of host cell molecules involved in bacterial adherence would help in the study since the techniques described in this thesis were limited to receptors of a proteinaceous nature. The exclusive interaction of *L. intracellularis* with proteins on the host cell surface has not been determined.

If the results of such experiments confirmed the involvement of host cell surface protein in bacterial adherence then, in the future, providing production of sufficient quantities of native rLsaA was optimised, previously described techniques, e.g. affinity chromatography, could be repeated in order to try and identify an interacting partner from the host cell surface for LsaA.

Although no homologues from the initmin/invasion family of proteins were found in *L. intracellularis*, other potential adhesins have already been identified. One possible candidate is a GroEL sequence identified by Dale *et al.* (1998). The production of heat shock proteins by several intracellular bacteria, including *Brucella abortus*, *Chlamydia trachomatis*, and *Legionella pneumophila*, is known to correlate with

virulence (Fernandez *et al.*, 1996). However, the role of these proteins in pathogenesis is not fully understood but members of this family have been shown to play the role of adhesins in other bacteria. Hsp60, a member of the GroEL family of chaperonins, is responsible for adherence of cells by *Clostridium difficile* (Heenquin *et al.*, 2001), *Helicobacter pylori* (Yamaguchi *et al.*, 1997), and the intracellular pathogen *Legionella pneumophila* (Garduno *et al.*, 1998b). Hsp60 is displayed on the surface of virulent strains of *Legionella pneumophila* (Garduno *et al.*, 1998a) and surface associated Hsp60 has been shown to play a role in invasion of HeLa cells by this bacteria (Garduno *et al.*, 1998b).

Once the sequence of the *L. intracellularis* genome is released it is likely that other potential adhesins will come to light which may further our understanding of the vital process of attachment/adherence to host cells for this pathogen.

8.1.2.2 Function of the TlyA family as regulators of transcription

Comparison of protein expression profiles from *H. pylori* SS1 and *tlyA* knockout, RS7 highlighted some distinct differences between the wild-type and mutant strains. In particular it was observed that several factors normally involved in *H. pylori* colonisation had been downregulated in the mutant strain, noted for its reduced ability to adhere to AGS cells *in vitro* and to colonise a mouse model of *H. pylori* infection (Martino *et al.*, 2001). Expression of both flagellin B and catalase was reduced in the mutant compared to the wild-type, whereas expression of urease subunits A and B, had been notably up-regulated in RS7. The results suggest that the phenotype of reduced adherence observed in the *tlyA* knockout could be due, not to a direct function of TlyA in adherence, but indirectly via transcriptional regulation of virulence factors including those involved in motility and survival mechanisms.

Such a function had been suggested previously by Hsu *et al* (2001) due to the presence of two functional domains in several members of the TlyA family with homology to the S4 RNA-binding domain and a methyltransferase, FtsJ. Therefore, *in vitro* evidence that at least one member of the TlyA family may function as a transcriptional regulator is an interesting and tantalising result that warrants further investigation.

There has been widespread and detailed analysis of *H. pylori* protein expression using 2D PAGE. It would therefore be possible to gain an even greater insight into the effect of the loss of TlyA on protein expression by focusing the separation across narrower ranges of pI to increase resolution of protein species. It is possible that catalase, urease and flagellin B are not the only factors affected by the loss of TlyA and increased resolution may identify further factors whose expression differs between the two strains.

The discovery that TlyA is capable of regulating host cell protein expression does not hold with its previously assigned cell surface location. For the majority of TlyA family members this was a theoretical placement based on their putative role in attachment/adherence of bacteria to host cells. However, in the case of *L. intracellularis* cell surface location of LsaA has been confirmed. If LsaA is not an adhesion the reason for its cell surface location remains is somewhat unclear. It was however hypothesised that the cell surface location of LsaA would put it in an ideal position to interfere with host cell protein expression, possibly to the benefit of *L. intracellularis* pathogenesis. The hypothesis was supported by previous observations, both *in vivo* and *in vitro*, that *L. intracellularis* is often found in close association with the mitochondria or in a perinuclear distribution. Both positions would place it in proximity of host cell ribosomes, the site of host protein synthesis, and therefore an ideal location from which to regulate mRNA translation and hence protein synthesis.

