2010

Identification and characterisation of immunoreactive antigens of mycoplasma hyopneumoniae

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Identification and Characterisation of Immunoreactive Antigens of *Mycoplasma hyopneumoniae*

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2010

Declaration of Authenticity

This thesis is submitted in accordance with the regulations of the University of Wollongong in fulfillment of the degree of Doctor of Philosophy. It does not include any material previously published by another person except where due reference is made in the text. The experimental work described in this thesis is original, and has not been submitted for a degree to any other university.

Jody Gorman

Acknowledgements

I started this thesis over 12 years ago and two jobs, two kids, two very patient supervisors, an ever-loving family, many supportive friends and one super-supportive, patient and loving husband later, I am FINALLY submitting. That makes for a very long list of acknowledgements!

Firstly I would like to thank Steven Djordjevic and Mark Walker - quite different in personality and supervisory roles but complementing each other perfectly as co-supervisors. Steve's passion for research is inspiring and Mark's rational calm a necessity to maintain focus and sanity during the throes of a PhD. A special thanks to Mark for the continual but gentle pressure to complete.

My family, immediate, extended and that I gained through marriage, you have all been so supportive and so loving and maintained an unwavering belief in me. Some of you probably didn't completely understand what I was doing or why but were supportive anyway. Thank you Mum and Dad for instilling in me a drive to succeed and an understanding of how much work it takes to do so … and of course for being the best parents anyone could ask for. My step-parents also deserve a special mention as they have been there since the early days and have shown endless love and support in their own way. Thank you also to my beautiful in-laws Ron and Ivy who are always there when I need them. I love you all so much and feel lucky to have you in my life.

To all my friends, too few words describe my gratitude for your support. My school friends Vic, Anne, Julie and Mel, you are a stabilising force in my life and have shown

endless support. My work friends Tam, Sue, Tracey, Alison, Linda and Kat, you believed in me and gave me just the right amount of encouragement and support. I'd also like to thank my long-time friends Kym, Sue, and Anna who I can't imagine life without, as well as my new friends (the mums' of my kids' friends) Alison, Jen and Rachel – I owe all of you hours of babysitting.

Lastly and very importantly I would like to thank my husband Mitch. You have been there since the beginning of it all. You have shown unwavering love and support and have hid any (warranted) frustrations you felt during my many attempts to finish – before my scholarship ran out, after my scholarship ran out, before my first "real" job started, in the month before Harrison was born and again before Ava, this time last year when I finally made some headway and again now. I know I turned out to be much more work than you had expected from the girl in the white uniform behind the counter at Bush's Meats, but I think I'm worth it! I love you and I thank you. We are a team in every endeavour.

A very big thank you to those mentioned and everybody else, who loved, supported, encouraged and helped me get to this point. I don't know what life without the incredible weight of an unfinished thesis is going to feel like but I suspect it will be pretty good!

I dedicate this thesis to my children Harrison and Ava.

Be everything you can be my beautiful babies!

List of Publications

Wilton, JL., Scarman, AL., Walker, MJ., and Djordjevic, SP. (1998) Reiterated repeat region variability in the ciliary adhesin gene of *Mycoplasma hyopneumoniae*. Microbiology. **144:**1931-43.

Matic, JN., **Wilton**, JL., Towers, RJ., Scarman, AL., Minion, CF., Walker, MJ., and Djordjevic, SP. (2003) The pyruvate dehydrogenase complex of *Mycoplasma hyopneumoniae* contains a novel lipoyl domain arrangement. Gene. **319:**99-106.

Djordjevic, SP., Cordwell, SJ., Djordjevic, MA., **Wilton**, JL., and Minion, FC. (2004) Proteolytic Processing of the *Mycoplasma hyopneumoniae* Cilium Adhesin. Infect Immun. **72:**2791-802.

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Wilton, JL., Jenkins, C., Cordwell, SJ., Falconer, L., Minion, CF., Oneal, DC., Djordjevic, MA., Connolly, A., Barchia, I., Walker, MJ., and Djordjevic, SP. (2009) Mhp493 (P216) is a proteolytically processed, cilium and heparin binding protein of *Mycoplasma hyopneumoniae*. Mol Microbiol. **71:**566-582.

Jenkins, C., Seymour, L., Deutscher, A., Falconer, L., Stewart, K., **Wilton**, JL., Minion, FC., Rhode, M., Eamens, G., Redacliffe, L., Walker, MJ., and Djordjevic, SP. (submitted) *Mycoplasma hyopneumoniae* triggers the deposition of fibronectin at the site of colonisation.

Abstract

M. hyopneumoniae is the causative agent of porcine enzootic pneumonia (PEP) and inflicts a severe economic burden on swine production. Colonisation involves adherence of *M. hyopneumoniae* to the porcine respiratory epithelia in a process believed to be mediated by surface proteins capable of binding target cells. This thesis involved the identification and characterisation of several immunoreactive *M. hyopneumoniae* antigens in an effort to increase the current understanding of the surface topography of this organism.

The first antigen identified in this research was the now well-characterised cilium adhesin of *M. hyopneumoniae*, P97. P97 was found in earlier research to contain two regions of reiterated repeats (RR1 and RR2). The repeats were found in this research to differ in number amongst strains of *M. hyopneumoniae* from different geographical localities and did not appear present in related porcine mycoplasmas *M. hyorhinis* or *M. flocculare*. Sequences like the proline rich tandem repeats of 5 and 10 amino acids found in RR1 and RR2 respectively are typically involved in protein-protein interactions. Proline rich repeats are usually highly immunogenic (Williamson, 1994) providing the recombinant 35 kDa antigen (encompassing the repeats) produced in this work with vaccine potential.

The second antigen cloned and characterised in this research was identified as a fragment of *M. hyopneumoniae* pyruvate dehydrogenase E1α (PdhA). Its intracellular location was indicated in trypsination studies of whole *M. hyopneumoniae* but its

identification in the insoluble phase as well as the aqueous phase of triton extractions raises the question of a dual function; a phenomenon reported for PdhA in *M. pneumoniae* (Layh-Scmitt *et al*., 2000) and for NrdF in *M. hyopneumonia* (Fagan *et al*., 1996).

The final antigen characterised in this research was identified as a 216 kDa adhesin-like protein of *M. hyopneumoniae* (P216). Four recombinant P216 antigens, covering 75% of the molecule, were cloned, expressed, purified and used to raise antisera in rabbits. P216 was found to be rapidly processed to cleavage fragments P120 and P85, both located on the surface of *M. hyopneumoniae*. A putative transmembrane domain was identified in P120 providing it with a mechanism for cell surface attachment. It has been hypothesized that P85 maintains its association with the cell surface through a KEKE motif and the two regions of glutamine repeats identified in its' sequence. Heparin binding of all three recombinant P216 fragments suggests that P120 contains at least two heparin-binding domains and P85 at least one, but further studies are required to define the exact location of these sites.

This research provided a foundation for continued research on P97 and P216, which remain the only two cilium adhesins of *M. hyopneumoniae* identified to date. Defining the colonisation mechanism of *M. hyopneumoniae* has implications for the treatment and prevention of disease, therefore research in this area provides an improved understanding of pathogenesis and potential vaccine development.

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List of Abbreviations

ABTS	2,2'-azino-bis(3-ethylebenzthiazoline-6-sulfonic acid) diammonium salt
ANGIS	Australian National Genomic Information Service
ATCC	American Type Culture Collection
bp	base pairs
CSPD	disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan}-4-yl)phenylphosphate
COG	clusters of orthologous groups
DAB	diaminobenzidine
DIG	digoxigenin
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
^{o}C	degrees celsius
2D	two-dimensional
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FCS	foetal calf serum
g	gram
g	g forces for centrifugation
GAGs	glycosaminoglycans
h	hour
IFN-γ	interferon gamma
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilo daltons
L	litre
LB	Luria Bertani
m	milli
M	molar
mA	milliamps
MALDI-TOF	matrix-assisted laser desorption ionization-time-of-flight
μ	micro
min	minute
mL	milliliter
MS	mass spectrometry
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
n	nano
NCBI	National Centre for Biotechnology Information
NrdF	R2 sub-unit of ribonucleotide reductase
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PBST	phosphate buffered saline with tween 20
PCR	polymerase chain reaction
Pdh	pyruvate dehydrogenase

PEP	porcine enzootic pneumonia
pH	picohenry
PRDC	porcine respiratory disease complex
PRR's	proline rich repeats
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
RR1	repeat region 1
RR2	repeat region 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
RT	room temperature
sec	second
TBE	tris-borate EDTA
TBS	tris buffered saline
TCA	tricarboxylic acid
TX-100	triton X-100
TX-114	triton X-114
V	volts
v/v	volume per volume
w/v	weight per volume

Chapter One

Mycoplasma hyopneumoniae

as the causative agent of

Porcine Enzootic Pneumonia

1.1. The Mycoplasmas

1.1.1. As a Genus of the Class Mollicutes

The Mollicutes are a single class in the Tenericutes division of the Prokaryote kingdom and consists of four orders, five families, and eight genera (Figure 1). They are a nutritionally fastidious group of microorganisms characterised by a small cell size, the lack of a rigid cell wall, simplified metabolic pathways and a small genome size with a low guanine (G) and cytosine (C) content (23-40 mol%) (Razin *et al*., 1998; Sirand-Pugnet *et al*., 2007). The Mollicutes share an intimate association with humans, animals, arthropods, insects, and plants as commensals, parasites or pathogens, and rely on their host for a vast array of nutrients as well as a constant environment (Miles, 1992). This association and host reliance is not surprising considering the Mollicutes possess the smallest genomes of any independently viable bacteria known (Razin, 1992; Halbedel and Stulke, 2007).

Mycoplasmas are by far the largest Genus of the Mollicute class and are the most thoroughly described. The mycoplasmas as a group are genetically diverse (Ferrell *et al*., 1989; Sirand-Pugnet *et al*., 2007). They are believed to be the products of reductive evolution of morphologically, biochemically, and genetically more complex eubacterial forms (Williams, 1983; Carson *et al*., 1992; Cleavinger *et al*., 1995; Sirand-Pugnet *et al*., 2007), specifically the *Bacillus-Lactobacillus-Streptococcus* subgroup of sporeforming bacteria, from which the mycoplasmas are believed to be derived (Carson *et al*., 1992). Loss of cell wall genes as well as simplified metabolic and regulatory

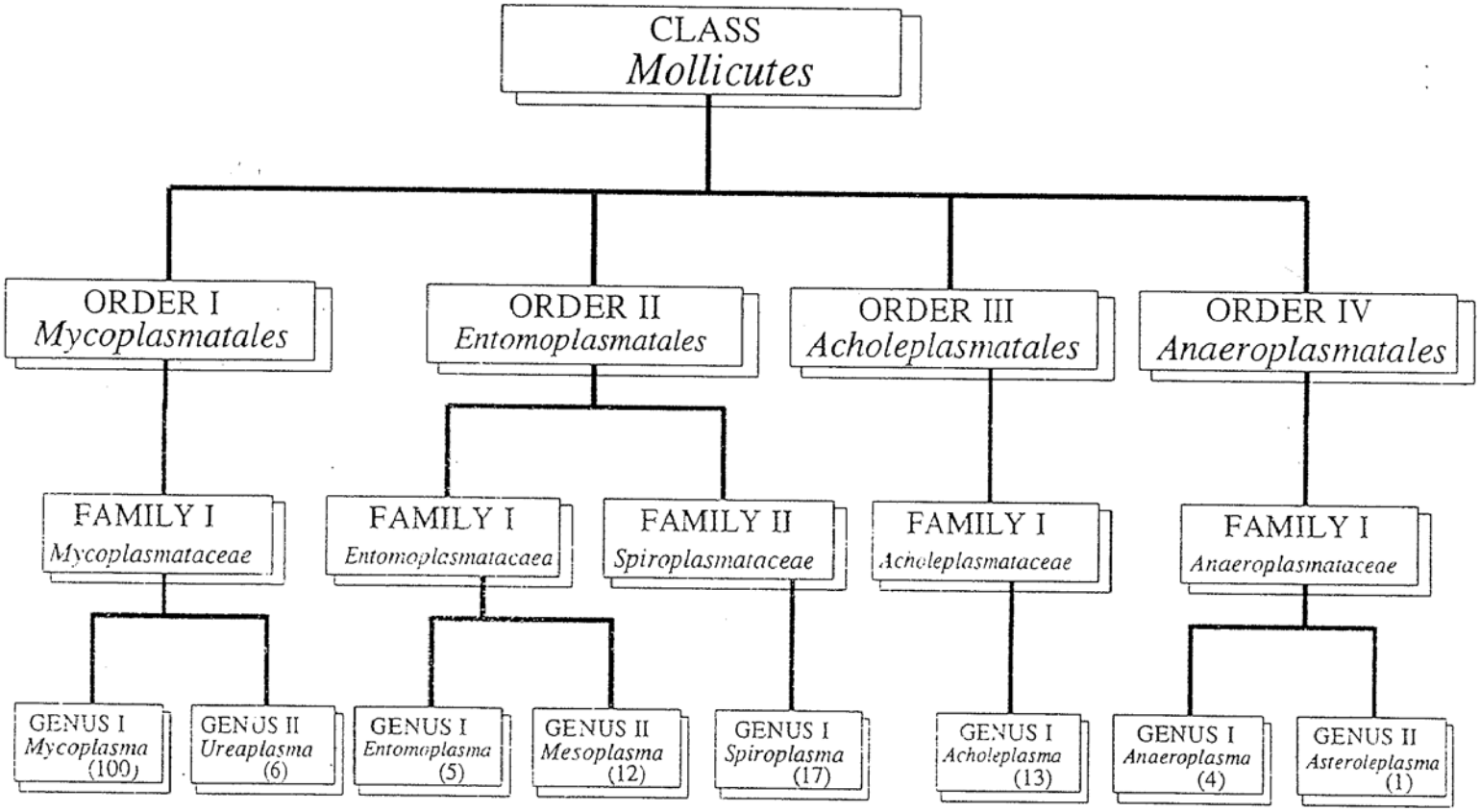


Figure 1. The taxonomic relationship within the class mollicutes. Numbers in brackets represent the current number of recognised species within each Genus (Adapted from Maniloff *et al.*, 1992)

pathways is the primary outcome of the reduction in genome size that accompanied their evolution (Sirand-Pugnet *et al*., 2007; Halbedel and Stulke, 2007). The main driving force for condensation of the genome from their eubacterial ancestors seems to be their parasitic way of life (Bork *et al*., 1995) which may account for the deletion of biosynthetic pathways. Without a cell wall, however, they are bound only by a plasma membrane (Razin, 1985), a distinctive feature the reason for which remains puzzling (Sirand-Pugnet *et al*., 2007).

Parasitism, when of a nutritionally exacting nature, as it is with the mycoplasmas, necessitates host and tissue specificity, as observed with the known mycoplasma species (Razin *et al*., 1998). Their limited biosynthetic capabilities and reliance on the host for the supply of many nutrients makes them difficult to culture in the laboratory. Their dependence upon the host for a wide spectrum of nutrients must be facilitated by proteins spanning their single limiting membrane involved in host cell attachment and nutrient transport (Cleavinger *et al*., 1995). The identification and characterisation of such proteins as well as an enhanced knowledge of the structure, organisation and function of the mycoplasma genome is critical in the determination of the survival and pathogenic strategies employed by the approximately 111 identified mycoplasma species (Minion *et al*., 2004).

1.1.2. Morphology and Ultrastructure

Being bound only by a plasma membrane, mycoplasmas are predominantly spherical in shape but may also be pear-shaped, flask-shaped with terminal tip structures or filamentous (Razin *et al*., 1998). The ability to maintain shape when lacking a rigid cell

wall suggests the presence of an internal membrane-bound cytoskeleton in mycoplasmas. To date there has been no significant homology reported between eukaryotic cytoskeletal proteins and bacterial proteins, but it is believed that interconnecting protein networks in wall-less bacteria are important for various cellular functions, including the maintenance of cell integrity, the asymmetric distribution of membrane proteins, colonisation of the host, gliding motility and cell division (Razin *et al*., 1998; Balish and Krause, 2006).

Evidence for the presence of a cytoskeleton in mycoplasmas has come largely from experiments on *Mycoplasma pneumoniae*. *M. pneumoniae* is a flask-shaped mycoplasma with a protruding tip-structure that functions as an attachment organelle. Meng and Pfister (1980) performed experiments that involved the removal of the cell membrane of *M. pneumoniae* and analysis of the "Triton-shell". In doing so they revealed a number of cytoskeleton-associated proteins that have since been characterised for their role in cell shape, cell division, cytadherence, motility, and the localisation of adhesins (review by Balish and Krause 2002; Balish and Krause, 2006). Although there has been a significant amount of research on the cytoskeleton of *M. pneumoniae*, only minimal research has been conducted on the structure of the cytoskeleton in other mycoplasmas.

Orthologs of the majority of the cytoskeleton-associated proteins of *M. pneumoniae* have been identified in the closely related *M. genitalium* (Herrmann *et al*., 1998) but significant differences have also been noted (Balish and Krause, 2006). Proteins with homology to the cytadherence associated proteins of *M. pneumoniae* have been identified in *M. gallisepticu*m (Papazisi *et al*., 2003) and *M. penetrans* (Sasaki *et al*.,

2002) but many show substantial divergence. Interestingly, some of these proteins were proline rich and exhibited repeat sequences and other motifs characteristic of cytoskeletal proteins in eukaryotes (Proft *et al*., 1995; Proft *et al*., 1996). Another interesting feature of some of the identified proteins was their ability to be phosphorylated (Dirsken *et al*., 1994). Protein phosphorylation is a mechanism used frequently by eukaryotes for regulating intracellular signalling.

1.1.3. Genome Structure and Organisation

The numerous species of mycoplasma are genetically very diverse and although they have features in common there is no consistent genetic organisation (Sirand-Pugnet *et al*., 2007). Genetic diversity of this nature may be attributed to a deficiency in DNA repair systems and a high level of DNA recombination (Dybvig, 1990; Razin *et al*., 1998; Minion *et al*., 2004). Mycoplasmas are therefore generally considered to be in a state of rapid evolution brought about by high mutation rates (Woese, 1987; Dybvig, 1990) as well as the more recent evidence suggesting chromosomal rearrangements and possibly gene transfer or acquisition (Sirand-Pugnet *et al*., 2007). The presence of insertion sequences, transposons and endogenous plasmids capable of contributing to the observed rate of evolution have been reported in a number of mycoplasma genomes (Sasaki *et al*., 2002; Papazisi *et al*., 2003; Westberg *et al*., 2004; Sirand-Pugnet *et al*., 2007a).

Mycoplasma genomes range in size from 580 kb (*M. genitalium*; Fraser *et al*., 1995) to 1350 kb (*M. penetrans*; Sasaki *et al*., 2002) with the majority of those completely sequenced being less than 1000 kb. In addition to being small, mycoplasma genomes

are also characterised by a low G+C content in the range 23 to 40 mol% (Sirand-Pugnet *et al*., 2007). The G+C distribution along the genome is uneven with many non-coding regions having a higher A+T content than the coding regions (sometimes reaching values as high as 90 mol%) and in contrast, regions encoding variable surface proteins are frequently G+C rich (Dybvig and Voelker, 1996; Muto *et al*., 1998; Sirand-Pugnet *et al*., 2007).

The A-T biased base composition of mycoplasmas has resulted in unique codon usage, one that favours the use of codons rich in A+T, particularly in the wobble (3') position (Muto and Osawa, 1987). As a consequence, mycoplasmal proteins have fewer glycine, proline, alanine and arginine residues than other bacterial proteins. Another outcome of the A+T bias in mycoplasmas is the reassignment of the UGA codon from a stop signal to a tryptophan residue (Samuelsson and Boren, 1992), thus limiting successful expression of full-length gene products in *E. coli*.

Little is known about transcription and translation initiation signals in mycoplasmas. What is known suggests that they only partially resemble the consensus sequences of their eubacterial counterparts (Razin *et al*., 1998; Musatovova *et al*., 2003). The mycoplasmal –10 region (Pribnow box) shows a greater homology with the consensus sequence than the –35 region if present (Markham *et al*., 1994; Weiner *et al*., 2000; Musatovova *et al*., 2003). A consensus –10 sequence has been identified in a number of mycoplasma species with a study of 32 genes in *M. pneumoniae* defining a conserved sequence in this region (Weiner *et al*., 2000) which was also identified in a more recent study of a number of *M. genitalium* genes (Musatovova *et al*., 2003). Conversely there has been no strong consensus sequence identified in the –35 region of the Mycoplasma

species studied which includes *M. pneumoniae* (Waldo *et al*., 1999; Weiner *et al*., 2000), *M. genitalium* (Musatovova *et al*., 2003), *M. hyopneumoniae* (Taschke *et al*., 1987; Futo *et al*., 1995), *M. capricolum* (Taschke *et al*., 1988) and *M. gallisepticum* (Markham *et al*., 1994). The region upstream of the -10 region has been reported to consist mainly of an A+T rich region (Christiansen *et al*., 1994; Futo *et al*., 1995) with the more recent suggestion that the -35 region may not be a necessity for RNA polymerase binding in mycoplasmas (Weiner *et al*., 2000; Musatovova *et al*., 2003). In some cases, a Shine-Dalgarno like sequence is identified upstream from the initiation codon (Cheng *et al*., 1996; Christiansen *et al*., 1994; Shibata *et al*., 1995; Yogev *et al*., 1991; Weiner *et al*., 2000) and although the sequences varied they always contained primarily A and G bases. In many cases, no recognisable transcription or translation initiation signals could be identified. The translation initiation codons for mycoplasmas are ATG or GTG but ATG is much more common. The termination codons are TAA or TAG but TAA is most common (Markham *et al*., 1994; Yogev *et al*., 1991; Sirand-Pugnet *et al*., 2007a).

1.1.3.1. Comparative Genomics

Because of their small genome size, mycoplasmas were once believed to be the simplest of organisms, but recent genome sequencing projects have indicated a degree of genomic complexity not seen in other living systems and a suggestion that they may in fact be able to adapt to new environments or hosts (Sirand-Pugnet *et al*., 2007). The genomes of *M. genitalium* (Fraser *et al*., 1995), *M. capricolum* (Bork *et al*., 1995), *M. pneumoniae* (Himmelreich *et al*., 1996; Dandekar *et al*., 2000), *M. pulmonis* (Chambaud *et al*., 2001), *M. penetrans* (Sasaki *et al*., 2002), *M. gallisepticum* (Papazisi *et al*., 2003),

M. synoviae (Vasconcelos *et al*., 2005), *M. hyopneumoniae* (Minion *et al*., 2004; Vasconcelos *et al*., 2005), *M. mobile* (Jaffe *et al*., 2006), *M. mycoides* (Westberg *et al*., 2006), *M. agalactiae* (Sirand-Pugnet *et al*., 2007), *M. arthritidis* (Dybvig *et al*., 2008) and the closely related *Ureaplasma urealyticum* (Glass *et al*., 2000) have been sequenced and characterised. Genetic data in such volume allows comparative genomics, providing extensive data on the structure and organisation of these mycoplasma species.