In vitro expression of a GFP-LsaA homologue in a semi-confluent INT-407 cell line would also support this hypothesis. Following 24 hrs post-transfection the fusion protein was often observed to be distinctly accumulating immediately surrounding the nucleus. Identical fusion protein where LsaA was replaced by TlyA_{HP} showed similar patterns of distribution within the mammalian cell. Whether or not TlyA from *H. pylori* has any effects on host cells *in vivo* would require further investigation. Disparities in protein expression identified between SS1 and RS7 suggest a role in bacterial protein expression for TlyA_{HP}. The localisation could be due to a common mechanism of action, the determinants of which are conserved between prokaryotes and eukaryotes.

In the future, culture of INT407 cells transformed with the pEGFPC1/LsaA construct could be selectively grown in the presence of G418 sulphate. Since the vector carries a neomycin resistance cassette, cells expressing the GFP-LsaA fusion protein will be unaffected. Once sufficient numbers of GFP-LsaA transfected INT407 cells had been cultured, whole cell lysate of transfected cells could be compared to that of an untransfected INT407 control by 2D PAGE, or an alternative proteomic procedure. Using this technique it may be possible to detect the effect, if any, of the presence of LsaA on host cell protein expression.

8.1.3 Immunogenic antigens of *L. intracellularis*

Analysis of *L. intracellularis* protein expression using 2D-PAGE proved to be a successful method of detecting previously unidentified immunogenic antigens. When used in combination with western blotting using sera from *L. intracellularis* infected swine it was possible to identify immunogenic antigens with significant homology to YopH, a well characterised *Yersinia* virulence determinant, and LipA, an extracellular lipase precursor from *Actinobacillus* sp..

Further repetition of the technique is necessary to conclusively confirm the expression of the YopH and LipA homologues by *L. intracellularis*. Once sequence of the *L. intracellularis* genome is released it will be interesting to further characterise homologues. The role of extracellular lipase, LipA, is unclear although it is possible it may play a role in establishing intracellular infection. The function of a YopH homologue in *L. intracellularis* pathogenesis is also untested at present. It is plausible that it plays a role in downregulation of the host immune response. Since molecular techniques are not available with which to manipulate the *L. intracellularis* genome and produce a *yopH* mutant, complementation of the existing *yopH* mutant in *Y. enterocolitica* with the homologue from *L. intracellularis* could be used to assess the role of this factor in virulence. Alternatively, a similar strategy could be used for YopH as for LsaA, whereby the recombinant protein is expressed from a plasmid in eukaryotic cells and the effects characterised. To add weight to the case for transcriptional regulation activities of the TlyA family of proteins it would also be interesting to examine the non-haemolytic *M. smegmatis* transformed with TlyA from *M. tuberculosis* created by Wren *et al.*, 1998. By carrying out 2D-PAGE analysis of

transformed and wild-type strains it would be possible to investigate whether the observed haemolytic phenotype was due to upregulation of haemolytic proteins in *M. smegmatis* opposed to inherent haemolytic properties of the TlyA protein.

This work was the first example of the application of immunoproteomics to the analysis of *L. intracellularis* protein expression. It has shown the potential as a useful tool in the further analysis of this difficult subject. Given the complicated nature of *L. intracellularis* culture, the provision of sufficient starting material is always a bottleneck in analysis of this nature. Indeed, the work in this study was performed using the lowest level of resolution possible in the system, a 7cm 1st dimension strip, and the minimal necessary concentration of protein. Further analysis of the *L. intracellularis* proteome using this technique would most certainly provide insights in the physiological pathogenesis of this unusual organism if higher quantities of starting material became available. It is possible to culture *L. intracellularis* in larger quantities than was practical in the scope of this thesis. In the future, the use of sera obtained from swine vaccinated with the live attenuated vaccine strain of *L. intracellularis* (DK-1790) could also be used to highlight the antigens providing protective immunity for further study. Such an approach yielded successful results when applied to *Mycobacterium tuberculosis* and the vaccine strain *M. bovis* bacillus Calmette-Guerin (BCG). Its application led to the identification of six proteins in *M. tuberculosis* that lacked a counterpart in the vaccine strain. Four of these were soluble enzymes and two were conserved hypothetical proteins (Jungblut *et al.*, 1999).

An identical approach was used to identify immunoreactive antigens from *Francisella tularensis* subsp. *Holarctica*, the common causal agent of tularaemia (Havlasova *et al.*, 2002). More than 80 immunorelevant antigens were detected by screening whole cell lysate, or more selective groups of proteins, from the live vaccine strain of *F. tularensis* with sera collected from patients with tularaemia. Amongst the spots that provided characteristic reactions were predominantly the 60 kDa and 10 kDa chaperonins.

8.2 Conclusions

The work in this thesis described an initial attempt at molecular characterisation of the obligate intracellular pathogen *L. intracellularis*. As with all intracellular bacteria, specific attachment to the host cell membrane precedes cellular invasion and is therefore a critical step in the pathogenesis of these organisms. Identification and characterisation of the *L.intracellularis* surface components responsible for adhesion and cell surface receptor would not only benefit the understanding of mechanisms of bacterial entry to cells, but could also aid in the development of a subunit vaccine against *L.intracellularis* that would target functional surface components.

Information regarding the pathogenesis of *L.intracellularis* is sparse. Only now are the molecular tools being developed to analyse such organisms. Publication of the *L.intracellularis* genome will increase the success of techniques such as immunoproteomics in identifying immunoreactive antigens used by this pathogen in its complex and unique mechanisms of pathogenesis.

Appendix

Appendix I – Plasmids

I.01 pRSRET expression plasmids

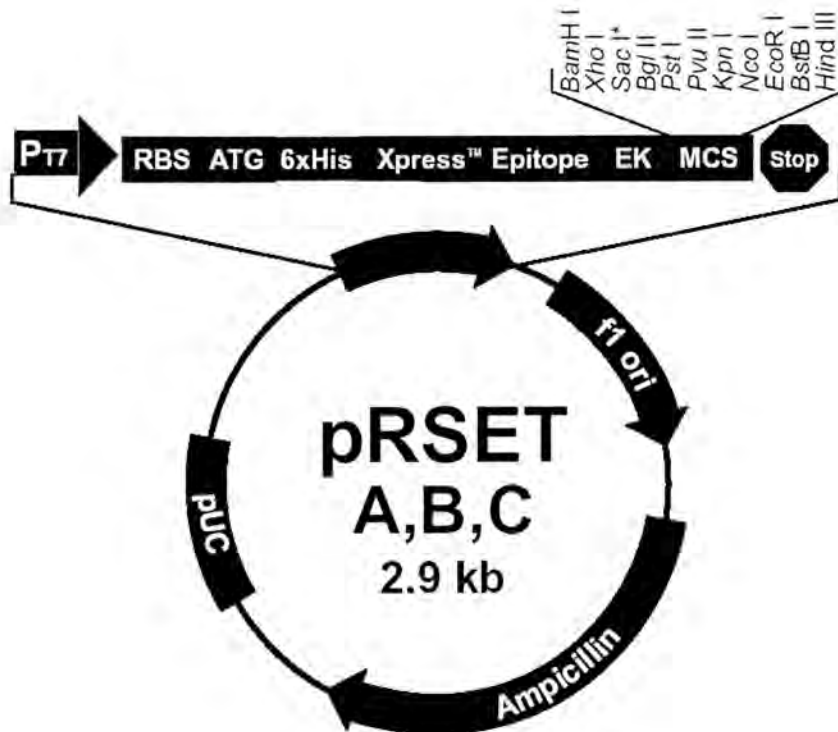


Figure I.01 pRSRET expression vectors PUC = *E. coli* origin of replication (colE1), Ampicillin = β -lactamase ampicillin resistance gene, f1 ori = phage single stranded origin of replication, P_{T7} = T7 promoter, RBS = ribosome binding domain, ATG = transcriptional initiation code, 6xHis = six histidine residue tag, Xpress™ epitope = anti-Xpress™ monoclonal antibody recognition sequence, EK = enterokinase recognition site, MCS = multiple cloning site, Stop = T7 transcription terminator. Adapted from website.