Comparative genomics of mycoplasmas is potentially difficult because of the rapid rate of evolution which can result in significant sequence divergence. Sirand-Pugnet and colleagues (2007) conducted a comparison between the 17 sequenced mollicutes (of which mycoplasmas make up the majority) and of a total of 729 clusters of orthologous groups (COG) of proteins identified, only 168 were found in all Mollicute genomes. A similar study by Souza *et al*. (2007) comparing twelve mycoplasma genomes (including *U. urealyticum*) presented a similar result, identifying 210 genes shared by the twelve genomes with most having a known function. Genes making up the greatest proportion categorically include 56% that belong to the information, storage and processing division (ie. replication, transcription, translation and associated processes), 21% related to metabolism and 11% to cellular processes. Together these data suggest that other than a limited core genome there is substantial diversity of COG's limited to a single or only a few species. An exception to this generalisation lies within the genomes of *M. genitalium* and *M. pneumoniae*, two closely related species. All of the 479 open reading frames identified in the *M. genitalium* genome were found within the *M. pneumoniae* genome, with conserved genetic organisation and transcriptional direction (Herrmann and Reiner, 1998).

Interestingly, the number of genes for which function remains undefined and the number that seem to be unique to each species are indicated in a number of mycoplasma genomic sequencing papers. In *M. hyopneumoniae* for example those numbers are 38% and 18% respectively (Minion *et al*., 2004) and similarly in *M. gallisepticum* approximately 33% have unknown function and 17% are unique (Papazisi *et al*., 2003). In *M. penetrans*, which reportedly has the largest genome sequence to date, 45% of genes were indicated as specific to that species (Sasaki *et al*., 2002). It is these species specific genes that are frequently the target of functional elucidation in an effort to prevent, treat and generally better understand the various diseases caused by this unique group of bacteria.

Also, of importance to this thesis, is the number of membrane proteins identified in these species. It has been estimated that the *M. genitalium* genome encodes somewhere in the vicinity of 140 membrane proteins (Fraser *et al*., 1995). For *M. pneumoniae*, 275 predicted gene products carry a transmembrane domain, which is equivalent to almost 50% of cell proteins being of membrane origin (Himmelreich *et al*., 1996). Motif analysis as a predictor of membrane-associated proteins identified 149 proteins from a total of 742 (20%) in *M. gallisepticum* with an additional 10% putative lipoproteins (Papazisi *et al*., 2003). Lipoproteins are a major component of mycoplasma membranes (Razin *et al*., 1998).

1.1.4. Membrane Proteins

Lacking a cell wall, mycoplasmas interact with their surrounding host environment by means of the plasma membrane, effectively mediating all necessary transport, sensory

and physical interactions. Genome sequencing projects have indicated that 30-50% of coding regions in mycoplasmas contain signal sequences and transmembrane domains indicative of membrane proteins (Fraser *et al*., 1995; Himmelreich *et al*., 1996; Papazisi *et al*., 2003). These membrane proteins may have an integral (strong) or peripheral (weak) association with the cell membrane. Proteins only peripherally associated with the cell membrane can be dissociated under mild non-denaturing conditions, such as the addition of EDTA or a change in pH or ionic strength. Integral membrane proteins on the other hand, can only be dissociated from the membrane under strong denaturing conditions or with detergents. Triton X-100 (TX-100) and Triton X-114 (TX-114) are two detergents commonly used to solubilise and extract integral membrane proteins from mycoplasma species (Kim *et al*., 1990; Riethman *et al*., 1987; Wise and Kim, 1987; Kahane *et al*., 1985).

Their minimal coding capacity once led to the suggestion that the majority of mycoplasma genes encoded housekeeping proteins and therefore they had a limited ability to respond to environmental or metabolic change (Wieslander *et al*., 1992). However, mycoplasmas excel as pathogens (Wise, 1993) and membrane associated proteins have long been implicated in essential roles in pathogenesis, including adherence (Klinkert *et al*., 1985) and evasion of the host immune system (Wise, 1993), as well as transport of essential nutrients across the membrane (Cirillo, 1979). For these reasons, it is often membrane-associated proteins that are targeted for scientific characterisation.

1.1.4.1. Mycoplasmal Adhesins

As parasites, mycoplasmas rarely invade host tissues; instead they cause disease by adhering securely to the epithelial cells of the host, most commonly in the respiratory and urogenital tracts. Adherence and subsequent colonisation is a prerequisite for successful infection. Several experiments involving the mutation of proteins involved in adherence, has demonstrated a loss of infectivity with the loss of cytadhering ability (Krause *et al*., 1982; Zhang and Wise, 1997; Dhandayuthapani *et al*., 1999; Fisseha *et al*., 1999; Minion *et al*., 2000; Bird *et al*., 2008). A myriad of surface proteins with proposed roles in adherence have been identified in mycoplasmas (Table 1.1).

Table 1.1. Surface proteins observed in a number of mycoplasma species.

Organism	**Protein/ Family**	**Lipid-modified**	**Associated Function**	**References**
M. agalactiae	Vpma	Yes	Unknown	Solsona *et al*., 1996; Bergonier *et al*., 1996; Tola *et al*., 1997; Glew *et al*., 2000; Glew *et al*., 2002 ; Chopra-Dewasthaly *et al*., 2008
M. arthritidis	MAA (MSA)	Yes	Cytadhesin and virulence	Washburn *et al*., 1998; Washburn *et al*., 2000 ; Dybvig *et al*., 2008
	Msp	No		Dybvig *et al*., 2008
M. bovis	Vsp	Yes	Adhesin, escaping host Ab inhibitory activity	Poumarat *et al*., 1994; Yogev *et al*., 1994; Behrens *et al*., 1994; Lysnyansky *et al*., 1996; Poumarat *et al*., 1999 ; Lysnyansky *et al*., 2001 ; Nussbaum *et al*., 2002
	PMB67	No	Immuno-dominant	Behrens *et al*., 1996
M. capricolum	Vmc	Yes	Unknown	Wise *et al*., 2006
M. fermentans	P29	Yes	Unknown	Theiss *et al*., 1993; Theiss *et al*., 1996; Leigh and Wise, 2002

Organism	Protein/ Family	Lipid-modified	Associated Function	References
M. gallisepticum	VlhA (PMGA)	Yes	Adhesin, haemagglutinin, escaping host antibodies	Yogev *et al*., 1994; Baseggio *et al*., 1996; Glew *et al*., 1998; Liu *et al*., 2001
	PvpA	Yes	Adhesin	Boguslavsky *et al*., 2000
	GapA	No	Adhesin	Winner *et al*., 2003
M. genitalium	MgPa (+ proteins MgPb and MgPc)	No	Adhesin	Fraser *et al*., 1995; Peterson *et al*., 1995; Iverson-Cabral *et al*., 2006; Ma *et al*., 2007
M. hominis	P120/P120'	Yes/No	Unknown	Christiansen *et al*., 1994; Ladefoged and Christiansen, 1998; Zhang and Wise 2001
	Vaa (P50)	Yes	Adhesin	Zhang and Wise, 1996; Henrich *et al*., 1996; Zhang and Wise, 1997; Henrich *et al*., 1998; Kitzerow *et al*., 1999; Boesen *et al*., 2001
M. hyopneumoniae	P94	No	Adhesin	Zhang *et al*., 1995; King *et al*., 1997; Hsu and Minion, 1997; Wilton *et al*., 1998; Minion *et al*., 2000
M. hyorhinis	Vlp	Yes	Escaping host Ab inhibitory activity	Rosengarten *et al*., 1991; Yogev *et al*., 1993; Rosengarten *et al*., 1993; Yogev *et al*., 1995; Citti *et al*., 2000
M. imitans	VlhA	Yes	Likely adhesin, haemagglutinin	Markham *et al*., 1999
M. mobile	Msvp	Some	Unknown	Jaffe *et al*., 2004
M. mycoides subsp. mycoides SC	Vmm	Yes	Unknown	Persson *et al*., 2002
M. penetrans	Mlp (P35)	Yes	Unknown	Neyrolles *et al*., 1999; Roske *et al*., 2001; Horino *et al*., 2003
M. pneumoniae	P1	No	Cytadhesin	Himmelreich *et al*., 1996 ; Kenri *et al*., 1999
M. pulmonis	Vsa	Yes	Adhesin	Bhugra *et al*., 1992; Bhugra *et al*., 1995; Simmons *et al*., 1996 ; Shen *et al*., 2000; Denison *et al*., 2005
M. synoviae	VlhA	Yes	Haemagglutinin, adhesin	Noormohammadi *et al*., 1997; Noormohammadi *et al*., 2000

The most well defined mycoplasmal adhesins are those of *M. pneumoniae* and *M. genitalium*. Both are flask-shaped human pathogens that use a tip-like structure to adhere to host cells, and both have had an array of identified proteins associated with adherence. The primary adhesin of *M. pneumoniae* is P1 (170 kDa), and its counterpart in *M. genitalium* is a 160 kDa protein known as MgPa. Layh-Schmitt *et al*. (2000), outlines a complex of membrane and cytoskeleton-like proteins, in addition to the P1 adhesin, that are required for adherence of *M. pneumoniae* to host epithelial cells. All the accessory proteins required for correct tip-structure formation contained proline rich repeats which have been implicated in protein-protein interactions. There were also other proteins identified in association with this adhesin (Balish and Krause, 2006), the majority of which are cytoskeleton-associated suggesting a functional interaction between membrane and cytoskeletal proteins.

Other adhesins, with reduced homology to the above-mentioned proteins, have also been identified. All seem to share the common feature of repeated sequences. In contrast to *M. pneumoniae* and *M. genitalium*, *M. hominis* is a human pathogen that does not use an attachment organelle to adhere to its host. It possesses a surface lipoprotein, P50, exhibiting repeating motifs (the *vaa* gene products) that has been designated as an adhesin (Zhang and Wise, 1996; Henrich *et al*., 1996). Phase variation in *vaa* gene expression in this adhesin has also been correlated with the ability of *M. hominis* to adhere to cultured cells (Zhang and Wise, 1997). Interestingly, Vaa exhibits coiled-coiled regions (Boesen *et al*., 2001) as does the abundant P29 surface protein of *M. fermentans* which is also a lipoprotein largely responsible for adherence (Leigh and Wise, 2002).

Multiple adhesin molecules of *M. hyopneumoniae* have been identified that are not lipoproteins. P97 is a well-characterised surface protein of *M. hyopneumoniae*. Cloning and characterisation of the P97 adhesin gene by Hsu *et al*. (1997) revealed an open reading frame coding for a 125 kDa protein with a hydrophobic transmembrane spanning domain. N-terminal sequence data from purified P97 mapped to amino acid position 195 of the translated ORF sequence suggesting that P97 is formed as a cleavage product from the 125 kDa precursor protein. Proteolytic processing of the cilium adhesin was investigated further by Djordjevic *et al*. (2004) to address the perplexing multiple immunoblot banding pattern of whole-cell antigen seen in earlier research (Zhang *et al*., 1995). In addition to the major cleavage event at amino acid position 195 there are a host of cleavage events yielding peptides of various sizes.

The role of the adhesin was confirmed by demonstration of its specific binding to cilia, and the successful inhibition of binding by heparin and fucoidan (Hsu *et al*., 1997). Two regions of repeats were identified in the carboxy-terminus of the protein (the first a 5 amino acid repeat and the second a 10 amino acid repeat) that showed no homology to repeats of other mycoplasma species. It was later shown that that the number of repeats varied among geographically diverse strains of *M. hyopneumoniae* (Wilton *et al*., 1998), that a minimum of eight 5 amino acid repeats are required for cilium binding (Minion *et al*., 2000) and that both regions of repeats bind heparin in a dose-dependant, saturable manner (Jenkins *et al*., 2006). The significance of these collective findings relates directly to the adherence of *M. hyopneumoniae* to host tissue and subsequently pathogenesis of the organism. The number of repeats in repeat region 1, has been shown to determine adherence to porcine epithelial cells in vitro (Minion *et al*., 2000). A second surface protein of *M. hyopneumoniae* with a proposed role in adherence was

identified more recently by Burnett *et al*. (2006). P159 is a proteolytically processed, heparin-binding protein of *M. hyopneumoniae* shown to promote adherence to eukaryote cells *in vitro*. A 146 kDa protein with adhesive potential was also reported by Stakenborg *et al*. (2006).

1.2. *Mycoplasma hyopneumoniae*

M. hyopneumoniae is the causative agent of porcine enzootic pneumonia (PEP) and was discovered over 30 years ago (Williams, 1983). It is found worldwide and costs producers hundreds of millions of dollars each year (Minion, 2002). *M. hyopneumoniae* primarily colonises the respiratory epithelium of swine but has also been re-isolated from the brain (Friis, 1974), kidney, lymph nodes (Le Carrou *et al*., 2006), liver and spleen (Marois *et al*., 2007). It colonises the respiratory tract without invasion to produce a chronic pneumonia by intimately associating itself with the cilia of epithelial cells of the trachea, bronchi, and bronchioles resulting in extensive loss of cilia (Zhang *et al*., 1994; Zhang *et al*., 1995). Toxins have been reported for *M. hyopneumoniae* (Geary *et al*., 1984; Geary *et al*., 1985) but the exact mechanism by which *M. hyopneumoniae* damages ciliated cells remains poorly understood.

Alone, *M. hyopneumoniae* is generally non-fatal to swine but secondary bacterial infection by pathogens such as *Actinobacillus pleuropneumoniae* and *Pasteurella multicoda* can result in death (Chung *et al*., 1994). Other bacterial pathogens commonly isolated from *M. hyopneumoniae* infected pigs include *Haemophilus parasuis* and *Streptococcus suis* (Meyns *et al*., 2006). *M. hyopneumoniae* infection is also a major contributor to the porcine respiratory disease complex (PRDC) which is a multifactorial

disease caused by a combination of both viral and bacterial agents that has serious implications for growing and finishing swine (Thacker *et al*., 2001; Chae, 2005). *M. hyopneumoniae* is found wherever pigs are raised (Minion *et al*., 2002) and is spread directly by contact between infected and susceptible pigs and indirectly by inhalation of the airborne microorganism (Williams, 1983; Czaja *et al*., 2002). It has also more recently been suggested that indirect contact exposure can result in *M. hyopneumoniae* infection (Fano *et al*., 2005).

Control of *M. hyopneumoniae* infections can be achieved with optimisation of management practices and housing conditions, the use of antimicrobials and vaccination (Maes *et al*., 2008) but is difficult to eradicate with re-infections occurring frequently (Minion *et al*., 2002; Stakenborg *et al*., 2005). The ability of *M. hyopneumoniae* to increase the severity of lung disease caused by viral pathogens elevates its importance as a swine pathogen even further (Thacker *et al*., 1999; Thacker, 2004).

1.2.1. Pathogenesis of disease caused by *M. hyopneumoniae*

Like many mycoplasmas, adherence of *M. hyopneumoniae* to ciliated epithelial cells of the porcine respiratory tract is required for successful colonisation and development of disease (Zeilinski and Ross, 1993). Once in the respiratory tract, *M. hyopneumoniae* resists the mucociliary clearance mechanism, penetrates the mucus blanket and attaches to the bronchial epithelial cells. It colonises the ciliated epithelium without penetration, instead situating itself at the apex of the cilia, in the interciliary space, or in contact with the microvilli (Blanchard *et al*., 1992; Jacques *et al*., 1992). Once positioned on the ciliated tufts among the cilia (Figure 2) it seems to be protected from the immune

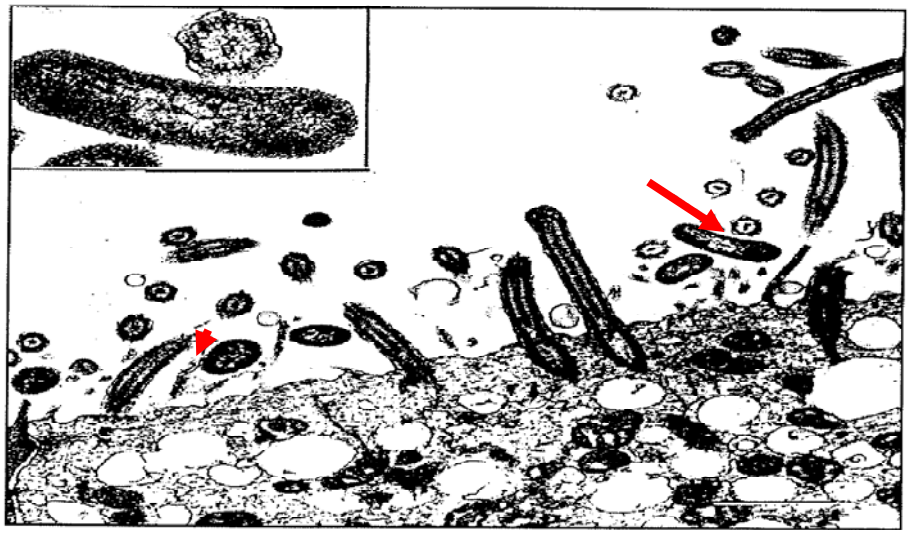


Figure 1.2. Electron micrograph showing *M. hyopneumoniae* (small round bodies) interacting with cilia (arrow) and microvillae (arrowhead) of porcine tracheal cells. Bar = 100nm. Inset is a higher magnification showing the apposition of mycoplasmas and ciliary membranes (taken from Zeilinski and Ross, 1993).

system and can persist for long periods. During infection, *M. hyopneumoniae* induces a reduction in ciliary activity and a gradual but significant loss of cilia as well as microcolony formation and an accumulation of *M. hyopneumoniae* relative to remaining cells (Jacques *et al*., 1992). There is recent evidence to suggest that the intracellular release of calcium in epithelial cells bound by *M. hyopneumoniae* is at least partially responsible for the loss of cilia (Park *et al*., 2002). In addition the organism modulates the immune system of the respiratory tract causing lung tissue damage and resulting in a predisposition to secondary infection (Thacker, 2006).

1.2.1.1. Attachment Mechanism

It is thought that *M. hyopneumoniae* attaches itself to the epithelial cell via fine thread like projections to proteinaceous constituents of the host (ie. receptor-ligand interactions are involved) (Zhang *et al*., 1995). This is evidenced largely by two observations. The first is the fact that *M. hyopneumoniae* only attaches to porcine respiratory ciliated cells, and the second is the results from an adherence assay using suspensions of porcine respiratory tract epithelial cells and *M. hyopneumoniae* with various chemicals and reagents. In these experiments, attachment was abolished when *M. hyopneumoniae* was pre-incubated with periodate and trypsin. In addition, attachment was reduced with dextran sulfate, ammonium sulfate, magnesium sulfate, and methionine, as well as with tetramethyl-urea, in the absence of salt, or at low temperatures (Ross and Young, 1993; Zeilinski and Ross, 1993).

These observations infer a host specific event mediated by proteins, carbohydrates and sulfur containing molecule(s) on the surface of the organism, and sulfur containing

molecule(s) in the host cell membrane. More recently, the binding of *M. hyopneumoniae* to diverse carbohydrate sequences in glycolipids suggests there are several different receptors existing in nature with a strong suggestion of sulfatide involvement (Zhang *et al*., 1994). Non-specific hydrophobic interactions have also been suggested to play a role in the attachment process (Ross and Young, 1993).

Debey and Ross, (1994), developed a tracheal ring culture assay to investigate ciliostasis and loss of cilia caused by *M. hyopneumoniae* strains of different passage. They determined that close association of *M. hyopneumoniae* and porcine tracheal cells was required for the induction of cilial damage and that cilial damage diminishes with increasing *in vitro* passage. Zhang *et al*. (1995) adapted a procedure that extended the work of Zeilinski and Ross (1993), in which cilia extracted from tracheal epithelial cells of swine were immobilised on ELISA plates and used in an ELISA procedure to detect, quantify and further characterise the attachment process. These authors used this assay in the identification and characterisation of *M. hyopneumoniae* adhesion.

1.2.2. Porcine Enzootic Pneumonia (PEP)

PEP is one of the most common and economically important diseases occurring in swine (Ross, 1986; Klinkert *et al*., 1986; Eamens, 1992). In major swine producing countries 35-50% of pigs of marketable age (Williams, 1983) and consistently greater than 50% of swine at slaughter (Minion, 2002) have pneumonic lesions typical of *M. hyopneumoniae* infection. With *M. hyopneumoniae* found in most intensive pig farms (Meyns *et al*., 2007), economic losses are estimated at $20 million a year in Australia

and $1 billion a year worldwide and affect large and small producers equally (Clarke *et al*, 1991; Minion, 2002).

PEP is a complex disease involving *M. hyopneumoniae* (as the primary agent), environmental factors, other pathogenic microorganisms and management practices. The disease varies with age at infection (Morris *et al*., 1995) as well as virulence of the strain causing infection (Vicca *et al*., 2003). It has been described as a chronic but mild lower respiratory tract infection, largely affecting piglets between 2 and 16 weeks of age (Kobisch *et al*., 1993). PEP is characterised by high morbidity and low mortality (Ross, 1986) with the morbidity shown recently to vary substantially between herds due to strain variation (Vicca *et al*., 2003). Once introduced into a herd, lack of appetite, pyrexia, acute dyspnoea, or even sudden death may be observed but the principal clinical sign is a non-productive cough (Williams, 1983; Ross, 1986; Noyes *et al*., 1990). Coughing generally appears 10-16 days after infection, but varies greatly in the field and may be infrequent or absent in some infected pigs (Ross, 1986; Maes *et al*., 2008). A recent behavioural study showed no significant differences in food intake, body temperature or time spent "active" between pigs infected with *M. hyopneumoniae* and non-infected pigs (Escobar *et al*., 2007).