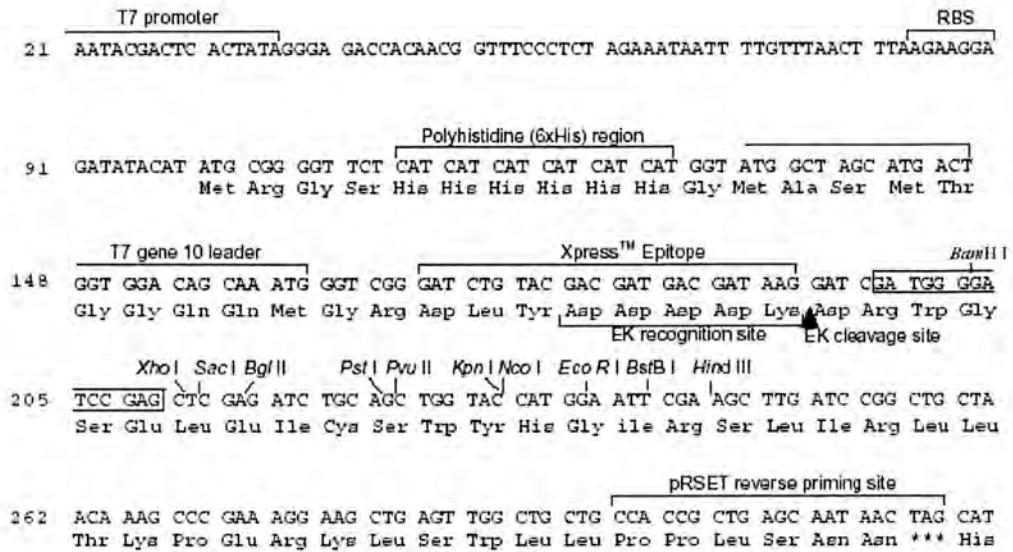
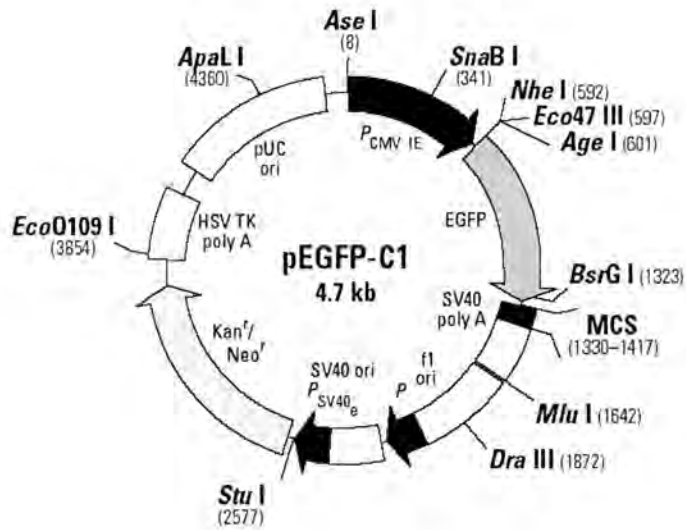


Figure I.02 pRSET-A MCS and variable regions pRSET-A is shown. Adapted from Invitrogen technical data sheets

I.02: pEGFPC1 GFP Expression vector

a)



b)

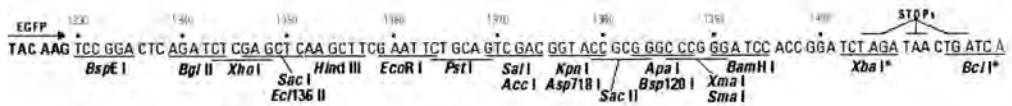


Figure I.03 pEGFPC1 a) vector restriction map. $P_{CMV IE}$ = Human cytomegalovirus (CMV) immediate early promoter, SV40 poly A = SV40 early mRNA polyadenylation signal, f1 ori = f1 single-strand DNA origin, SV40 = SV40 origin of replication, P_{SV40_E} = SV40 early promoter, Kan^r/Neo^r = Kanamycin/neomycin resistance gene, HSV TK = Herpes simplex virus (HSV) thymidine kinase (TK) polyadenylation signal, pUC ori = pUC plasmid replication origin. **b) MCS.** Sequence and restriction map of the MCS in pEGFPC1. Adapted from Clontech website.

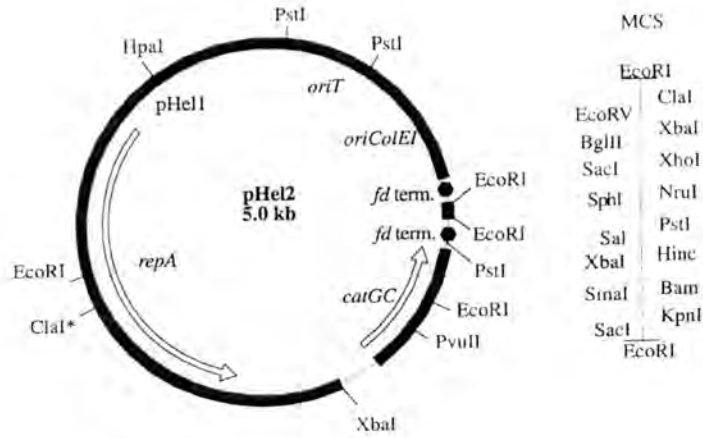
I.03: pHel2 *H.pylori* / *E.coli* Shuttle Vector

Figure I.04: Adapted from Heuermann and Hass (1998). *repA* = initiation of replication, *catGC* = chloramphenicol resistance cassette, *oriT* = origin of transfer from plasmid RP4, *oriCOLE1* = replication of origin of plasmid pBR322, MCS is the multiple cloning site of the vector pIC20R, * = recognition site is modified in *dam*⁺ *E. coli* host cells and is resistant to cleavage by corresponding restriction enzyme.

I.04 pGBKT7 expression vector

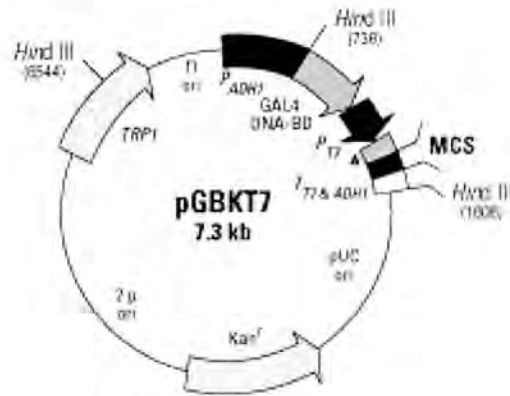


Figure I.05 pGBKT7 vector map. f1 ori = f1 single-strand DNA origin, Kan^r = Kanamycin resistance gene, pUC ori = pUC plasmid replication origin, P_{ADH1} = constitutive ADH1 promoter, GAL4 DNA-BD = GAL4 DNA binding domain, P_{T7} = T7 promoter, T_{T7&ADH1} = T7 and ADH1 transcription termination signals, TRP1 = nutritional selection in yeast, 2μ ori = plasmid replication origin (*S.cerevisiae*).