Another characteristic sign of PEP is the sequential development of microscopic and macroscopic lung lesions (Livingston *et al*., 1972; Ross, 1986), shown in Figure 3. These lesions generally take 2 to 3 weeks to appear after experimental infection (Sorenson *et al*., 1997; Kwon *et al*., 2002). Appearing first are microscopic lesions which are characterised by an infiltration in the peribronchiolar and perivascular areas with mononuclear cells and the subsequent production of several interleukins and tumor

necrosis factor (Asai *et al*., 1993, 1994; Rodriguez *et al*., 2004; Choi *et al*., 2006). As the infection progresses, gross lesions, purple to grey in colour, develop mainly bilaterally in the apical, cardiac, intermediate and the anterior parts of the diaphragmatic lobes of the lung (Maes *et al*., 2008) and are characterised by extensive mononuclear cuffing and advanced lymphocytic hyperplasia (Livingston *et al*., 1972; Ross, 1986). These lesions can however resolve 12-14 weeks after a pure *M. hyopneumoniae* infection (Maes *et al*., 2008).

If secondary bacterial infection occurs, debility is severely increased through lack of appetite, laboured breathing, enhanced coughing, elevated temperatures and exhaustion (Ross, 1986). In a study of *M. hyopneumoniae* infection in pigs (Sorensen *et al*., 1997), it was found that *Pasteurella multocida* was present in the lungs of the majority of pigs in the study 4 weeks post inoculation, and that those pigs with *P. multocida* had significantly larger lung lesions. Most pigs with PEP are not debilitated in any major way but usually appear unthrifty, have a dull coat, and show retarded growth even though their appetites are normal (Ross, 1986). Pointon *et al*. (1985) showed that growth rates were reduced by 12-16% in pigs infected with *M. hyopneumoniae*, and that feed conversion was reduced by 14% in the early stages of growth. A pig with 20% lung lesions during its lifetime was found to weigh 25kg less, or take 25 days more to reach market age, than a pig unaffected by enzootic pneumonia (Noyes *et al*., 1990). A comprehensive study of almost 7000 pigs from 14 farms conducted over a period of 8 years aimed to evaluate the relationship between lung lesion data and animal growth during fattening (Pagot *et al*., 2007). Despite a number of variables between the clinical trials from which this data was gathered, a growth loss indicator of 0.7% per one point of pneumonia score was calculated. It is important to note that lung lesions were not

solely attributable to *M. hyopneumoniae*, with a number of other bacteria and viruses identified.

Economic losses as a result of *M. hyopneumoniae* infection are largely associated with poor feed conversion and therefore reduced growth rate and consequently lower market price of the carcass as well as increased susceptibility to infections by other organisms and corresponding treatment costs (Ross, 1999; Sibila *et al*., 2007). This cost can be minimised if factors such as secondary invaders, environmental conditions, strain variation, and poor animal husbandry practices are addressed (reviewed by Maes *et al*., 2008).

1.2.2.1. Treatment and Control of Disease

Treatment and control of PEP is made difficult by the fact that it is a multi-factorial disease. Treatment by antibiotic therapy is expensive, complicated by the presence of residues in meat and of limited success; therefore prevention tends to be the desired treatment. Piggery design, production system, ventilation and climate, stocking density, maintenance of group size, prevention of other diseases and condition of breeding stock are important considerations in disease prevention (Williams, 1983; Eamens, 1992; Maes *et al*., 2008). The identification and removal of infected pigs is critical, and identifying sources of PEP-free pigs for stock replacement can be of huge benefit (Williams, 1983; Eamens, 1992). Many of these approaches have been found to be relatively effective in the control of PEP but require considerable changes in management practices and/or housing of animals.

A number of antimicrobials have been identified to be effective against *M. hyopneumoniae*. The most frequently used are the tetracyclines and macrolides but others including lincosamides, pleuromutilins, fluoroquinolones, florfenicol, aminoglycosides and aminocyclitols are potentially active against *M. hyopneumoniae* (Maes *et al*., 2008). In general, antimicrobials are unable to eliminate disease, but reduce the clinical signs and the mortality rate associated with it. Control of disease using antimicrobials is largely dependant on the antimicrobial regimen, which when given for 1 to 3 weeks starting 1 week prior to disease onset, has been shown to minimise the severity of disease and the infection load (Thacker *et al*., 2004).

Quinolones and tetracycline antibiotics were shown to have activity against *M. hyopneumoniae* as well as other secondary bacteria (Hannan *et al*., 1989). Danofloxacin is a novel fluoro-quinolone and was shown to be highly active against a number of mycoplasma species tested *in vitro* (Cooper *et al*., 1993). For *M. hyopneumoniae*, it was shown to be more active than other antimicrobials tested, including tylosin and oxytetracycline. In a study by Hannan *et al*. (1997), valnemulin (Econor®) showed exceptional activity against *M. hyopneumoniae*, having 100 fold greater activity than tiamulin (a related agent), and 20 fold greater activity than enrofloxacin. Doxycycline and oxytetracycline are antimicrobial agents that work synergistically against *M. hyopneumoniae* as well as the common secondary bacteria causing infection in the porcine lung, *P. multocida* and *Actinobacillus pleuropneumoniae* (Bousquet *et al*., 1997). A more recent field study of 720 pigs in 6 naturally infected swine herds assessed the efficacy of a single dose of Tulathromycin, a novel triamilide antimicrobial. The cure rate was found significantly higher and the mortality rate significantly lower for pigs treated with Tulathromycin compared to those treated with

saline (Nutsch *et al*., 2005). The value of this same drug was demonstrated for a natural outbreak in a Slovenian herd by Hellman *et al*. (2008).

1.2.2.2. Resistance to Reinfection: Cell Mediated vs Humoral Immunity

M. hyopneumoniae is able to persist for long periods by evading the host immune system and it has been suggested that infection results in a suppression of both humoral and cell-mediated immunity. Infection reduces the ability of lymphocytes to produce antibodies and inhibits macrophage mediated cytosis, particularly in the early post-infection period (Maes *et al*., 1993). Therefore, active immunity of pigs to *M. hyopneumoniae* is acquired slowly and only becomes stronger after repeated natural challenge (Maes *et al*., 1996). Despite the many studies that have focused on the immune response of swine to natural or experimental infection by *M. hyopneumoniae*, this topic remains poorly understood.

A study by Kobisch *et al*. (1993) investigated *M. hyopneumoniae* infection in pigs, specifically the duration of the disease and resistance of animals to reinfection. They showed that under experimental conditions, *M. hyopneumoniae* infected swine acquired immunity as a result of primary challenge, which explains why pigs that are exposed regularly to the organism do not display clinical signs of the disease even when they live in a chronically infected herd. The immune mechanisms behind this resistance are yet to be characterised. Sheldrake *et al*. (1993) suggested that protection was largely afforded by cell-mediated immunity and local secretion of IgA rather than humoral immunity, because there doesn't seem to be any correlation between antibody titre and protection from disease (Kobisch *et al*., 1993; Djordjevic *et al*., 1997; Thacker *et al*.,

1998). It has been suggested more recently that the increased cytotoxic and phagocytic ability of macrophages and the increased chronic inflammation observed after *M. hyopneumoniae* infection is important for the development of pneumnonic lesions (Sarradell *et al*., 2003). Although mechanisms of cell mediated immunity are poorly understood, these results infer its importance in development of disease. In addition, Bandrick *et al*. (2008) demonstrated for the first time that lymphocytes passively transferred from vaccinated sows to their piglets via colostrum are able to multiply and participate in specific and functional responses against *M. hyopneumoniae*.

The humoral immune response has been strongly implicated as one of the agents affording protection from *M. hyopneumoniae* infection. A study by Wallgren *et al*. (1998) showed that convalescent sows are susceptible to *M. hyopneumoniae* infection during the final stages of pregnancy because of the transfer of antibodies from the blood to the colostrum. A transfer of protective immunity from sow to offspring was also demonstrated when maternal antibodies were detected but no clinical signs of disease were observed in piglets 2 weeks old. A decline in maternal immunity was however observed with an increase in age of piglets, as had been reported previously (Fu *et al*., 1990). Protection in piglets has also been demonstrated for sows with high antibody levels in their colostral milk (Rautiainenand Wallgren, 2001; Martelli *et al*., 2006). It is believed that protection against *M. hyopneumoniae* infection is dependent on a balance between the immune status of the animal and the pathogen load (Wallgren *et al*., 1998).

1.2.3. Vaccination Against PEP

M. hyopneumoniae infection can be controlled partially by improved management practices and treatment regimes. Vaccination also plays an important role in the control of disease with many countries vaccinating greater than 70% of swine herds (Haesebrouck., 2004; Maes *et al*., 2008). Many studies have demonstrated that the vaccination of pigs can improve the average daily weight gain in infected herds (Jenson *et al*., 2002). A recent review reported a 2-8% increase in average daily weight gain, a 2-5% improvement in feed conversion ratio, a shorter time to reach slaughter weight, reduced clinical signs, a decrease in lung lesions, lower treatment costs and possible improvement in mortality rate as major reasons for vaccination (Maes *et al*., 2008).

Vaccination relies on the use of commercial bacterin vaccines which have been shown to induce only partial protection with significant variation in beneficial effects between herds. A number of factors have been suggested as contributors to this variation including improper vaccine storage conditions and immunisation technique, age at vaccination, vaccine schedule (single or double dose and timeframes involved for multiple doses), housing system and group size, antigenic differences between field strains and vaccine strains, presence of disease at time of vaccination (caused by *M. hyopneumoniae* or other bacterial or vial infections), interference of response by maternally derived antibodies, and the high heterogeneity between *M. hyopneumoniae* isolates (Jenson *et al*., 2002; Haesebrouck *et al*., 2004; Maes *et al*., 2008).

Field success of vaccines evidences the potential of mycoplasma species to be strong immunogens (Ross and Young, 1993) but a lack of knowledge of the pathogenic

mechanisms and virulence factors remains a limiting component in the development of highly effective vaccines (Maes *et al*., 2008). Very few studies defining pathogenic mechanisms of *M. hyopneumoniae* have been conducted and although mechanisms of adherence are beginning to be elucidated, much remains unknown about adherence and the interaction of *M. hyopneumoniae* with host cells (Minion *et al*., 2004).

Although commercial vaccines are widely applied worldwide, their inability to provide complete protection and prevention of colonisation sees the search for a vaccine with improved efficacy continue. New generation sub-unit antigen vaccines and DNA vaccines that can be delivered easily (single dose, aerosol, feed-based) and cheaply are actively being researched. Immunisation protocols and the choice of adjuvant are also important considerations of any vaccine and can impact significantly on the immune response generated.

1.2.3.1. Commercial Vaccines

There are a number of commercial vaccines against *M. hyopneumoniae* available, all killed whole cell, or bacterin preparations. These include: Suvaxyn (Europe) which is also called Respifend (outside Europe) and is produced by Fort Dodge; Stellamune Uno (Europe) also known as Respisure One (outside Europe) supplied by Pfizer Animal Health, Inglevac M. hyo produced by Boehringer Ingleheim, and Hyoresp by Merial. The preferential mode of delivery of these vaccines is intramuscular and relies on the adjuvant in the preparation to stimulate an immune response in the animal that is significant enough to provide protection.

A study by Murphy *et al*. (1993) assessed the efficacy of intra-muscular and aerosol vaccination of pigs against *M. hyopneumoniae* using the commercially available vaccine Suvaxyn. The vaccine was unable to induce local and systemic antibody responses as detected by ELISA, regardless of the administration route. However, when given intramuscularly, clinical signs of disease and lung lesions were substantially reduced upon challenge with virulent *M. hyopneumoniae*, but when given as an aerosol it afforded no protection against disease. In another study, Respisure was shown to decrease the prevalence of lung lesions at slaughter by 50% and increase slaughter weight by 1 kg (Doohoo and Montgomery, 1996). A recent trial by Moreau *et al*. (2004) involving more than 28,000 pigs, evaluated the effects of Inglevac M. hyo on average daily weight gain in herds infected with *M. hyopneumoniae*. Vaccinated pigs showed a higher average daily weight gain of 42 g and the mortality rate was decreased by 1.5%. The efficacy of Respisure and Inglevac M. hyo was compared in a trial conducted by Baccaro *et al*. (2006). No difference was observed in average daily weight gain between the two but Respisure showed a greater reduction in prevalence and severity of lung lesions. Hyoresp was also reported as effective at improving performance and reducing the prevalence and severity of lung lesions (Kyriakis *et al*., 2001).

Although all commercial vaccines have shown a consistent reduction in lung lesions, none have demonstrated a significant reduction in the prevalence of disease (Dayalu *et al*., 1992; Pejsak *et al*., 1992; Bilic *et al*., 1992; Blagovic *et al*., 1992; Scheidi *et al*., 1994; Dohoo *et al*., 1996; Haesebrouck *et al*., 2004; Meyns *et al*., 2006; Sibila *et al*., 2007). Colonisation of the lung by *M. hyopneumoniae* seems to persist and transmission is only marginally reduced. In addition, the high cost of commercial vaccines and labour

intensive mode of delivery govern the economic feasibility depending on pork market prices (Moreau *et al*., 2004).

1.2.3.2. Experimental Vaccines

The results of many *M. hyopneumoniae* whole cell and extract vaccine trials have been published. There are many factors that need to be considered for the development of an efficacious vaccine including composition, dose and dosing schedule, adjuvant choice and route of administration.

Early experimental *M. hyopneumoniae* vaccines were prepared from whole cell extracts. Without adjuvant the whole cell vaccines offered no protection (Goodwin and Whittlestone, 1973). Some protection against PEP was observed when whole-cell antigens were administered in formulation with Freund's complete adjuvant or a mixture of Bayol/Arlacel adjuvants (Goodwin and Whittlestone, 1973), but adverse reaction to them rendered their use unacceptable. There are now more modern adjuvant mixtures available that don't produce such adverse side affects but are still able to induce an appropriate and lasting response. The Auspharm adjuvant (Auspharm Int. Ltd. Australia) is an example of such, which when mixed with formalin-killed *M. hyopneumoniae* cells and delivered intraperitoneally demonstrated significant protection against virulent challenge of *M. hyopneumoniae* in pigs as measured by a decrease in lung score (Sheldrake *et al*., 1991). Needleless intradermal vaccination with a commercial bacterin was also shown to be feasible and protective when it resulted in significantly higher antibody titres than pigs vaccinated intramuscularly (Jones *et al*., 2005).

Oral microencapsulated *M. hyopneumoniae* vaccines were tested in pigs for their ability to prevent PEP and were shown to provide some protection against disease (Weng *et al*., 1992). An extension of this method which incorporated co-spray drying to prepare an oral microsphere vaccine containing *M. hyopneumoniae* antigens also demonstrated protection, with significantly reduced lung lesions at slaughter in vaccinated pigs compared to non-vaccinated controls (Lin *et al*., 2003). Djordjevic *et al*. (1997) used a range of adjuvant formulations in conjunction with a size-pool of *M. hyopneumoniae* proteins (in the range 75-80 kDa range) to demonstrate significant reductions in lung damage caused by experimentally induced PEP. Protection was also demonstrated in a trial by Okada *et al*. (2000) in which the use of *M. hyopneumoniae* inactivated vaccine prepared from sedimented whole cells and cell-free culture supernatants was evaluated. Protection against *M. hyopneumoniae* infection was provided more consistently with the culture supernatants than with the sedimented whole cells, which gave varied protection.

1.2.3.3. Recombinant DNA and Sub-unit Antigen Vaccines

Several DNA and sub-unit antigen vaccines have been developed and trialled in animals. A recombinant 74.5 kDa DnaK-like protein, was tested in a vaccine trial as an *E. coli* lysed cell supernatant formulated in Freund's incomplete adjuvant (Faulds *et al*., 1990). At low dosage (100 μg), the severity of *M. hyopneumoniae* infection was reduced by 66%, but at 1000 μg per dose the severity of infection increased. The protein was subsequently purified (≈ 95%) and tested in pigs at 100 μg per vaccine dose and when given intranasally, the severity of infection was reduced by up to 80%. Despite the large intra group variation observed, its potential as a vaccine antigen is

reinforced by the fact that stress proteins such as DnaK and DnaJ are often antigenic, and can be upregulated during host colonisation (Garsia *et al*., 1989; McKenzie *et al*., 1991; Qoronfleh *et al*., 1993). An *M. hyopneumoniae* heat shock protein was incorporated into plasmid pcDNA3 for assessment of its potential as a DNA vaccine antigen (Chen *et al*., 2003). The levels of serum IgG, proliferation of splenocytes, and the levels of T-cell-specific cytokines all significantly increased in mice immunised with the recombinant plasmid. This represented both a cellular and humoral immune response and therefore suggests its potential as a vaccine.

The *M. hyopneumoniae* ribonucleotide reductase, an enzyme that controls the cellular concentration of deoxyribonucleotides, has been used as a vaccine component for a number of experimental trials. An 11 kDa antigen of the R2 sub-unit of ribonucleotide reductase (NrdF) fused to β-galactosidase was shown to provide some protection against PEP in a pig trial (Fagan *et al*., 1996). The NrdF antigen expressed as a fusion product with β-galactosidase was purified by preparative SDS PAGE and given at a dose of 1 mg per pig in one of four different adjuvants. After experimental challenge with *M. hyopneumoniae*, the pigs were assessed for the effects of vaccination on lung score. The mean lung score of vaccinated animals was significantly reduced when compared to that of the unvaccinated control pigs ($P < 0.05$), regardless of the adjuvant used.

The promising results of this early trial with the NrdF antigen led to further investigation of its use as a vaccine antigen. The gene encoding NrdF was introduced into a *Salmonella typhimurium aroA* expression system and generated a strong lung IgA antibody response in immunised mice (Fagan *et al*., 1997). The same attenuated strain

expressing the recombinant NrdF antigen was then used to orally immunise swine against PEP (Fagan *et al*., 2001). Immunisation primed the immune system to elicit a significant ($P < 0.05$) secretory IgA response against the NrdF antigen in the respiratory tract, caused blood lymphocytes to proliferate significantly ($P < 0.05$) following stimulation with *M. hyopneumoniae* whole cell extracts 14 days after vaccination, and generally increased daily weight gains and reduced lung pathology when compared to controls. More recently, the immunogenicity of NrdF was evaluated in mice immunised orally with attenuated *Salmonella typhimurium aroA* harbouring recombinant prokaryotic and eukaryotic expression vectors (Chen *et al*., 2006). Neither vector was able to induce a mucosal immune response in the study, but route of administration can impact on successful initiation of mucosal immunity so an alternative route may provide a better response. The eukaryotic expression vector was able to produce a significant level of IFN-γ compared to control groups when stimulated with NrdF indicating its ability to induce a cell-mediated response. These data strongly suggests that the NrdF antigen may be a useful component in a vaccine against PEP.

Another recombinant antigen trialled in pigs for its vaccine potential is the *M. hyopneumoniae* adhesin. King *et al*. (1996) expressed and purified the *M. hyopneumoniae* adhesin Mhp1 (P97) as a fusion protein with glutathione S-transferase (GST). The fusion protein (50 μg) was complexed with Freund's adjuvant and given intramuscularly in two boosts, fourteen days apart. Although the adhesin has a confirmed role in pathogenesis, and is a protein recognised by naturally and experimentally *M. hyopneumoniae* infected pigs, it was unable to significantly reduce coughing and lung lesions in infected animals when given as a vaccine preparation. The RR1 fragment of P97 (cilial binding fragment) was fused to *Pseudomonas* exotoxin A

(PE) and was trialled in both mice and pigs (Chen *et al*., 2001). An RR1 specific IgG response in mice saw the PE-RR1 given in conjunction with a conventional *M. hyopneumoniae* vaccine in pigs. A two-fold higher antibody titre against RR1 was found in the pigs immunised with PE-RR1 in addition to the commercial vaccine compared to those vaccinated with the commercial vaccine alone. Several other trials using P97 fragments as immunising antigens showed similar results indicating a cell-mediated immune response in addition to the systemic immune response (Shimoji *et al*., 2003; Conceicao*et al*., 2006; Chen *et al*., 2006).

Vaccine formulations composed of single antigens such as those described above have largely demonstrated only partial protection, hence the suggestion of a multivalent vaccine as an improved alternative. The most recent trial by Chen *et al*. (2008) evaluated the immune response in mice to cocktail DNA and/or recombinant protein vaccines composed of potential protective antigens of *M. hyopneumoniae*. The cocktail vaccines were composed of five immunogenic *M. hyopneumoniae* antigens; P97, P97R1, NrdF, P36 and P46, all shown to have high sequence homology among different strains. Intramuscular immunisation with the DNA cocktail vaccine induced a strong Th1 immune response whereas the protein cocktail vaccine induced both a humoral and Th1 response. The combination vaccine generated both strong humoral and Th1 responses and requires further investigation via challenge experiments in pigs.

1.3. Aims and Objectives

Further studies are required for the improvement of vaccines and vaccine strategies against *M. hyopneumoniae*. It is well documented that a comprehensive understanding of an organism's pathobiology and the molecular mechanisms underlying its pathogenicity are required for the rational design of effective vaccines. A new generation sub-unit antigen vaccine incorporating immunoreactive surface antigens of *M. hyopneumoniae* is the ultimate goal of this research. The specific aims of this thesis involve the cloning and characterisation of immunoreactive *M. hyopneumoniae* antigens with potential for this purpose. Elucidating the role of surface antigens of *M. hyopneumoniae* has the added potential to increase the current understanding of adherence and the pathogenic mechanisms of this organism.

Chapter Two

General

Materials and Methods

2.1. Materials

This section summarises the important biological materials used or produced in the research component of this thesis and indicates their source.

2.1.1. Biological Material

Bacterial strains and plasmids used in this study are given in Tables 2.1 and 2.2 respectively. All mycoplasma strains used in the study are given in Table 2.3.

Table 2.1. Bacterial strains used in this study.

Bacterial Strain	**Source**	**Use**
Escherichia coli JM109	Laboratory	Plasmid host strain
Escherichia coli BL21(DE3)(pLysS)	Novagen	Host strain for creation of *M. hyopneumoniae* expression library
INVαF' One Shot™	Invitrogen	TA cloning host strain
Escherichia coli XL1-Blue MRF' kan	Stratagene	pPCR-Script cloning host strain
Escherichia coli M15[pREP4]	Qiagen	His-tag expression strain
Escherichia coli DH5α	Invitrogen	Transformation strain for Mhp493 mutagenesis clones (ORF545A&B)
Escherichia coli Top 10	Invitrogen	Champion cloning host strain

Table 2.1. Plasmids used in this study.