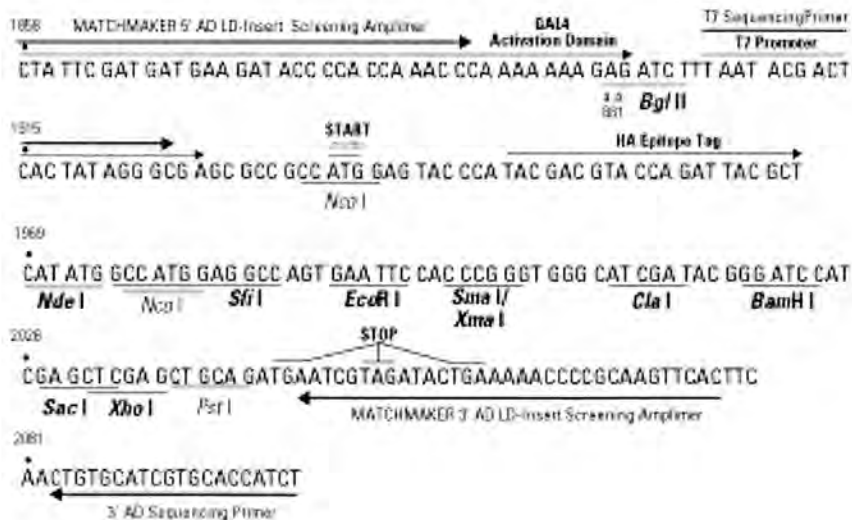


Figure I.06 pGBKT7 MCS. Adapted from Invitrogen technical data sheets

I.05 pGADT7 expression vector

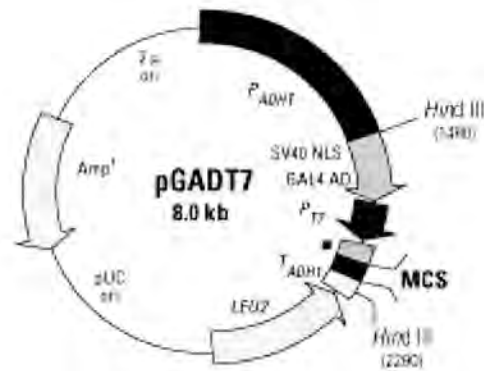


Figure I.07 pGADT7 vector map. f1 ori = f1 single-strand DNA origin, Kan^r = Kanamycin resistance gene, pUC ori = pUC plasmid replication origin (*E.coli*), P_{ADH1} = constitutive ADH1 promoter, GAL4 AD = GAL4 activation domain, P_{T7} = T7 promoter, T_{T7&ADH1} = T7 and ADH1 transcription termination signals, LEU2 = nutritional selection in yeast, 2μ ori = plasmid replication origin (*S.cervisae*).



Figure I.08 pGADT7 MCS. Adapted from Invitrogen technical data sheets

1.06 PCR 2.1-TOPO



Figure I.03 pCR[®]2.1 TOPO cloning vector PUC ori = *E.coli* origin of replication (colE1), Ampicillin = β -lactamase ampicillin resistance gene, Kanamycin = kanamycin resistance gene, f1 ori = phage single stranded origin of replication, P_{lac} = lac promoter, lacZ α = encodes the first 146 amino acids of β -galactosidase. Complementation in *trans* with the Ω fragment gives active β -galactosidase for blue-white screening.

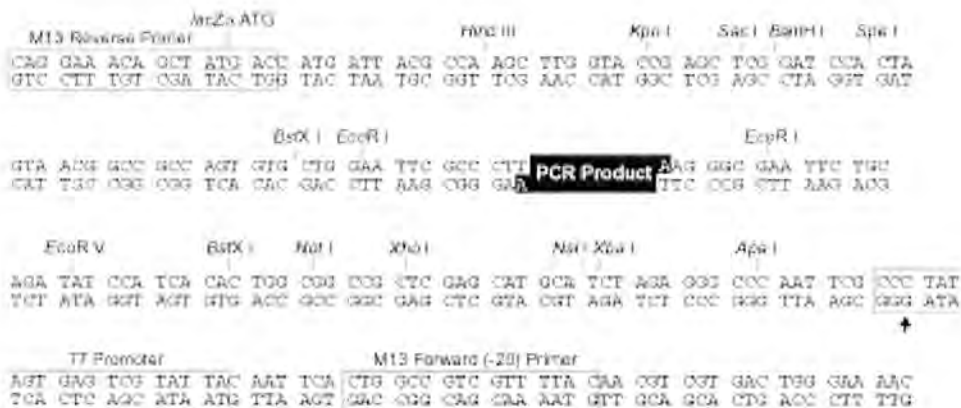


Figure I.04 pCR[®]2.1 TOPO TA cloning site Adapted from Invitrogen technical data sheets

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