Plasmid	**Source**	**Use/Description**
PET23a-c	Novagen	Construction of *M. hyopneumoniae* expression library
pCR®2.1	Invitrogen	TA cloning
pPCR-Script	Stratagene	pPCR-Script cloning
pQE9	Qiagen	His-tag expression
pET161	Invitrogen	Champion TOPO cloning
pAS1	A. Scarman	28 kDa adhesin antigen expression clone (in pET23c)
pJW1	This work	5' end terminal adhesin clone (in pCR®2.1)
pJW2	This work	35 kDa adhesin antigen expression clone (in pQE9)
pJWRR1-15	This work	Repeat region clones (in pCR®2.1)
pJW3	This work	PdhA clone (in pPCR-Script)
pJW4	This work	PdhA expression clone (in pPCR-Script)
pJW5	This work	PdhA expression clone (in pQE9)
pJW6	This work	rP27 clone (in pPCR-Script)
PJW7	This work	rP27 expression clone (in pPCR-Script)
pJW8	This work	rP27 expression clone (in pQE9)
ORF545 A & B	F. Chris Minion	Mutagenised Mhp493 clones (in pCR2.1)
pJW9	This work	$F1_{P216}$ expression clone (in pET161)
pJW10	This work	$F2_{P216}$ expression clone (in pET161)
pJW11	This work	$F3_{P216}$ expression clone (in pET161)

Table 2.3. Mycoplasma species and strains used in this study.

Species	**Strain**	**Source**
M. hyopneumoniae	J (NCTC 10110)	A. Pointon
M. hyopneumoniae	232	T. Young
M. hyopneumoniae	Beaufort	AMRC
M. hyopneumoniae	Sue	AMRC
M. hyopneumoniae	YZ	M. Kobisch
M. hyopneumoniae	C1735/2	AMRC
M. hyopneumoniae	OMZ407	AMRC
M. hyorhinis	field strain 1	AMRC
M. hyorhinis	field strain 2	AMRC
M. hyorhinis	GDL	AMRC
M. hyorhinis	BTS7	AMRC
M. flocculare	MS42	AMRC

- Most of the mycoplasmas used in this study were obtained from A. Pointon at the Australian Mycoplasma Reference Collection (AMRC), South Australian Department of Primary Industries, Central Veterinary laboratory, Adelaide.

2.2. Methods

This section contains methods that are common to more than one chapter of this thesis. Unless otherwise specified all media, solutions and buffers were made with sterile water from the Milli-Q water purification system, and all reagents used were of analytical grade. Recipes for these buffers and solutions can be found in Appendix A. Maps for the vectors used for cloning are given in Appendix B.

2.2.1. Culture Methods

2.2.1.1. Bacterial Cell Culture

Unless otherwise specified, bacterial strains were grown on Luria Bertani (LB) agar or in LB broth (Sambrook *et al*., 1989). All incubations were at 37°C and liquid incubations were agitated in an orbital shaker at 250 rpm. Where appropriate LB media was supplemented with ampicillan to a concentration of 100 μg/mL, kanamycin to a concentration of 50 μg/mL, chloramphenicol to a concentration of 32 μg/mL, 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-gal) to a concentration of 40 μg/mL, and isopropyl-β-D-thiogalactopyranoside (IPTG) at a 10 mM final concentration.

2.2.1.2. Mycoplasma Cell Culture and Harvest

Culture of the porcine mycoplasmas has been described previously (Sheldrake and Romalis, 1992). Both field and laboratory adapted strains of *M. hyopneumoniae*, *M. hyorhinis* and *M. flocculare* were grown in modified Friis (1977) media at 37°C and

harvested during log phase when in the pH range 7.2-6.9 as indicated by a colour change. Mycoplasmas were harvested by centrifugation at 7000 g for 20 min and the pellet was washed 3 times with Phosphate buffered saline (PBS).

2.2.2. Methods for DNA Analysis

2.2.2.1. Extraction of Genomic DNA from Mycoplasma

Genomic DNA was extracted from mycoplasma cultures using a phenol chloroform method. After three washes with PBS, cells (≈ 0.6 g) were resuspended in 4 mL of 10% sucrose. Sodium dodecyl sulfate (0.4 mL of a 10% solution) was added to lyse the cells upon mixing by inversion. The addition of 50 µL RNase (10 mg/mL) and incubation at 37°C for 30 min followed. Proteinase K (250 µL of a 1 mg/mL solution) was then added and the cell suspension incubated at 37°C for 1 h. An additional overnight incubation at 55°C was also required before the phenol chloroform extractions were performed.

Phenol/chloroform/isoamylalcohol (25:24:1) was added in equal volume to the cell suspension and the resulting solution mixed gently but well by inversion in serum separator tubes (Becton-Dickinson) containing silica gel polymer. Phase separation by centrifugation (in a Beckman GP benchtop centrifuge) at 1,750 *g* for 15 min followed. The top layer was removed and the extraction repeated 3-4 times or until clear. An equal volume of chloroform was then added and mixed by inversion before being centrifuged as before. The resulting DNA solution was dialysed against Tris EDTA (TE) buffer

overnight and the DNA precipitated by the addition of 0.7 volumes of isopropanol. DNA concentration was determined by measuring absorbance at 260 nm.

2.2.2.2. Extraction of Plasmid DNA from Bacteria

Small-scale purification of plasmid DNA was performed using the Pharmacia Biotech Flexiprep Kit. Overnight cultures (1.5 mL) were grown and pelleted by centrifugation (30 sec at maximum speed) in Eppendorf tubes. Cell lysis followed according to the manufacturer's instructions using kit solutions I (isotonic solution containing RNase for resuspension of cells), II (alkaline solution for cell lysis) and III (potassium acetate solution for neutralisation and precipitation of proteins, chromosomal DNA and cell debris). The resulting cell suspension was then centrifuged at maximum speed for 5 min and isopropanol was added to the supernatant to precipitate the plasmid DNA. Pelleted DNA was then bound to Sephaglas™ FP and washed by resuspension and centrifugation with firstly the wash buffer supplied in the kit and then ethanol (70%). Bound plasmid DNA was eluted in TE buffer or water and the concentration determined by reading absorbance at 260 nm.

Larger-scale purification of DNA was performed on 200 mL culture pellets using the QIAGEN Plasmid Midi Kit according to the manufacturer's instructions. Cell lysis used kit buffers P1 (Tris EDTA containing RNase A for resuspension), P2 (Sodium hydroxide and SDS to lyse the cells) and P3 (Potassium acetate for precipitation and neutralisation). Centrifugation at 20,000 g for 30 min at 4°C followed and the supernatant was removed and recentrifuged for 15 min. The supernatant was then promptly applied to a QIAGEN tip-100 to which the plasmid DNA bound. The

QIAGEN-tip was washed twice with buffer QC (containing sodium chloride, MOPS and isopropanol) and eluted with buffer QF (containing sodium chloride, Tris and isopropanol). The eluted DNA was precipitated with 0.7 volumes of isopropanol, washed with ethanol (70%) and resuspended in TE or water. Again concentration was determined by reading the absorbance at 260 nm.

2.2.2.3. Genomic DNA Digestion

Mycoplasma chromosomal DNA (1 μg) was digested using 4 units of enzyme and 4 μL of restriction enzyme buffer (10X) in a final volume of 40 μL with water. The samples were mixed and incubated at 37°C overnight before being placed immediately at -20°C for storage. The samples were subject to agarose gel electrophoresis for confirmation of complete digestion.

2.2.2.4. Plasmid DNA Digestion

Plasmid DNA (400 ng) was digested with 2 units of enzyme and 2 μL of appropriate restriction enzyme buffer (10X stock) in a 20 μL volume with water. The samples were mixed and incubated at 37°C for a minimum of 2 h, heated to 65°C for 5 min and kept at -20°C until analysis by agarose gel electrophoresis.

2.2.2.5. Oligonucleotide primers

All oligonucleotide primers were synthesised by Sigma-Aldrich in a 50 nmol quantity as a desalted preparation. They were resuspended in 100 µL TE buffer and stored at -20°C until required. These were thawed and diluted in MQ water for use.

2.2.2.6. Polymerase Chain Reaction (PCR)

All amplifications were performed in a 50 µL reaction mixture containing 200 ng of chromosomal DNA, 10 pmol of each oligonucleotide primer, 200 µM deoxyribonucleotide triphosphates, 2 units of polymerase (*Taq* or *Pfu*), 10X PCR buffer (as supplied with the polymerase) and 4 mM magnesium chloride. Reaction mixtures were incubated at 94°C for 2 min for 1 cycle, followed by 25-35 cycles of denaturation at 94°C for 20 sec, primer annealing at 50-60°C for 20 sec, and extension at 70°C for 1½ -2 ½ min. A further cycle of denaturation at 94°C for 20 sec, primer annealing at 59°C for 20 sec and a final extension at 70°C for 2½ min was also included in the cycle parameters. The products were visualised on agarose slab gels stained with ethidium bromide.

2.2.2.7. Agarose Gel Electrophoresis

For general electrophoresis, 0.9 to 1.5% agarose (w:vol) was prepared in 1X TAE and poured into a gel frame, the gel comb inserted, and the agarose allowed to set at room temperature for 30-60 min. Once set, gels were placed in mini sub gel or wide mini sub gel electrophoresis chambers (BioRad) and covered with 1X TAE. Bromophenol Blue

(BPB) loading dye was added to the samples for loading. Once loaded, gels were electrophoresed at a constant voltage of 80-100 V until the dye front had migrated approximately 2 cm from the end of the gel. They were then placed in a weak ethidium bromide solution (1 μg/mL in 1X TAE) for 15-30 min for DNA visualisation by UV light.

2.2.2.8. Purification of DNA from Agarose

Purification of DNA from agarose was achieved using the Bresa-Clean™ DNA purification kit (Bresatec). DNA fragments were separated in agarose, stained with ethidium bromide and excised from the agarose using a new scalpel blade while being viewed under UV light. Extraction of the DNA followed as specified in the manufacturer's instructions. Briefly, 3 volumes of Bresa-Salt™ was added to the gel slice in an Eppendorf tube and incubated for 5 min at 55°C or until completely melted. Bresa-Bind™ was then added (5 μl plus 1 μL/μg DNA) and incubated for 5 min at room temperature to bind DNA. The Bresa-Bind™/DNA complex was pelleted and then washed once with Bresa-Wash™. The complex was pelleted again and the wash solution was aspirated off and the pellet air-dried. DNA was then recovered by resuspension of the Bresa-Bind™/DNA complex in TE or water and incubating at 45 to 55°C for 5 min. The Bresa-Bind™ is again pelleted and the DNA in the supernatant removed immediately.

2.2.2.9. Preparative Agarose Gel Electrophoresis for DNA Fractionation

M. hyopneumoniae (strain Beaufort) DNA (50 μg) was digested with restriction enzyme *Eco*RI or *Hin*dIII (250 units) in a 500 μL reaction volume containing RNase A (10 μg). The digestions were incubated at 37°C for 4 h before being stopped by the addition of 10 μL of EDTA (0.5 M). An ethanol precipitation of the digested DNA followed. To each digestion reaction, 50 μL of 3 M sodium acetate (pH 7.0) and 1 mL of cold ethanol (100%) was added. The DNA was allowed to precipitate at -80°C for a minimum of 1 h and was then centrifuged at maximum speed in a benchtop microfuge at 4°C for 30 min. The supernatant was removed and the DNA pellet air-dried. Each pellet was resuspended in 50 μL of TE to give a total of 500 μg of digested DNA in a volume of 500 μL, which was to be loaded onto the agarose column. BPB loading dye was added to the digested DNA prior to loading.

An agarose gel (0.8% in 0.5X TBE) was poured and allowed to set for 4 h at room temperature in the casting unit (37 mm) of a Model 491 Prep Cell (BioRad). For electrophoresis the unit was assembled as specified by the manufacturer using 0.5X TBE as the running buffer. The digested DNA preparation was loaded onto the top of the gel column and electrophoresed through the gel at 50 V constant voltage. Fractions (1 mL) were collected at the rate of 50 μL/min for a period of 60-72 h using an ISCO Foxy 200 automated fraction collector. Preliminary agarose gels (1%) were run of every 5th fraction collected after the dye front. Fractions within the desired size range were then concentrated using centrifugal concentrators (Filtron, 30 K) by pooling every two samples and reducing the volume (2 mL) to 100 μL.

2.2.2.10. Southern and Colony Hybridisations

Southern and colony hybridisations were performed using the DIG system (Boehringer Mannheim) as detailed in the manufacturer's instructions. Probes were either 3' end labelled oligonucleotides or random primed PCR products also prepared according to the manufacturer's instructions.

For Southern hybridisation analysis (Southern, 1975), digested DNA was electrophoresed in agarose at 10 V overnight. The resulting gel was submerged in 0.25 M HCl for 10 min and rinsed in water for 1 min. It was then washed 2 x 15 min in Denaturation solution and a further 2 x 15 min in Neutralisation solution before being transferred to a Hybond N+ membrane (Boehringer Mannheim). The transfer was a wet capillary transfer set up overnight using 10X SSC. For colony hybridisation single colonies were patched onto an agar plate and grown overnight at 37°C. Colonies were "lifted" off the plate and directly onto a Hybond N+ membrane. The membranes were placed on buffer soaked filter paper for denaturation (2 x 15 min), neutralisation (2 x 15 min) and a rinse with 2X SSC.

All membranes were baked at 80°C for 30 min for cross-linking and probed in roller bottles in a Hybaid oven. Membranes were pre-hybridised for at least 1 h in DIG Easy Hyb at 42°C and then probe was added overnight for hybridisation. In the morning membranes were washed 2 x 5 min at RT in 2X SSC containing 0.1% SDS and then 2 x 15 min at 58°C in 0.5X SSC containing 0.1% SDS. They were then blocked for 1 h in maleic acid buffer containing 5% skim milk. After blocking DIG anti-Fab fragments were added to the blocking buffer for 30 min at RT. Two washes of 15 min each in

maleic acid buffer containing 0.3% Tween® 20 followed prior to development with CDP-Star™.

2.2.2.11. Cloning into pPCR-Script

The pPCR-Script vector is a component of the pPCR-Script™ Amp Cloning Kit (Stratagene), which is designed to simplify the cloning of blunt-ended DNA fragments such as those amplified by *Pfu* polymerase. A plasmid map of pPCR-Script is given in appendix B and cloning into this particular vector was performed as described in the manufacturer's instructions. Briefly, a ligation reaction containing 10 ng of cloning vector, 500 ng of insert DNA, 5 nmol of rATP, 5 Units of *Srf*I, 4 Units of T4 DNA ligase and 1 µL of 10X reaction buffer in a final volume of 10 µL was prepared. The ligation reaction was mixed gently and incubated at room temperature for 1 h. It was then heated at 65°C for 10 min before being placed on ice until transformation into *Escherichia coli* XL1-blue MRF' Kan supercompetent cells (Stratagene).

The competent cells (40 µL) were thawed on ice prior to transformation. Once thawed, β-mercaptoethanol was added to the cells to a final concentration of 25 mM and swirled gently every 2 min for a total of 10 min. The ligation mix (2 µL) was then added, mixed in gently, and the cells incubated on ice for 30 min. After the incubation, the cells were given a heat pulse at 42°C for 45 sec and placed back on ice for 2 min. Pre-warmed (42°C) SOC medium (450 µL) was added to the transformation mixture, which was then incubated at 37°C for 1 h with shaking at 225-250 rpm. After incubation, the transformation mixture was plated onto LB Ampicillin/Kanamycin agar plates

containing X-gal and IPTG and incubated overnight at 37°C. White colonies were selected for screening by colony and Southern hybridisation analysis.

2.2.2.12. Cloning into pQE9 for expression in *E. coli*

The cloning vector pQE9 (Qiagen) was used as an expression vector to allow easy purification of recombinant antigens when expressed in *E. coli* M15[pREP4] cells (Qiagen). To maximise the efficiency of the ligation reactions carried out, vector (pQE9) and insert DNA were purified from agarose using the Bresa-Clean™ method after being double digested with appropriate enzymes. The relative concentrations of the two were determined by reading the absorbances at 260 nm. Ligation reactions were then prepared in a 20 µL reaction volume containing vector and insert DNA in a ratio of 1:4. The reaction mix was incubated at 65°C for 1 min, 37°C for 10 min, room temperature for 10 min, and then finally 10°C for 10 min before the addition of 10X ligation buffer (2 µL) and T4 DNA ligase (4 Units). The ligation reaction was mixed gently and left at 10°C overnight. The ligation mix was stored on ice or at -20°C until transformation.

If frozen competent cells purchased from Qiagen were being used then the transformation would proceed as described in the QIAexpressionist handbook. Otherwise, ligation mix (1 µL) was added to a 50 µL aliquot of pre-prepared *E. coli* M15 (pREP4) cells (Qiagen) in 10% glycerol. The cells were mixed gently with a pipette tip before being pulsed at 2.5 kV in a BIORAD Gene Pulser. The electroporated cells were then placed in 1 mL of LB media and incubated at 37°C with shaking for 1 h.

After incubation, the transformation mixture is plated onto LB Ampicillin/Kanamycin agar plates and incubated overnight at 37°C. Colonies are selected and cultured for screening by plasmid extraction and digestion.

2.2.2.13. Cloning from N-terminal Sequence Data

Degenerate oligonucleotide probes were designed and used to probe *Eco*RI or *Hin*dIII digested chromosomal DNA in Southern hybridisation analysis using the DIG System (Boehringer Mannheim). Digested DNA (strain Beaufort) was fractionated on an agarose column using a 491 Prep Cell and the fragment to be cloned was identified by Southern analysis with the degenerate oligonucleotide probe. Fragments (in a 20 µL reaction) were made blunt ended by incubation with Klenow fragment (1-5 U) and dNTPs (5 µL of 0.1 mM stock) at 30°C for 15 min. The reaction was stopped by the addition of 1 µL of 0.5 M EDTA. The blunt ended fragments were then cloned into pPCR Script as outlined in the manufacturer's instructions (Stratagene). Positive clones were identified by Colony hybridisation using the DIG System (Boehringer Mannheim) and 3' end labelled degenerate oligonucleotides.

2.2.2.14. DNA Sequence Analysis

Purified plasmid DNA (Qiagen) or agarose purified PCR product was used for sequencing. All sequencing was accomplished using synthetic oligonucleotide primers supplied commercially (Sigma) and the BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions were performed exactly as specified by the

Perkin-Elmer Corporation Protocol for BigDye™ Terminator Cycle Sequencing using the Perkin-Elmer GeneAmp 9600 and the Perkin-Elmer ABI PRISM 377.

For each sequence reaction, 4 μL of Terminator ready reaction mix, 300-500 ng of plasmid DNA or 30-180 ng of PCR product, 3.2 pmol of primer, and water to give a final volume of 10 μL was mixed in 0.2 mL GeneAmp tubes. The tubes were then placed in the thermocycler programmed as follows; rapid thermal ramp to 96°C, 96°C for 10 sec, rapid thermal ramp to 50°C, 50°C for 5 sec, rapid thermal ramp to 60°C, and 60°C for 4 min. The cycle was repeated 25 times before the cycler rapid thermal ramps to 4°C and holds.

Excess dye terminators were removed by an ethanol precipitation procedure. For each reaction a 1.5 mL Eppendorf tube containing 2 μL of 3M sodium acetate (pH 4.6) and 50 μl of 95% ethanol was prepared. The entire 10 μL contents of the reaction tube was added to the prepared Eppendorf tube, vortexed and placed on ice for 10 min. Each tube was then centrifuged at maximum speed for 30 min. The ethanol was aspirated off and the pellet was rinsed by adding 250 μL of 70% ethanol and centrifuging once again at maximum speed for 5 min. The ethanol solution was again aspirated off and the pellet air-dried. The dried sample was resuspended in 6 μL of loading buffer, vortexed and spot-centrifuged, heated at 90°C for 2 min, and placed on ice until loaded.

2.2.2.15. DNA sequence Assembly and Bioinformatics Analysis

DNA sequences were assembled using the computer programs Factura (Perkin Elmer) and Autoassembler (Perkin Elmer). Assembled sequences were analysed using MacVector (Version 7.2, Accelrys) and the package from the University of Wisconsin Genetics Group (GCG) version 7, accessed via the Australian National Genomic Information Service (ANGIS, University of Sydney). Alignments were performed and formatted using the ClustalW and Boxshade programs accessible from the EMBNET website (http//www.ch.embnet.org). Potential transmembrane regions were identified in the predicted P216 sequence using the TmPred algorithm also accessed from the EMBNET website. Coiled coil regions were predicted using the coils program provided by the Expasy Proteomics Server at http://ca.expasy.org/tools/. Compute pI/Mw and ProtScale (for the generation of hydrophilicity plots using the Kyte and Doolittle method, 1982) were among other programs accessed at the same site. ProfileScan (www.isrec.isb-sib.ch/software/PFSCAN_form.ht) was used to search for statistically significant peptide motifs. KEKE regions (Realini *et al.*, 1994) were identified using an algorithm kindly supplied by Prof. Rechsteiner (University of Utah, Salt Lake City, Utah).

2.2.3. Methods for Protein Analysis

2.2.3.1. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE (Laemmli, 1970) was carried out under reducing conditions using 10 to 15% separating gels and 4% stacking gels. Protein samples were reduced prior to

loading with 1:4 (vol:vol) Laemmli reducing solution (RS) and heated for 10 min at 95°C. BPB loading dye (2 µL) was added to the sample. Electrophoresis was carried out using a Mighty Small II (Hoefer Scientific) electrophoresis apparatus at 25mA constant current per gel, or a Protean II mini gel system (Biorad) at 100V constant voltage, and run until the dye front was eluted. Both were used according to the manufacturer's instructions.

Some gels were stained with Coomassie Brilliant Blue (CBB) while others were used for Western transfer onto polyvinylidene diflouride (PVDF) membranes.

2.2.3.2. Preparation of Antisera

Preparation of porcine hyperimmune serum against *M. hyopneumoniae* has been described previously (Scarman *et al*., 1997). In brief, *M. hyopneumoniae* free pigs were challenged with a preparation of *M. hyopneumoniae* emulsified in Freund's complete adjuvant and boosted one month later with the same preparation in Freund's incomplete adjuvant. Serum titres were monitored by immunoblot analysis and ELISA, until an anti-*M. hyopneumoniae* response was confirmed.

Rabbit hyperimmune serum was raised against recombinant proteins. Purified recombinant proteins were dialysed against PBS containing 5% glycerol and concentrated using polyvinyl-pyrrolidone (Sigma) if required. Approximately 50 mg of purified protein in a volume of 250 µL was emulsified with an equal volume of Freund's incomplete adjuvant and given subcutaneously at two sites. Booster immunisations were similarly delivered three weeks later. Serum responses were

confirmed by immunoblot against purified protein and whole *M. hyopneumoniae* cell lysates.

2.2.3.3. Immunoblot Analysis

For specific and sensitive detection of proteins Western transfer (Burnette, 1981) was performed. This involved the transfer of electrophoretically separated proteins from an acrylamide gel to a polyvinylidene difluoride (PVDF) membrane. The procedure used was a wet transfer using a TE series Transphor electrophoretic apparatus (Hoefer Scientific) set at 15-30 V overnight in Western transfer buffer. Proteins bound to the membrane were then detected by the immunoblotting procedure described by Blake *et al*. (1984).

Membranes were initially blocked for 1 h in PBS containing 5% skim milk and 0.05% Tween® 20 before the addition of primary antibody. Primary antibody (pig hyperimmune at 1/400 or rabbit hyperimmune at 1/200) was diluted in PBS containing 1% skim milk and 0.05% Tween® 20 and incubated with the membrane for 1 ½ h. The membrane was then washed (3 x 10 min) in PBS containing 0.1% skim milk and 0.05% Tween® 20. Second antibody (goat anti-pig or sheep anti-rabbit at 1/1000 dilution) was added to the membrane in the same diluent as used for the primary antibody and incubated for a further 1 h. The membrane was washed again (3 x 10 min) before development with freshly prepared DAB substrate solution.

2.2.3.4. Two-Dimensional Gel and Immunoblot Analysis

Two-dimensional gel electrophoresis was essentially carried out as described previously (Djordjevic *et al*., 2003 and 2004). First dimension immobilised pH gradient (IPG) strips (Pharmacia-Biotechnology) were prepared for focussing by submersion in rehydration buffer overnight. Proteins were extracted from *M. hyopneumoniae* by sonication in lysis buffer. *M. hyopneumoniae* cells were resuspended in 1 mL lysis buffer for each 0.1 g bacterial pellet. A Microson Ultrasonic sonicator (Misonix) was used to disrupt the pellet (6 x 30 sec pulses) before ultracentrifugation at 50 000 g for 2 h at 15°C (TLA 100.3 rotor, Beckman). *M. hyopneumoniae* whole cell protein (0.5-1.0 mg) was diluted with sample buffer (MSS or SSS) to a volume of 50 to 100 µL for application to the anodic end of each IPG strip. Isoelectric focusing was run with the Immobiline DryStrip kit in a Multiphor II electrophoresis unit (Pharmacia-Biotechnology) for 200 kVh at 20°C. IEF strips were subsequently prepared for second dimension SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in equilibration buffer. Equilibrated strips were placed onto Pharmacia ExcelGel gels (12 to 14% acrylamide) for molecular mass separation of *M. hyopneumoniae* proteins. Electrophoretic conditions consisted of 200 Volts for 1.5 h followed by 4 hours at 600 Volts. Gels were maintained at 5°C throughout. Gels were fixed in 40% methanol and 10% acetic acid prior to staining in Colloidal Coomassie overnight. Destaining followed in a 1% acetic acid solution. When blotting was to be performed, proteins were transferred to PVDF membranes prior to staining using a Hoefer TE70 transfer unit at 200 mAmps until the voltage doubled from 7 to 14 V (approximately 3 h). Immunoblot analysis followed as described in section 2.2.3.3.

2.2.3.5. N-terminal Protein Microsequencing

N-terminal protein microsequencing was carried out at at APAF, Macquarie University from western blots on an Applied Biosystems Procise Sequencer using a method described previously by Nouwens *et al*. (2000).

2.2.3.6. Protein Expression and Purification

LB medium containing appropriate antibiotics were inoculated with overnight culture of recombinant bacteria (5% of culture volume) and incubated at 37°C with shaking. These were induced for expression with IPTG (to a final concentration of 1 mM) after reaching an OD_{600} of approximately 0.6. Induction samples (1 mL) were taken initially at 0, 1, 2, 3 and 4 h and expression assessed by SDS PAGE. Cells were generally harvested for purification from 4 h induction cultures by centrifugation at 4,000 x g for 20 min. Purification of the recombinant 6xHis-tagged protein was achieved using Ni-NTA resin under denaturing conditions as outlined in the manufacturer's instructions (Qiagen). Briefly, bacterial pellets were resuspended in buffer B (containing 8 M urea) to approximately 5 mL per g wet weight and gently rocked for a minimum of 30 min at RT until lysis was complete and culture appeared translucent. Cleared lysate was obtained by centrifugation of the lysed cells at 10,000 g for 30 min. Ni-NTA superflow resin (50%) was added to the cleared lysate at approximately 1 mL per 4 mL cleared lysate and binding encouraged by rocking at RT for 1 h. The lysate-resin mixture was loaded onto an empty column and allowed to gravity pack. The flow-through was collected and not discarded until a sample had been tested for binding by SDS PAGE. The column was then washed with 4 bed volumes of wash buffer C prior to elution of

6x His-tagged protein with 2 bed volumes of elution buffer D and 4 bed volumes of elution buffer E. These conditions acted as a start point for purification and were optimised for individual *E. coli* recombinants after assessment by SDS PAGE.

2.2.3.7. Trypsin Treatment of *M. hyopneumoniae* Cells

M. hyopneumoniae cells were resuspended in sterile PBS (200 mg wet weight/mL) and aliquots (300 μL) were introduced into sterile Eppendorf tubes. Cell suspensions of *M. hyopneumoniae* and stock solutions of trypsin were pre-incubated to 37°C. Trypsin was added to the cell suspensions (in a volume less than 15 μL) to give concentrations ranging from 0.1 μg/mL to 1000 μg/mL. The suspension was gently mixed and incubated at 37°C for 20 min. Immediately after incubation the cells were lysed in Laemmli reducing solution and heated at 95°C for 10 min. Cell lysates were analysed by SDS PAGE and immunoblotting.

2.2.3.8. Triton X-100 and X-114 Extractions

Triton extractions were performed similarly to the procedure described by Bordier (1981). For Triton X-100 extraction, pelleted *M. hyopneumoniae* (strain Beaufort) cells (200 mg wet weight) were resuspended in Triton buffer and incubated at 37°C for 30 min. The cell suspensions were then centrifuged at 14,000 x g for 30 min at 4°C. The aqueous phase was removed and the pellet was resuspended in the Triton buffer and centrifuged as before. This resulted in a pellet and two aqueous phases. For Triton X-114 extraction, 200 mg of cells was resuspended in 2% Triton X-114 in TBS and rotated

at 4°C for 2 ½ h. The cell suspensions were then centrifuged at 10,000 x g for 5 min. The pellet was kept as the insoluble phase. The supernatant was incubated at 37°C for 10 min and then centrifuged at 10,000 x g for 5 min again for phase separation. An aqueous and detergent phase resulted. All phases were reduced with Laemmli buffer for SDS PAGE and Immunoblot analysis.

Chapter Three

Cloning and Characterisation of the Strain J Cilium Adhesin

3.1. Introduction

Development of a cheap and efficacious vaccine against PEP is hindered by a lack of knowledge of the pathogenic mechanisms employed by *M. hyopneumoniae*. Vaccines currently available commercially are killed whole cell preparations and are expensive to produce, require a labour intensive mode of delivery and vary in efficacy. Several trials using *M. hyopneumoniae* antigens in a vaccine formulation have shown promising results (Faulds *et al*., 1990; Fagan *et al*., 1997, Djordjevic *et al*., 1997, Fagan *et al*., 2001; Chen *et al*., 2003). The antigens generally targeted for this purpose reside in the cell membrane because they mediate all transport, sensory and physical interactions with the host environment. There is therefore a need to better understand the surface topography of *M. hyopneumoniae*.

Previous studies (Djordjevic *et al*., 1997, Djordjevic *et al*., unpublished results) have shown that *M. hyopneumoniae* antigens fractionated by preparative SDS PAGE vary in their ability to provoke a protective immune response in pigs challenged with *M. hyopneumoniae*. A preparative SDS PAGE ELISA profiling technique developed by Scarman *et al*. (1997) identified the primary immunoreactive antigens of *M. hyopneumoniae*. The profile was divided into groups of proteins based on molecular size, namely Fraction 1 (> 150 kDa), Fraction 2 (85 to 150 kDa), Fraction 3 (70 to 85 kDa), Fraction 4 (50 to 70 kDa), Fraction 5 (39 to 50 kDa), and Fraction 6 (< 39 kDa). Fractions 2 and 3 were the most immunoreactive fractions containing antigens of 76, 78, 80, 82, 94, 106, and 114 kDa. The pooled antigens of Fractions 2 and 3 were trialed in pigs as parenteral vaccines and resulted in a significant reduction in lung pathology of diseased animals (Djordjevic *et al*., unpublished data; Djordjevic *et al*., 1997). This trial

demonstrated the potential of immunoreactive antigens of *M. hyopneumoniae* as vaccine candidates.

In addition to being potentially protective, immunoreactive antigens of *M. hyopneumoniae* have also been shown to be largely surface located (Djordjevic *et al*., unpublished results). Previous genome sequencing studies have indicated that approximately 50% of genes residing in the *M. pneumoniae* and *M. genitalium* genomes encode proteins that possess transmembrane domains i.e. lipoproteins, ABC transporters, cell envelope proteins and adhesins (Fraser *et al*., 1995, Himmelreich *et al*., 1996). To identify immunoreactive surface proteins of *M. hyopneumoniae*, porcine hyperimmune *M. hyopneumoniae* antisera was used to screen whole cell lysates and to recover clones from randomly generated expression libraries of *M. hyopneumoniae* strain J. One of the clones (pAS1) expressed an immunoreactive antigen of 28 kDa. Antisera raised against the 28 kDa antigen, identified a protein of 94 kDa when used to probe a whole cell lysate of *M. hyopneumonia*e in an immunoblot. The 94 kDa antigen was a primary immunoreactive antigen of Fraction 2. This antigen was chosen for further analysis as a component of a pool of antigens that afforded a significant reduction in lung pathology when administered as a vaccine (Djordjevic *et al*., 1996).

Upon sequencing pAS1 it was revealed that the carboxy-terminal portion of a previously identified *M. hyopneumoniae* adhesin had been cloned. A number of other features of the adhesin identified in honours thesis research (Wilton, J. 1996) led to its in-depth investigation in this study. One of those features was the presence of two regions of tandem repeats within the sequence. The first repeat region (RR1) consisted of repeating units 15 bp long (encoding the amino acid sequence A(T)-K-P-E(V)-A(T))

arranged as nine tandem repeats. The second (RR2) consisted of units 30 bp long (encoding the amino acid sequence G-A(E,S)-P-N(S)-Q-G-K-K-A-E) arranged as five tandem repeats. Antigenic variation is a common phenomenon of mycoplasmal proteins and is believed to aid in surface diversity and maintenance of disease caused by *M. hyopneumoniae*. Antigenic variation can be achieved in a number of ways but usually involves repetitive sequences. The presence of these repeats in the strain J adhesin under investigation here warranted further research.

Although a recombinant adhesin antigen is expressed from pAS1, N-terminal amino acid sequence data confirmed it to encompass only RR2. It was determined by Minion *et al*. (2000) that RR1 of the adhesin mediates adherence of *M. hyopneumoniae* to swine cilia. Ideally a recombinant adhesin antigen with vaccine potential would encompass both regions of tandem repeats. The cloning, expression and purification of such an antigen and its ability to be recognised by sera from pigs that have been naturally infected with *M. hyopneumoniae* were therefore investigated.

3.2. Methods

Unless otherwise specified in the methods section below, all experimental procedures relating to this chapter were performed as described in Chapter 2 (General Methods).

3.2.1. Random Cloning and Preliminary Characterisation of the 3' end of the Strain J Adhesin Gene

3.2.1.1. Cloning of the 3' end of the *M. hyopneumoniae* strain J Adhesin Gene

M. hyopneumoniae strain J genomic DNA was extracted as described previously (Fagan *et al*., 1996) and digested with *Eco*RI. DNA was size fractionated through 10-40% sucrose gradients and fragments from 0.5-10 kb were ligated separately into *Eco*RI digested pET23a, b and c vectors (Novagen) which had been treated with calf alkaline phosphatase to prevent self-ligation. Ligation products were transformed into competent *E. coli* BL21(DE3)(pLysS) cells (Novagen), the cells inoculated into fresh LB medium and incubated for 2 h at 37°C prior to plating onto LB agar containing chloramphenicol for overnight incubation at 37°C. Colonies were then blotted onto nitrocellulose, placed on Whatman 3MM filter paper saturated with LB medium and 10 mM IPTG and incubated at 37°C for 4 h. The blots were then treated with 5.0% (w/v) SDS to lyse the cells, gently washed with PBS, blocked by incubation in 5.0% (w/v) non-fat milk in TBS and immunostained using porcine hyperimmune *M. hyopneumoniae* antiserum which had been extensively adsorbed against *E. coli* proteins as described previously (Ro *et al*., 1994). Potential positive clones were recovered from master plates and the immunoreactivity of recombinant antigens was confirmed by Western blotting.

3.2.1.2. Protein Expression and Purification of the 28 kDa Adhesin Antigen.

Recombinant *E. coli* BL21(DE3)(pLysS) cells containing pAS1 were grown to mid-logarithmic phase in LB medium containing the appropriate antibiotics prior to the addition of IPTG (10 mM). After further growth for 4 h, cells were harvested, lysed by heating to 95°C in Laemmli reducing mixture (Laemmli, 1970), and sonicated using a Branson Sonifier (10 x 30 sec, 70% duty cycle over a 5 min period at 4°C) to shear genomic DNA. Whole cell lysate (2 mL loading volume, 0.5 g wet weight) was loaded onto a preparative Model 491 Prep Cell (BioRad) column (37 mm internal diameter) containing a 12% polyacrylamide matrix with 4% polyacrylamide stacking gel and proteins separated by electrophoresis for 24 h at 40 mAmps constant current. Aliquots (15 μL) of fractions (5 mL) were analysed by immunoblotting using porcine hyperimmune antiserum against *M. hyopneumoniae* and fractions containing the immunoreactive 28 kDa antigen were pooled and concentrated by ultrafiltration (Amicon).

3.2.1.3. Preparation of Antisera Against the 28 kDa Adhesin Antigen

Polyacrylamide gels (12%) prepared with a single sample well, were loaded with prep cell-purified 28 kDa antigen (approximately 50 mg). Gels were stained with Coomassie blue and the region of gel containing the antigen band excised, frozen in liquid nitrogen, and ground with a mortar and pestle to a fine powder. Antigen was emulsified with Freund's incomplete adjuvant and then administered intramuscularly to two New Zealand White rabbits (delivering 0.75 mL to each of the hind legs and 0.25 mL to each

front leg). Booster inoculations, delivered one month later, consisted of the same dose of the pulverised polyacrylamide/antigen emulsified with Freund's incomplete adjuvant.

3.2.2. Cloning and Sequence Analysis of the 5' end of the Strain J Adhesin Gene

Two homologous *M. hyopneumoniae* adhesin gene sequences (accession numbers U27294 and U50901) were used to design primer Adh5'F. Primer Adh5'R was designed using DNA sequence derived from pAS1. Primers Adh5'F and Adh5'R (Appendix C) were used to amplify the 5' end of the *M. hyopneumoniae* strain J adhesin. A 50 µL reaction mixture comprised 200 ng chromosomal DNA, 200 µM deoxyribonucleotide triphosphates, oligonucleotide primers Adh5'F and Adh3'R (200 nM each), 2 units of *Amplitaq* gold DNA polymerase (Perkin Elmer), 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8) and 4 mM magnesium chloride. The reaction mixture was incubated at 94°C for 120 sec for 1 cycle, followed by 35 cycles of denaturation at 94°C for 20 sec, primer annealing at 59°C for 20 sec, and extension at 70°C for 90 sec. A final cycle consisting of 94°C for 20 sec, 59°C for 20 sec and 70°C for 150 sec followed. Amplification products from three independent PCR reactions were used to clone the 5' end of the gene using a TA cloning kit (Invitrogen). Ligation products were transformed into *E. coli* strain INVαF' and plated onto LB-agar containing X-Gal. Plasmid DNA was extracted from white colonies, and the presence of a 2.0 kb *M. hyopneumoniae* fragment was confirmed by restriction endonuclease digestion and agarose gel electrophoresis. A 2 kb fragment derived from each of three independent cloning experiments was sequenced (in both directions) initially using universal primers followed by a set of five

forward and four reverse primers designed from the two adhesin sequences deposited in the database.

3.2.3. Nucleotide Sequence Accession Number of the J Strain Adhesin

The DNA sequence for the *M. hyopneumoniae* strain J adhesin gene has been submitted to the GenBank database and assigned the accession number AF001398.

3.2.4. Cloning and Sequence Analysis of the Reiterated Repeat Regions

PCR was used to amplify an 820 bp DNA fragment encompassing RR1 and RR2 and was performed in a 50 μL reaction mixture containing 200 ng chromosomal DNA, 200 μM deoxyribonucleotide triphosphates, oligonucleotide primers RRF and RRR (Appendix C) at 200 nM, 2 units of *Amplitaq* gold DNA polymerase (Perkin Elmer), 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8) and 4 mM magnesium chloride. The reaction mixtures were incubated at 94°C for 120 sec for 1 cycle, followed by 25 cycles of denaturation at 94°C for 20 sec, primer annealing at 59°C for 20 sec, and extension at 70°C for 90 sec. A final cycle consisting of 94°C for 20 sec, 59°C for 20 sec and 70°C for 150 sec followed. Three independent PCR reactions were performed for *M. hyopneumoniae* strains J, 232, Beaufort, Sue, C1735/2 and OMZ407 and the amplification product from each PCR was cloned using the TA cloning kit. DNA encoding the reiterated repeat regions was sequenced in both directions initially using universal primers and with internal primers designed from sequence data generated from pAS1.

3.2.5. Southern Hybridisation Analysis Using a Radiolabelled Probe

*Eco*RI digested *M. hyopneumoniae*, *M. hyorhinis*, and *M. flocculare* genomic DNA were electrophoretically separated, blotted and probed using an 820 bp PCR fragment amplified using primers RRF and RRR. The PCR product was labelled with ^{32}P by nick translation according to standard procedures (Sambrook *et al*., 1989). Radiolabelled blots were stringently washed 4 times for 30-60 min at 65°C. The first two washes consisted of a solution of 1 mM EDTA, 40 mM $NaHPO_4$ and 5.0% (w/v) SDS, pH 7.2, and the second two washes consisted of a solution of 1 mM EDTA, 40 mM $NaHPO_4$ and 1.0% (w/v) SDS, pH 7.2.

3.2.6. Sub-unit Antigen ELISA

In the subunit antigen ELISA, 96-well microtitre plates (Flow laboratories) were coated with 100μL of purified protein diluted to a concentration of 1 μg/mL in carbonate coating buffer. These were incubated overnight at RT in a moisture chamber. Antigen coated plates were washed 5 times in PBS containing 0.05% Tween 20 using a SLT 96PW plate washer. Primary antibody (pig sera) was diluted 1/100 in PBS containing 2% skim milk (w/v) and 0.05% Tween 20, 100 μL was added to each well, and the plates were returned to the moisture chamber for 2 h at RT. Plates were washed again in PBS containing 0.05% Tween 20 before the addition of anti-pig HRP conjugate (diluted 1/1000 in PBS containing 2% skim milk (w/v) and 0.05% Tween 20) was added to each well (100 μL). The plates were then incubated in the moisture chamber for 1h at RT. The plates were washed again before development with ABTS substrate solution. Plates

were agitated and read over time at 405 nm on an automated plate reader (Labsystems Multiscan® BICROMATIC).

3.3. Results

3.3.1. Preliminary Analysis of the *M. hyopneumoniae* Strain J Adhesin

When sequenced, the clone expressing the immunoreactive 28 kDa antigen (pAS1) was determined to encode the carboxy-terminal portion of a *M. hyopneumoniae* adhesin from strain J (Wilton, J. Honours thesis, 1996). Interestingly, two regions of tandem repeats were identified in the cloned portion of the adhesin gene. The 28 kDa antigen was being expressed from an internal methionine residue in the sequence and encoded the second region of repeats within the adhesin (Figure 3.1). Antiserum was raised against the purified 28 kDa antigen (anti-28 kDa sera) and used in this research to further characterise this gene product.

3.3.1.1. Surface location of the *M. hyopneumoniae* Strain J Adhesin

The cilium adhesin antigen was expected to be located on the surface of *M. hyopneumoniae* (Zhang *et al*., 1995). An immunoblot of trypsin treated *M. hyopneumoniae* whole cells probed with antisera raised against the terminal 28 kDa of the adhesin confirmed this expectation for J strain *M. hyopneumoniae*. As can be seen in Figure 3.2 (A), the adhesin is readily digestible by trypsin, declining in intensity when subjected to trypsin concentrations of 0.1 to 1.0 μg/mL (lanes 1 to 5) and disappearing completely at greater concentrations. A second product of 30 kDa detected by the antisera can also be seen in the immunoblot and follows the same pattern as the 94 kDa

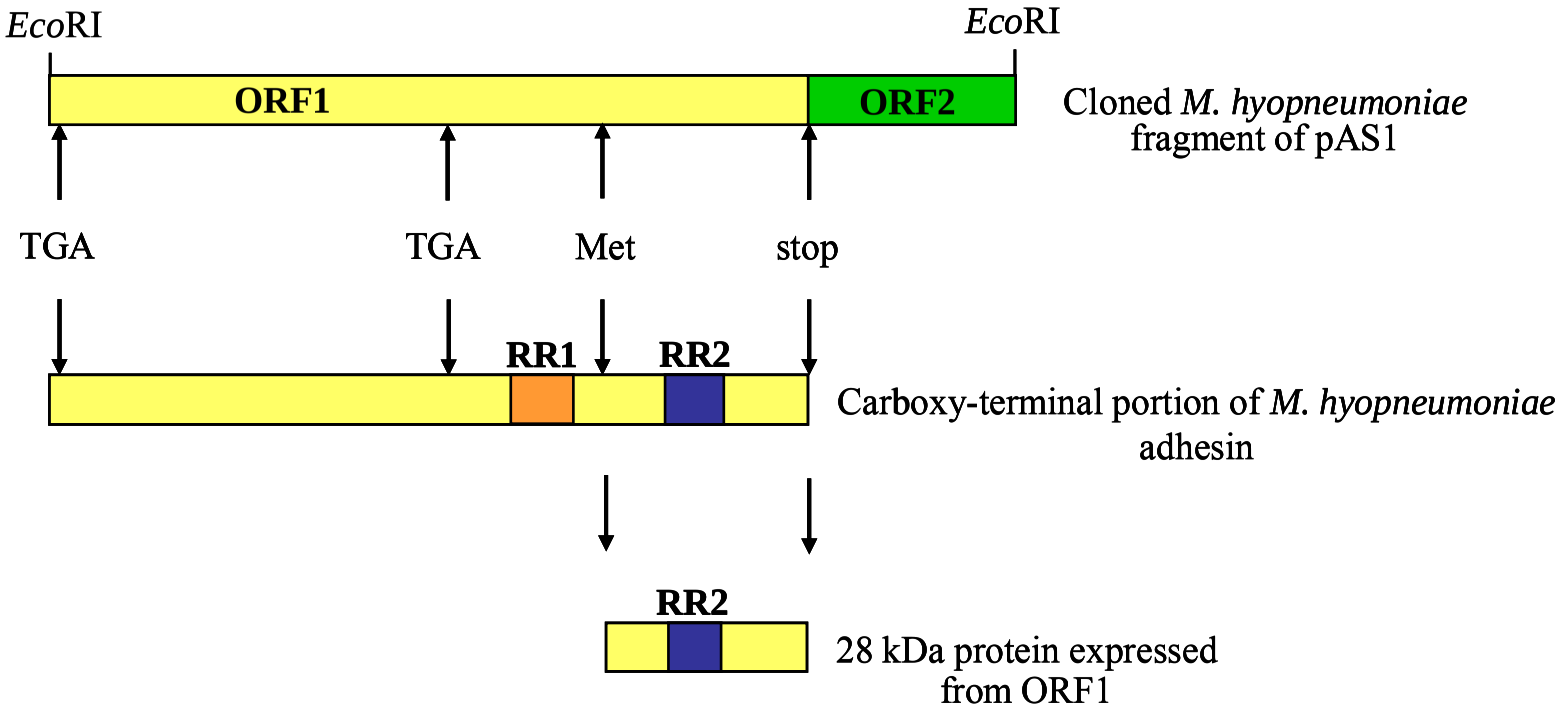


Figure 3.1. Gene map of the cloned *M. hyopneumoniae* fragment of pAS1. The *Eco*RI fragment of pAS1 contained two open reading frames, ORF1 and ORF2 shown in yellow and green respectively. ORF1 encoded the carboxy-terminal 1700 bp of the *M. hyopneumoniae* adhesin. The adhesin contained two regions of tandem repeats, designated RR1 and RR2 as shown by the hatched boxes. Two UGA codons were identified in ORF1 preventing full-length expression in *E.coli* but the internal methionine (met) residue located between RR1 and RR2 allowed expression of a 28 kDa antigen encoding RR2.

protein. This antigen may be a breakdown or cleavage product of the carboxy-terminus of the protein.

The integrity of the whole *M. hyopneumoniae* cells used in the experiment was confirmed by reprobing the immunoblot with rabbit antiserum raised to a purified preparation of recombinant *M. hyopneumoniae* lactate dehydrogenase (A.L. Scarman, G.J. Eamens, and S.P. Djordjevic, unpublished results). Lactate dehydrogenase (36 kDa) has been shown to reside in the cytosol in *M. hyopneumoniae* (Strasser *et al*., 1991). An antigen of approximately 36 kDa was observed with equal staining intensity in all preparations of trypsin-treated whole cells confirming that neither trypsin nor the procedure used caused cell lysis (data not shown).

3.3.1.2. Strain Variation in the Adhesin Antigen

To confirm the expression of the adhesin antigen in a number of geographically diverse strains of *M. hyopneumoniae*, whole cell lysates of seven strains of *M. hyopneumoniae* were probed in an immunoblot with rabbit anti-28 kDa sera. Whole cell lysates of the related porcine mycoplasmas *M. hyorhinis* and *M. flocculare* were also probed with the antisera. The result is shown in Figure 3.2 (B). Antigens of 30 and 94 kDa were identified by the anti-28 kDa sera in whole cell lysates of *M. hyopneumoniae* strain J, as seen in the whole cell lysates of the trypsin treated cells. However, only a single antigen was observed in *M. hyopneumoniae* strains 232, Beaufort, Sue, C1735/2 and OMZ407. A similar sized antigen can also be seen for strain YZ, but it has an additional (faint) band at approximately 75 kDa. The lesser reaction observed for C1735/2 can be explained by a reduced loading as determined by an identical coomassie stained gel

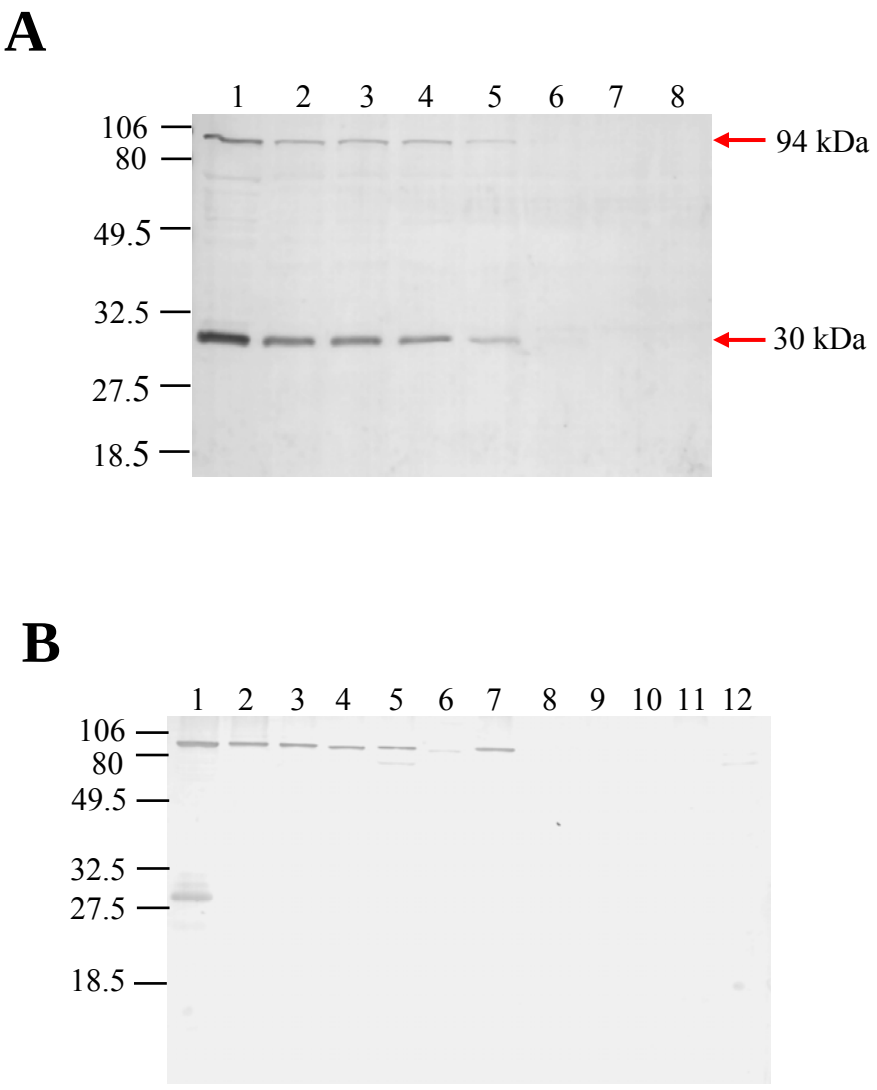


Figure 3.2. Surface location and strain variation of the cilium adhesin. (A) Surface location of the adhesin antigen was determined through the treatment of whole *M. hyopneumoniae* (strain J) cells with trypsin. The immunoblot shows cell lysates of *M. hyopneumoniae* whole cells treated with 0, 0.1, 0.3, 0.5, 1, 2, 3, and 5 μg of trypsin per ml (lanes 1-8 respectively) as separated in 12% polyacrylamide, transferred to PVDF, and reacted with anti-28 kDa sera. (B) Anti-28 kDa serum was also reacted with whole cell lysates of 12 porcine mycoplasma strains. The immunoblot shows *M. hyopneumoniae* strains J, 232, Beaufort, Sue, YZ, C1735/2, and OMZ407 (lanes 1 – 7 respectively), *M. hyorhinis* strains field strain 1, GDL, BTS-7, and field strain 2 (lanes 8 –11 respectively), and *M. flocculare* type strain MS42 (lane 12), were electrophoresed in 12% polyacrylamide and transferred to PVDF for their reaction with antisera. Molecular markers are given in kDa.

(data not shown). Also evident in the immunoblot was size variation (94 to 97 kDa) in the adhesin antigen amongst the geographically diverse strains of *M. hyopneumoniae*. Importantly, the protein also seems unique to *M. hyopneumoniae* with only very weak cross-reactivity being observed with the whole cell lysate of *M. flocculare* strain MS42. No cross-reactivity was evident in the whole cell lysates of the four *M. hyorhinis* strains probed.

3.3.1.3. Repeat Region Variability Leading to Strain Variation in the Adhesin

An alteration in the number of repeats between strains was the hypothesised cause of the observed size variation in the adhesin antigen amongst the strains of *M. hyopneumoniae* used in this study. The regions containing RR1 and RR2 from the geographically diverse strains of *M. hyopneumoniae* were subsequently subjected to PCR and Southern blot analysis. Figure 3.3 (A) shows the carboxy-terminal end of the adhesin containing RR1 and RR2 and approximate location of the primers used for PCR amplification.

A DNA fragment spanning the two regions of repeats was successfully amplified from genomic DNA of all seven strains of *M. hyopneumoniae* (Figure 3.3 (B), lane 1-7). The single amplification product ranged in size from 750 to 900 bp among strains. A similar fragment was unable to be amplified from genomic DNA of four *M. hyorhinis* strains or a single type strain of *M. flocculare* (Figure 3.3 (B), lanes 8-12). This suggested that the 94 kDa adhesin gene was not present in the genomes of these two phylogenetically related porcine mycoplasmas which inhabit the respiratory tract of pigs (Stemke *et al*., 1992). The result was confirmed when the 820 bp J strain amplification product was used to probe *Eco*RI digested genomic DNA of the same mycoplasma species (Figure

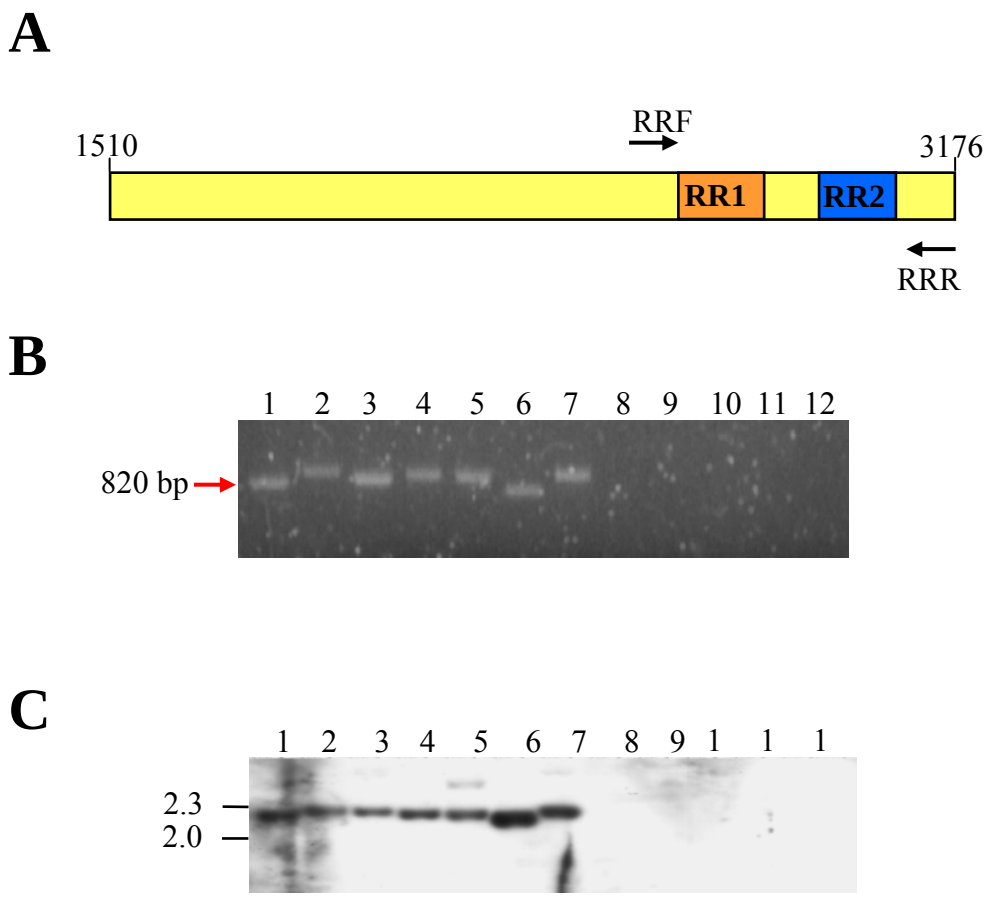


Figure 3.3. Repeat region variability in the adhesin antigen. The diagram in (A) shows the cloned carboxy terminus of the *M. hyopneumoniae* adhesin, indicating the position of the repeat regions (RR1 and RR2) and approximate location of the primers (RRF and RRR) used to PCR amplify a fragment spanning both sets of repeats from 12 porcine mycoplasmas. PCR products (visualised on a 1.5% agarose gel with ethidium bromide staining) of an amplification using primers RRF and RRR are shown in (B). A fragment ranging in size from 750-900 bp was amplified from *M. hyopneumoniae* strains J, 232, Beaufort, Sue, YZ, C1735/2, and OMZ407 (lanes 1 – 7 respectively). No fragment was amplified from *M. hyorhinis* strains field strain 1, GDL, BTS-7 and field strain 2 (lanes 8 –11 respectively), or *M. flocculare* type strain MS42 (lane 12). The 820 bp fragment spanning RR1 and RR2 from *M. hyopneumoniae* strain J was used to probe EcoRI digested genomic DNA from the same porcine mycoplasmas in a Southern blot (C) and a similar pattern was observed. Sizes in bp and kb are indicated on the left for (B) and (C) respectively.

3.3 (C)). Under stringent conditions, the PCR fragment was only able to hybridise to the seven strains of *M. hyopneumoniae*. A predominant hybridisation product ranging in size from 2.15 to 2.30 kb was observed amongst the *M. hyopneumoniae* strains suggesting that different strains of *M. hyopneumoniae* may produce an adhesin with variable numbers of oligonucleotide repeats within RR1 and RR2. Several weakly hybridising bands were also observed with DNA from strains 232, YZ, C1735/2 and OMZ407 and a single very faint additional band was observed in the strain J DNA (data not shown).

3.3.2. Cloning and Sequence Analysis of the 5' end of the *M. hyopneumoniae* Strain J Cilium Adhesin Gene

To facilitate a comparison of the gene encoding the 94 kDa *M. hyopneumoniae* antigen with genes encoding adhesins P97 (from strain 232) and Mhp1 (from strain P5722), it was necessary to clone and sequence the 5' end of the J strain adhesin gene. To do so, the 5' end of the gene encoding the 94 kDa protein was amplified by PCR using primers Adh5'F and Adh5'R, cloned into pCR®2.1 and sequenced. A diagram of the adhesin gene showing the 3' region already cloned and the 5' region to be cloned.

3.3.2.1. Cloning of the 5' end of the Gene Encoding the J Strain Adhesin

The 5' end of the adhesin gene to be amplified by PCR primers Adh5'F and Adh5'R is shown in Figure 3.4. Because *Taq* polymerase, which has no proofreading function, was used as the amplification enzyme, three independent PCR reactions and clonings were

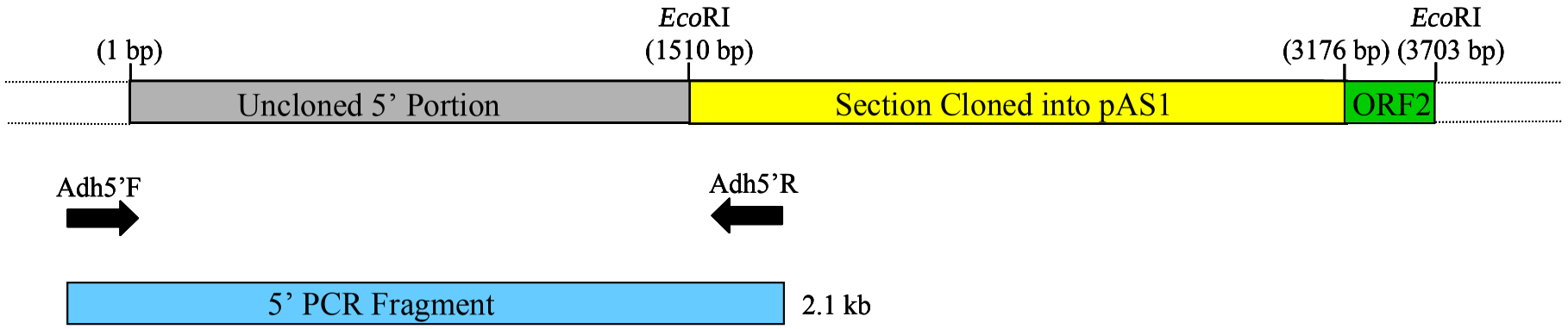


Figure 3.4. Gene map of the adhesin showing primers used for amplification of a 5' fragment of the gene. The original cloned portion of the adhesin gene is shown in yellow, the green box represents ORF2 of the pAS1 clone, and the grey box indicates the uncloned portion of the adhesin gene. The large black arrows indicate the primers (Adh5'F and Adh5'R) used to amplify the 5' fragment (blue box) for sequence completion.

required to ensure sequencing was performed without errors. Figure 3.5 (A) shows the PCR products as electrophoresed in 1% agarose and stained with ethidium bromide. On the gel, the PCR product appears as a band of approximately 2.1 kb. Amplication with *Taq* polymerase allowed the PCR products to be cloned directly into pCR®2.1 (Invitrogen). As a cloning vector with A overhangs, pCR®2.1 exploits the T overhangs left by *Taq*. The procedure performed to the manufacturer's instructions was very efficient, with greater than 80% of the clones screened carrying inserts (data not shown).

Figure 3.5 (B) shows an ethidium bromide stained agarose gel of the three independent positive clones that were used for sequencing. They were extracted by QIAGEN midi columns from their *E. coli* hosts, and digested with *Eco*RI for visualisation of the *M. hyopneumoniae* insert DNA. The pCR®2.1 vector is 3.9 kb in size and can be seen in the digestions just below the 4.4 kb marker. The insert is seen as two fragments of 1.8 kb and 0.3 kb due to the presence of an *Eco*RI site within the *M. hyopneumoniae* sequence. The three clones were sequenced and compared for errors before a final 5' adhesin gene sequence was assembled.

3.3.2.2. Sequence Analysis of the J Strain Adhesin

The previously sequenced 3' end of the adhesin gene was assembled with the sequence of the 5' end to give the complete J strain adhesin DNA sequence. The gene encoding the J strain adhesin of 3279 bp translated to a protein of 1093 amino acids. TGA codons are located at amino acid positions 72, 546 and 766 and are bolded. The two repeat regions are shown in orange and blue as repeating units. The predicted

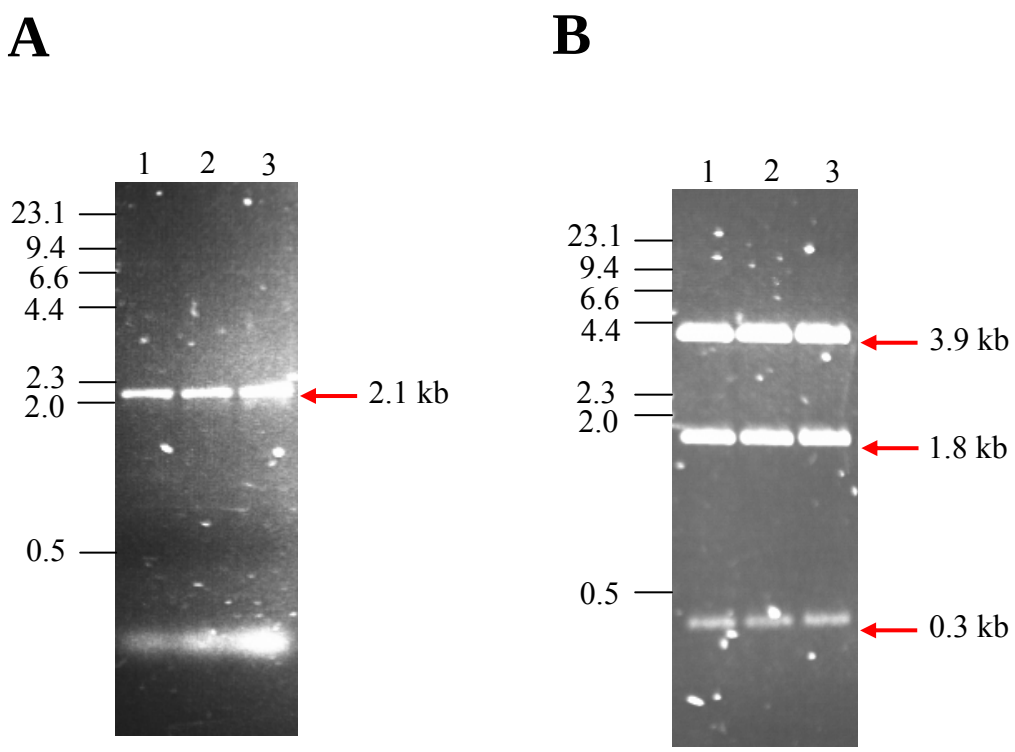


Figure 3.5. PCR amplification and cloning of the 5' end of the adhesin gene. (A) Shows the 2.1 kb amplification product from three independent PCR reactions as electrophoresed in 1% agarose and stained with ethidium bromide. The amplification products in (A) were used in three separate cloning experiments. A representative positive clone for each is shown in (B) digested with *Eco*RI, electrophoresed in 1% agarose and stained with ethidium bromide for visualisation. Three fragments are seen representing the vector (3.9 kb) and 5' adhesin insert (1.8 and 0.3 kb) DNA. Molecular size markers are given in kb on the left.

size of the 94 kDa antigen based on the complete sequence is 123 kDa, which is similar to the calculated size reported for P97 and Mhp1 (124.9 and 124.2 kDa respectively). The first 32 amino acids of the 94 kDa antigen had features consistent with a prokaryotic signal sequence including the presence of a cluster of positively-charged amino acids (K) followed by a stretch of hydrophobic residues ending with a signal peptidase II cleavage site (data not shown). The absence of a cysteine residue immediately after the cleavage site or anywhere within the deduced amino acid sequence discounted the possibility that the 94 kDa antigen was a lipoprotein (Wieslander *et al*., 1992).

3.3.3. Computational Analysis of the Deduced Amino Acid Sequence of the J Strain Adhesin

The deduced amino acid sequence of the J strain adhesin was subject to a number of computational analyses using various programs on the EMBnet website and the MacVector software. From the deduced sequence, the J strain adhesin was determined to be 1092 amino acids in length with an estimated pI of 8.81. It was rich in the amino acids lysine and leucine (13.2% and 9.6% respectively) and had very few tryptophan, methionine and histidine residues (0.5%, 0.6% and 0.7% respectively). The entire molecule was free from the amino acid cysteine.

The adhesin is a highly hydrophilic molecule with a small region of hydrophobicity at the N-terminus (Figure 3.6 (A)). That small but significant region of hydrophobicity was hypothesised as a possible transmembrane domain using the TMpred program on the ISREC ProfileScan server (Figure 3.6 (B)). A score of 1752 for an inside to outside

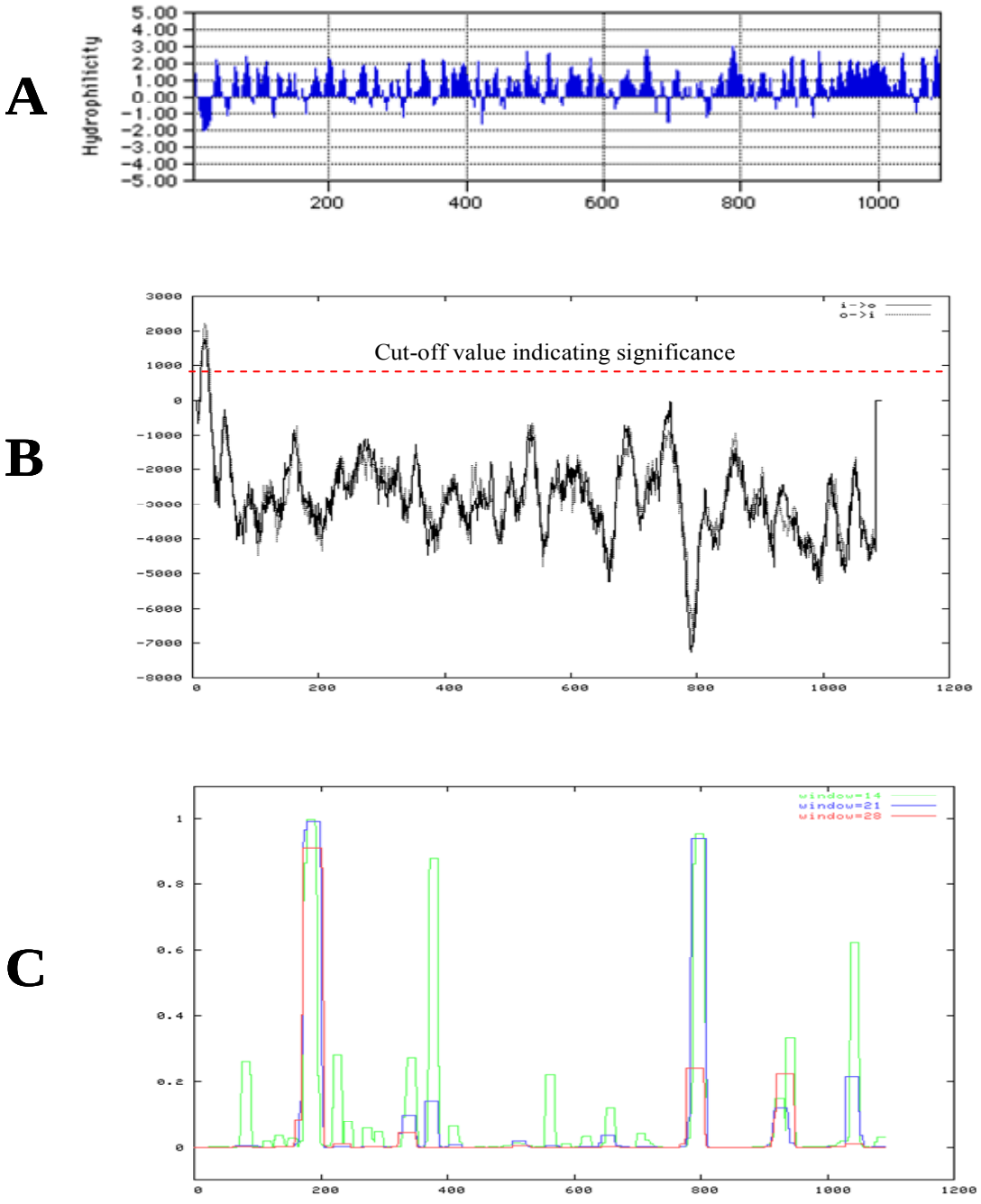


Figure 3.6. Computational analysis of the deduced amino acid sequence of the strain J adhesin. The hydrophilicity (A) plot was determined using MacVector. A graph of the transmembrane domain prediction is given in (B) as determined using the TMPred program on the ISREC ProfileScan server. Also determined using the ISREC ProfileScan server and the COILS program was the potential coiled-coil domains (C) of the adhesin which have been predicted at window sizes of 28 (red), 21 (blue) and 14 (yellow).

helices (amino acids 13 to 29) and 2202 for an outside to inside helices (amino acids 10 to 31) suggests a significant transmembrane domain (cut-off value for significance is 500). In addition to this observation, two significant coiled-coil regions were predicted using COILS on the ISREC ProfileScan server. Both were at a window size of 21 residues, the first at amino acid position 172 to 204 with a probability of 0.89 and the second at amino acid position 778 to 806 with a probability of 0.58 (Figure 3.6 (C)). A number of other coiled-coil domains were also identified at window sizes of 28 and 14 residues as can be seen in Figure 3.6 (C) but these were less significant.

3.3.4. Alignment of P94 with P97 and Mhp1

The predicted amino acid sequence of the 94 kDa antigen was aligned with the amino acid sequences of Mhp1 and P97 for comparative analysis (Figure 3.7). The 94 kDa antigen had 95.7% sequence homology with Mhp1, and 93.3% sequence homology with P97, when aligned at the amino acid level. The majority of differences reside in repeat regions 1 and 2 (spaced as repeating peptide units in orange and blue respectively). The J strain adhesin contained nine copies of the 5 amino acid repeat and five copies of the 10 amino acid repeat whilst Mhp1 (strain P5722) had twelve copies of the 5 amino acid repeat and five copies of the 10 amino acid repeat (King *et al*., 1996). P97 (strain 232 L127) had fifteen copies of the 5 amino acid repeat and only four copies of the 10 amino acid repeat (Hsu *et al*., 1997).

This data supports the earlier suggestion that differences in the number of repeats are the cause of the observed size variation in the adhesin among different strains of *M. hyopneumoniae*. PCR and Southern blot analysis of a region spanning the repeats in the

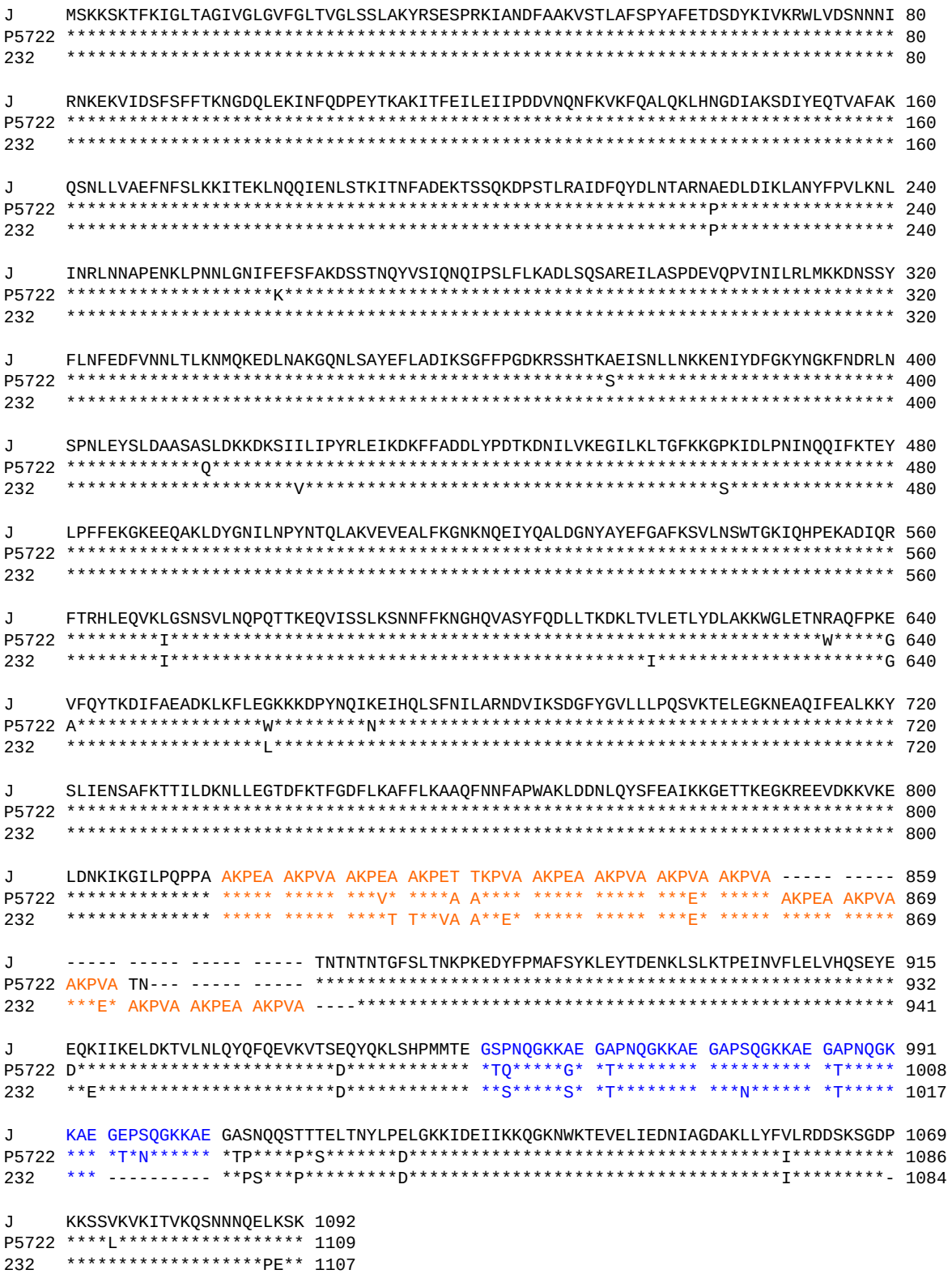


Figure 3.7. Protein alignment of the J strain adhesin with adhesins P97 from strain P5722 and MHP1 from strain 232. Amino acid numbers are given to the right, homologous amino acids are denoted by an asterick (*), the dashed lines represent deletions, and repeat regions are spaced as repeating peptide units. RR1 is shown as orange text and RR2 as blue text.

strains of *M. hyopneumoniae* used in this study indicate that this is the case for the *M. hyopneumoniae* strains that have not yet been sequenced (Figure 3.3). Cloning and DNA sequence analysis of the repeat regions from these strains would confirm this likely hypothesis. Important to note is the fact that the repeat regions are rich in proline. Many proline-rich sequences have been demonstrated to be involved in binding processes, a large number of which are located on the cell surface (Williamson, 1994; and references therein).

3.3.5. Cloning and Sequence Analysis of the Repeat Regions from Geographically Diverse Strains of M. hyopneumoniae

A PCR product spanning RR1 and RR2 was amplified from the DNA of *M. hyopneumoniae* strains Beaufort, Sue, YZ, C1735/2 and OMZ407 using primers RRF and RRR (see Figure 3.3 (A) and (B)). Like the 5' end of the adhesin gene, the PCR products were amplified with *Taq* polymerase. They were therefore each cloned three times independently, into pCR®2.1 for sequencing. After each independent clone was sequenced, the data was aligned and the final sequence assembled with confidence. The sequences were then aligned with that of the already sequenced J strain, and strains 232A and P5722 from the ANGIS database. Figure 3.8 shows the alignment of the deduced amino acid sequences.

The deduced amino acid sequences showed considerable variation, particularly in the number of tandem five amino acid repeats of RR1. As outlined earlier, strain J possessed 9 copies of the 5 amino acid repeats of RR1 and 5 copies of the 10 amino acid

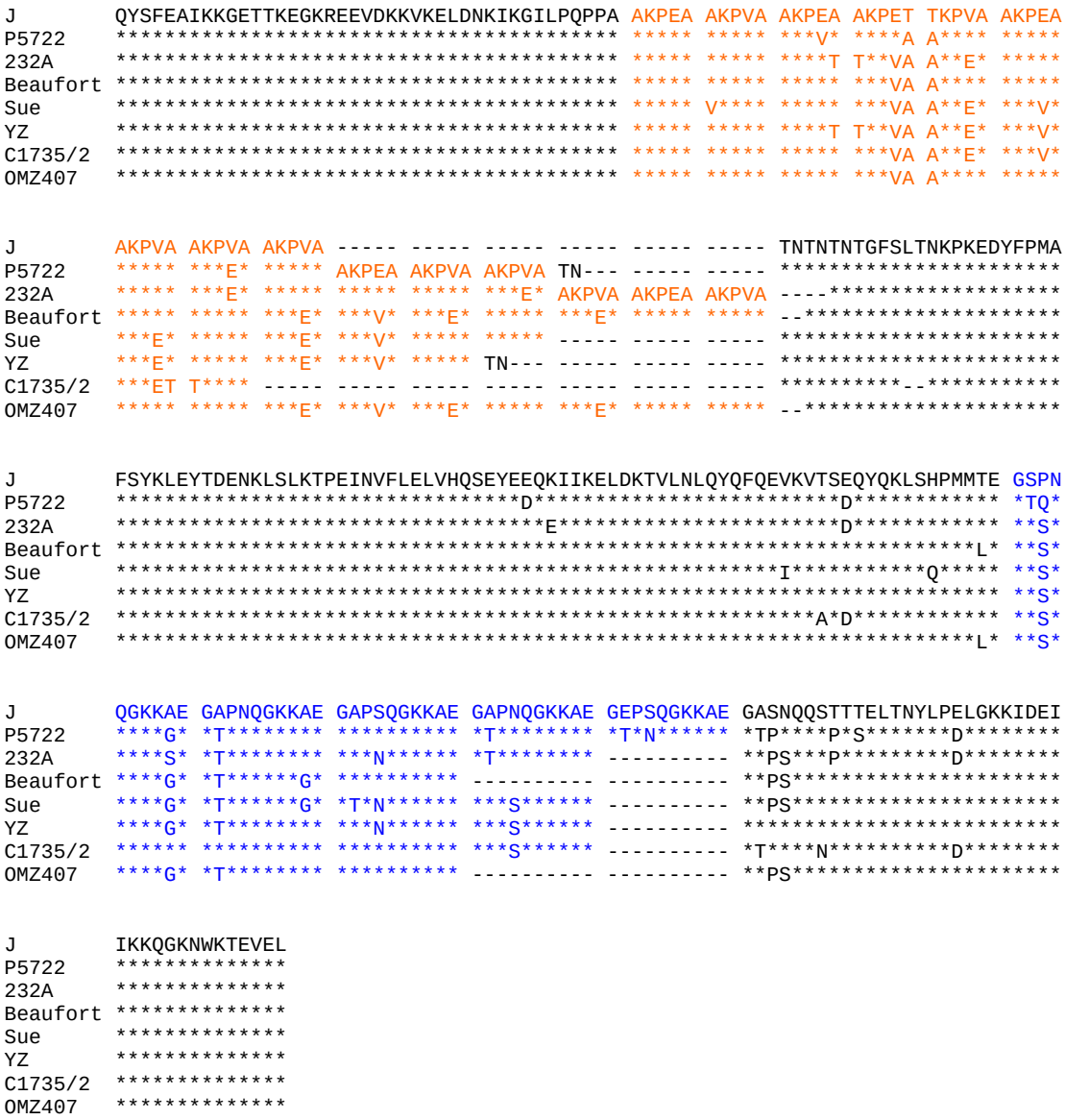


Figure 3.8. Predicted amino acid sequences of PCR amplification products spanning RR1 and RR2 from eight strains of *M. hyopneumoniae* originating from different geographic localities. Repeat regions are spaced as oligopeptide units with homologous amino acids denoted by an asterick (*) and deletions represented by a dashed line (-). RR1 is shown as orange text and RR2 as blue text.

repeat of RR2. Australian strains Beaufort and OMZ407 possessed 15 tandem copies of the five amino acid repeat in RR1 and 3 tandem copies of the ten amino acid repeat in RR2 similar to previously sequenced strain 232A. Australian strain Sue possessed 12 tandem copies of the five amino acid repeat in RR1 and 4 tandem copies of the ten amino acid repeat in RR2 as for the previously sequenced strain P5722. French strain YZ possessed 11 tandem copies of the five amino acid repeat and 4 tandem copies of the ten amino acid repeat in RR1 and RR2 respectively. Finally, Australian isolate C1735/2 possessed the fewest number of tandem copies (8) of the five amino acid repeat in RR1 and contained 4 tandem copies of the ten amino acid repeat in RR2.

3.3.6. Expression of an Adhesin Antigen Encompassing RR1 and RR2

As an immunodominant surface antigen of *M. hyopneumoniae* with a significant role in binding, the adhesin is an ideal target for vaccine development. Although a recombinant adhesin antigen has been expressed, it only contains RR2 and doesn't take advantage of the standard expression and purification systems available. RR1 mediates the adherence of *M. hyopneumoniae* to swine cilia (Minion *et al*., 2000) and being rich in proline means it has high immunogenic potential. It would therefore be ideal to express a recombinant antigen encompassing both repeat regions. There are no TGA codons present in the carboxy-terminal 547 amino acids of the adhesin encompassing RR1 and RR2 making expression of this particular portion of the adhesin in *E.coli* possible.

The pQE expression system (Qiagen) was chosen for cloning because it tagged the antigen with six histidine residues to allow its purification by nickel affinity column.

Fragments were cloned initially into pPCR-Script and sequenced prior to sub-cloning into pQE9 for expression.

3.3.6.1. Primer Design and PCR Amplification

Up to this point, all analysis of the adhesin has been performed on the non-pathogenic strain J of *M. hyopneumoniae*. The region of the adhesin to be expressed was amplified from strains J and Beaufort because expression from a virulent strain of *M. hyopneumoniae* was preferred as a vaccine antigen. Primers were designed from the J strain sequence, the forward (AdhHisF) just inside the TGA at amino acid position 766, and the reverse (AdhHisR) just inside the stop codon at amino acid position 1093. It is a region encompassing both sets of tandem repeats as indicated in Figure 3.9 (A).

Primers AdhHisF and AdhHisR (Appendix C) were used to amplify a product of approximately 0.9 kb from strains J and Beaufort as shown in Figure 3.9 (B). The PCR products were cloned separately into pCR-Script for sequencing. A sequence correct J and Beaufort strain clone was chosen for sub-cloning into pQE9 for expression.

3.3.6.2. Cloning for Expression

For directional cloning into pQE9, the sequence positive pCRScript clones and pQE9 vector were digested with *Bam*HI and *Hin*dIII. The digested products were electrophoresed in agarose and the *Bam*HI/*Hin*dIII digested pQE9 vector and insert DNA as released from the pPCR-Script clones were gel extracted (Bresa-Clean) for ligation. The ligation mix was used to transform M15 [pREP4] cells. Positive clones were determined by plasmid extraction and digestion (Figure 3.10 (A)). The vector

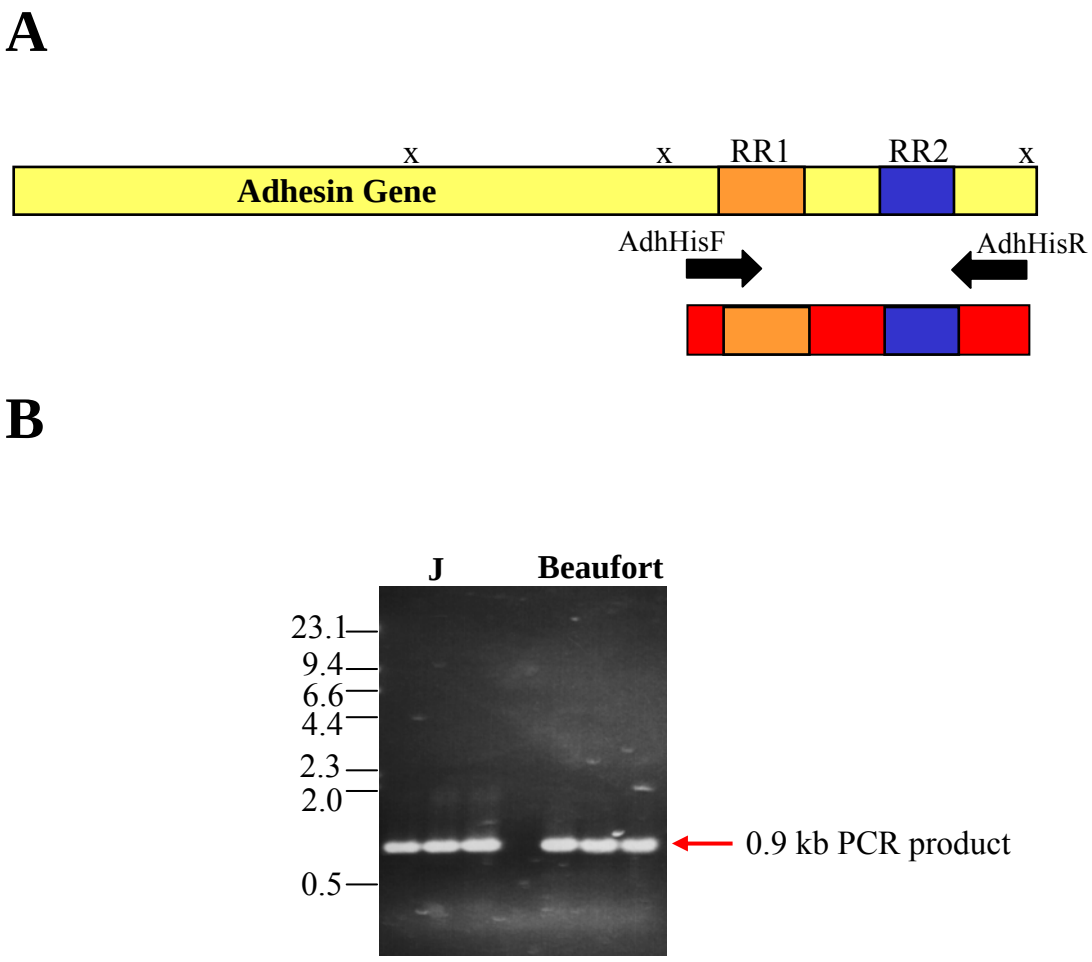


Figure 3.9. Amplification of an adhesin gene fragment for cloning and expression. (A) Gene diagram of the adhesin. TGA codons are given as X's above the gene and the repeat regions RR1 and RR2 are indicated. The region to be amplified by primers AdhHisF and AdhHisR is represented by a red box and encompasses both regions of repeats. (B) Ethidium bromide stained agarose gel (1%) of *M. hyopneumoniae* strain J and Beaufort amplification products for cloning. Markers are given in kb on the left and the 0.9 kb PCR product is indicated by a red arrow.

(pQE9) can be seen at 3.4 kb and the *M. hyopneumoniae* insert at 0.9 kb for both strain J and Beaufort clones.

Preliminary inductions were performed on both strain J and Beaufort expression clones. Growth comparisons of induced and non-induced cultures over a four hour induction period indicated growth was unaffected by induction of expression of the recombinant adhesin antigen (data not shown). A single Beaufort clone was chosen for expression and purification on a larger scale. Purification of the recombinant protein from a 4 h induction culture (200 mL) of this clone was monitored by SDS PAGE analysis. The result is shown in Figures 3.10 (B).

The recombinant 35 kDa antigen is observed in high concentration in the cell lysate (lane 1), as expected, which confirmed expression. A small amount of the recombinant antigen was detected in the flow-through fractions (lanes 2 and 3) indicating that the resin may have been saturated or the protein was not given sufficient time to bind to the resin. Wash fractions (lanes 4 and 5) also show a small amount of recombinant antigen amongst the non-specifically bound contaminant proteins. Elution was performed as a two-step process using buffers differing in pH. The recombinant protein is not completely released from the column until the addition of the elution buffer with the lower pH (lanes 8 to 12). The process in general was a success but required optimisation of conditions.

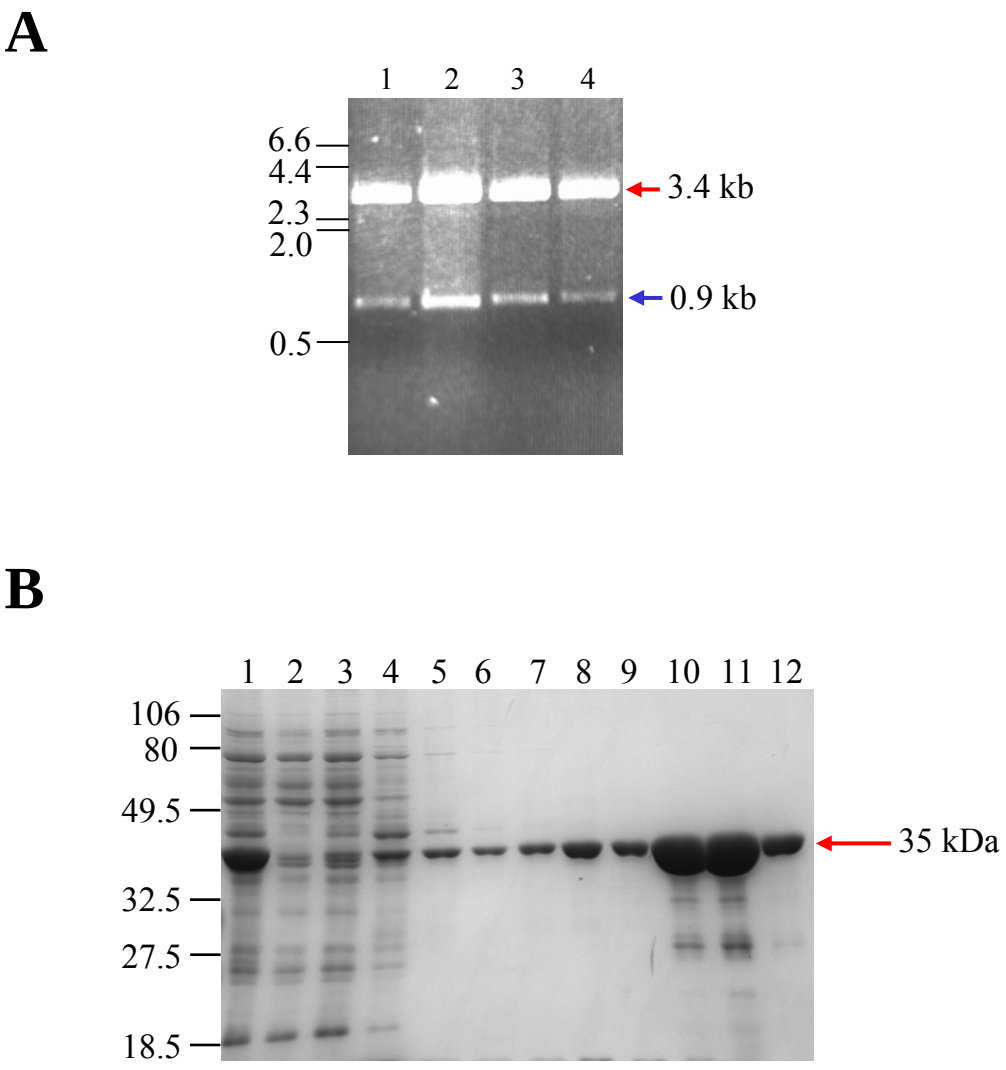


Figure 3.10. Cloning the adhesin gene fragment for expression and purification. (A) Ethidium bromide stained agarose gel (1%) of digested (*Bam*HI and *HindIII*) plasmid DNA from strains J (lanes 1 and 2) and Beaufort (lanes 3 and 4) pQE9 clones. Vector (pQE9) DNA is shown by a red arrow at 3.4 kb and adhesin insert DNA by a blue arrow at 0.9 kb. Markers are given in kb on the left. (B) SDS PAGE of nickel affinity purification fractions showing an induced whole cell lysate in lane 1, flow through fractions in lanes 2 and 3, wash fractions in lane 4 and 5, and elution fractions in lanes 6 to 12 (6-8 at pH 5.9 and 9-12 at pH 4.5). Molecular weight markers are given on the left and the recombinant 35 kDa protein is indicated by a red arrow on the right.

3.3.7. Immunogenicity of the Adhesin Antigen

As an immunoreactive, surface antigen of *M. hyopneumoniae*, the adhesin was a good candidate for development as a vaccine antigen. Sera from pigs designated *M. hyopneumoniae* positive by ELISA, were able to recognise the recombinant His-tagged adhesin antigen in Western blots, indicating the production of antibodies against the adhesin in naturally infected animals. Sera from six *M. hyopneumoniae* positive pigs and three *M. hyopneumoniae* negative pigs was used to probe the 35 kDa recombinant adhesin in a decaprobe Western. The result is shown in Figure 3.11. Sera from all six *M. hyopneumoniae* positive pigs identified the adhesin antigen, but differed in intensity. Sera from four pigs showed a reasonably strong reaction while the other two reacted only weakly. Unexpectedly, sera from two of the three *M. hyopneumoniae* negative pigs also showed a weak reaction with the recombinant 35 kDa antigen which could reflect the different disease profiles followed by different animals.

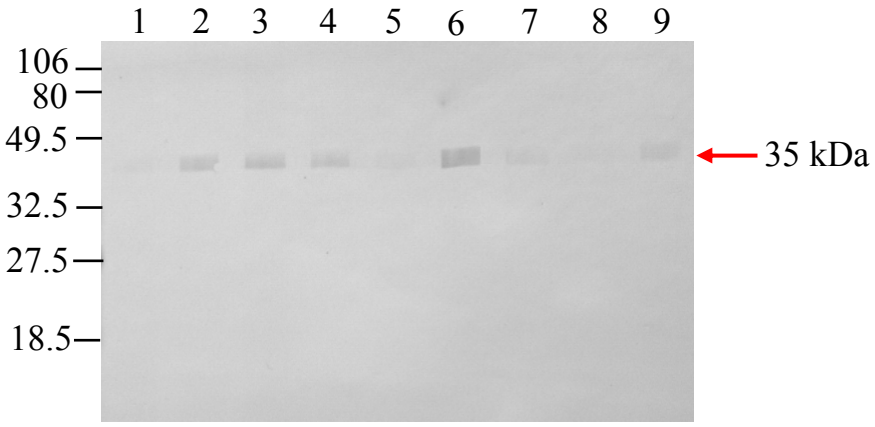


Figure 3.11. Potential of the adhesin as a vaccine antigen. Decaprobe Western blot of recombinant 35 kDa adhesin antigen reacted with sera from pigs positive (lanes 1 to 6) and negative (lanes 7 to 9) for *M. hyopneumoniae* infection as determined by diagnostic ELISA. Markers are shown on the left in kDa and the 35 kDa adhesin antigen by a red arrow.

3.4. Discussion

Immunoreactive clone pAS1 was discovered in honours research (Wilton, J. 1996) to encode the 3' end of the strain J ciliary adhesin (P94) of *M. hyopneumoniae* when sequence data generated from the clone was subjected to a homology search of Genbank (using the ANGIS facility). The two sequences with which it showed significant homology were P97 (Hsu *et al*., 1997), from pathogenic stain 232, and Mhp1 (King *et al*., 1997), from strain P5722. Earlier, Zhang *et al*. (1995) had described a 97 kDa *M. hyopneumoniae* (strain 232) adhesin (P97) which resided in a "fuzzy" layer external to the surface of the organism when (i) a monoclonal antibody (MAb F1B6) recognising the 97 kDa protein in several strains of *M. hyopneumoniae* inhibited the adherence of *M. hyopneumoniae* to cilia (ii) purified P97 adhered to cilia and blocked the subsequent adherence of intact *M hyopneumoniae* cells and (iii) proteins to which the monoclonal antibody reacted were located on the surface of *M. hyopneumoniae* cells. Mhp1 has been designated the P5722 strain equivalent and P94 the J strain equivalent of this protein.

Identified within the strain J adhesin and that of strains 232 and P5722 were two regions of tandem repeats. The first region of repeats (RR1) contained a five amino acid repeat (A(T)-K-P-E(V)-A(T)) that was repeated 9 times in the highly-passaged non-pathogenic strain J of *M. hyopneumoniae*, 15 times in the lower passage and virulent strain 232, and 12 times in the P5722 strain of unknown virulence. The ten amino acid repeat (G-A(E,S)-P-N(S)-Q-G-K-K-A-E) of RR2 was less variable between the strains with strains J and P5722 carrying 5 copies, and 232 only 4 copies. When the entire J strain adhesin gene was sequenced (through cloning of the 5' end of the gene), it was

determined that there was 95.7% and 93.3% homology (at the amino acid level) with Mhp1 and P97 respectively. The majority of differences between the sequences resided in the repeat regions.

The presence of a ciliary adhesin gene in the highly passaged, avirulent J strain of *M. hyopneumoniae* with significant homology to adhesin gene sequences from a low passage, virulent strain of this pathogen encouraged further investigation into differences in the number of repeats amongst a panel of *M. hyopneumoniae* strains from different geographic localities. Using PCR and cloning techniques, the repeat regions of five additional strains (Beaufort, Sue, YZ, C1735/2 and OMZ407) were sequenced. Whilst the number of repeats in RR2 remained between 3 and 5 copies, the number of repeats in RR1 ranged from 8 to 15. Transposon insertional mutagenesis of P97 by Hsu *et al*. (1997) found that a Tn*1000* insertion 109 residues upstream of the beginning of RR1 (residue 704) abolished ciliary and monoclonal antibody binding yet the presence of a transposon insertion (Tn*1000*) in RR2 (residue 1004) of P97 failed to abolish either ciliary binding or diminish the ability of MAb F1B6 to bind to P97. This suggests that RR2 is not essential for either of these functions and defines the biologically important region(s) of the molecule between these two transposon insertion sites spanning 300 residues (amino acids 704-1004) in the carboxy-terminus of P97. Although RR1 only comprises approximately 25% of the amino acids spanning the two transposon insertion sites, proline-rich repeats (which reside in RR1 and RR2) are often highly immunogenic (Williamson, 1994) suggesting that RR1 may comprise an epitope(s) recognised by MAb F1B6 crucial for ciliary binding. Although RR2 does not appear to be essential for ciliary binding, it has been retained in all strains of *M. hyopneumoniae* examined.

Like the ciliary adhesin of *M. hyopneumoniae*, P65 is a surface protein of *M. pneumoniae*, an extracellular pathogen that specifically adheres to the ciliated epithelial lining of the human respiratory tract, containing proline rich repeats, displaying size polymorphism and in addition possessing an RGD sequence (Proft *et al*., 1995). The two repeats (D-P-N-A-Y and D-P-N-Q-A-Y) in the NH_2-terminal portion of P65 form a proline rich acidic domain and are responsible for the observed size polymorphism in immunoblots of whole cell lysates of two different strains of *M. pneumoniae*, one a clinical isolate and the other a high passage laboratory strain. The high passage strain FH of *M. pneumoniae* contained 12 D-P-N-A-Y repeats whilst the clinical isolate contained only 9 copies. The function of P65 remains unknown but it is found at the surface of the tip-organelle of *M. pneumoniae* in close association with P30, a protein that has been implicated in cytadherence (Jordan *et al*., 2001, Seto *et al*., 2001, Seto and Miyata, 2003, Krause and Balish, 2004).

Surface proteins displaying reiterated repeats are increasingly being reported in mycoplasmas (Proft *et al*., 1995; Zhang & Wise 1996, Washburn *et al*., 1998; Persson *et al*., 2002; Tu *et al*., 2005) and phylogentically related Gram-positive bacteria (Wren, 1991; Li *et al*., 1992; Zheng *et al*., 1995; Wästfelt *et al*., 1996). The MB antigen from *Ureaplasma urealyticum* comprises 42.5 repeats comprising a six amino acid motif G-K-E-Q-P-A which resides in the carboxyl terminal portion of the molecule, is antigenic and surface exposed, and is responsible for antigenic size variation. Furthermore, it has been postulated that different antigen sizes may be associated with a specific pathological manifestation (Zheng *et al*., 1995)

Regions of tandem repeats similar to those displayed in *M. hyopneumoniae* are commonly involved in antigenic variation at the surface of pathogenic organisms and are believed to aid in surface diversity and maintenance of disease. When anti-28 kDa sera, recognising the carboxy-terminus of the J strain adhesin, was used in Western blots against whole cell lysates of geographically diverse strains of *M. hyopneumoniae*, a predominant antigen ranging in molecular mass from 94 to 97 kDa was observed in all seven isolates. The size differences in the antigen among the strains could be explained by differences in the number of repeats in RR1 and RR2. Weak cross-reactivity was observed with the *M. flocculare* whole cell lysate also present on the blot but there was no cross-reactivity evident in the whole-cell lysates of the four *M. hyorhinis* strains present. This result suggested that the adhesin was specific to *M. hyopneumoniae* which was confirmed in PCR and Southern hybridisation analysis. An 820 bp hybridisation probe spanning RR1 and RR2, produced a predominant hybridisation fragment ranging in molecular size between 2.15-2.3 kb when used in Southern blots of *Eco*RI digests of DNA from the seven strains of *M. hyopneumoniae* used in this study. No hybridisation fragments were evident in DNA from the *M. hyorhinis* or *M. flocculare* isolates examined.

Studies by King *et al*. (1996) suggested that Mhp1 may be duplicated in the genome of *M. hyopneumoniae* strain P5722. Similarly, Hsu *et al*. (1997) suggested that P97 was duplicated in *M. hyopneumoniae* strain 232A. In addition to the predominant band of 2.15 to 2.30 kb observed in Southern blots of *Eco*RI digests of *M. hyopneumoniae* DNA used in this study (when probed with a PCR product spanning RR1 and RR2), several weakly hybridising bands were also observed. Faint bands were seen with DNA from strains 232, YZ, Sue and C1735/2 suggesting that multiple copies of RR1 and/or RR2

may be present, however, none were observed in Australian isolates Beaufort and OMZ407 and a single very faint band was observed for strain J. If there were duplications of the adhesin gene varying in the number of repeats in RR1 and RR2, these should have been detected by the PCR cloning strategy. This data provides circumstantial evidence that ciliary adhesin genes with a variable number of repeat regions do not exist as multiple copies in *M. hyopneumoniae*. This was confirmed when the genome sequence of *M. hyopneumoniae* was released (Minion *et al*., 2004). P97 was found encoded in an operon with a second gene encoding a 102 kDa protein designated P102. BlastP analysis of the genome revealed six paralogs of P97 with greater than 30% amino acid identity over 70% of its length. There were also six P102 paralogs identified within the genome. Only minimal homology of the P97 paralogs at the DNA level was observed which would have prevented their hybridisation in Southern experiments. The function of the paralogs is unknown but interestingly only P97 contains RR1 required for cilial binding.

The amino acid proline is present in both repeats found in RR1 and RR2 in the *M. hyopneumoniae* adhesin. Proline rich repeats (PRRs) within proteins are often found as multiple tandem repeats of variable length in both prokaryotes and eukaryotes and are usually highly immunogenic (Williamson, 1994). Many bacterial outer membrane proteins associated with transport functions, binding cytoskeletal proteins, binding peptidoglycan and intracellular signalling have been demonstrated to contain PRRs critical in performing such tasks (Williamson, 1994 and references therein). The involvement of PRRs in binding occurs in a non-stoichiometric, but functionally important way and has a unique ability to bind rapidly and tightly to receptor molecules (Williamson, 1994).

It was confirmed by Minion *et al*. (2000) that RR1 of the adhesin mediates the adherence of *M. hyopneumoniae* to swine cilia. With the numbers of repeats varying between strains and the hypothesis that differences in the number of repeats may influence binding, Minion and colleagues produced a series of RR1 repeat peptides containing different numbers of repeating units and tested their ability to adhere to swine cilia. They achieved this by cloning the RR1 sequences in frame with lacZ and expressing R1-β-galactosidase fusion proteins. The fusion proteins were used in a previously developed cilium adherence assay (CAA) with adherence blocking monoclonal antibodies. Results indicated that eight RR1 repeating units were required for cilium binding. All *M. hyopneumoniae* strains sequenced had at least 8 repeats so no correlation between the number of RR1 repeats and virulence could be made.

As an immunodominant surface antigen of *M. hyopneumoniae* with a significant role in binding, the adhesin is an ideal target for vaccine development. A 35 kDa recombinant adhesin encompassing both regions of repeats (RR1 and RR2) was successfully expressed from pQE9 in *E. coli* M15 [pREP4] cells. Expression from the pQE system allowed simple and rapid purification of the adhesin antigen using nickel affinity chromatography. The recombinant 35 kDa adhesin antigen was recognised in Western blots by serum from pigs determined to be *M. hyopneumoniae* positive by ELISA. Although variation in recognition between animals was evident in both experiments reinforcing the different disease profiles followed in different animals, the ability of sera from naturally infected pigs to recognise the recombinant adhesin supports the idea of its incorporation in future sub-unit antigen vaccine preparations. Ultimately the potential of the recombinant adhesin as a vaccine antigen can only be assessed in a challenge and vaccination trial in pigs.

Chapter Four

Cloning and Characterisation of an Immunoreactive Antigen of *M. hyopneumoniae*

4.1. Introduction

Monoclonal antibodies produced against the *M. hyopneumoniae* ciliary adhesin were able to block the adherence of *M. hyopneumoniae* to the epithelial cell membrane of cilia by approximately 65% (Zhang *et al*., 1995). The inability of these monoclonal antibodies to completely block adherence suggests that other surface proteins are involved in binding to epithelial cilia and consequently in pathogenesis. A lack of knowledge of the surface topography of *M. hyopneumoniae* exists and is believed to be a factor preventing the development of new generation vaccines. An understanding of *M. hyopneumoniae* adherence has been greatly enhanced with detailed studies of the cilium adhesin (Zhang *et al*., 1995; Hsu *et al*., 1997; Hsu and Minion 1998; Wilton *et al*., 1998; Minion *et al*., 2000; Djordjevic *et al*., 2004) but research on other mycoplasmal pathogens indicate that adherence is a complex process between mycoplasma and host involving multiple proteins (Layh-Schmitt *et al*., 2000, Papazisi *et al*., 2002).

In order to identify other *M. hyopneumoniae* proteins with potential involvement in the adherence process, two-dimensional immunoblots with convalescent sera pooled from pigs infected with *M. hyopneumoniae* were performed (Djordjevic *et al*., unpublished results). N-terminal sequence data was generated for multiple immunoreactive proteins identified in the blots as a start point for further analysis. This chapter describes the cloning and characterisation of one of those proteins. At the time this research began the genome sequence of *M. hyopneumoniae* had not been released so the protein was believed to be novel when N-terminal sequence data showed no matches in a homology search with various ANGIS databases. The N-terminal amino acid sequence data was

used to generate a degenerate oligonucleotide probe for cloning using a novel technique involving DNA fractionation.

4.2. Methods

Unless otherwise specified in the methods section below, all experimental procedures relating to this chapter were performed as described in Chapter 2 (General Methods).

4.2.1. Preparation of Antisera Against the Recombinant Antigen

Polyacrylamide gels (12%) were prepared and loaded with purified antigen. Gels were stained with Coomassie blue and the region of gel containing the antigen was excised, frozen in liquid nitrogen, and ground with a mortar and pestle to a fine powder. Antigen was emulsified with Freund's incomplete adjuvant and then administered intramuscularly to two New Zealand White rabbits (delivering 0.75 mL to each of the hind legs and 0.25 mL to each front leg). Booster inoculations, delivered one month later, consisted of the same dose of the pulverised polyacrylamide/antigen emulsified with Freund’s incomplete adjuvant.

4.3. Results

4.3.1. Selection of the Immunoreactive Antigen and N-terminal Sequencing

The gene encoding the antigen of interest was cloned after being selected as one of several for further analysis based on its immunoreactivity with convalescent sera in a two-dimensional immunoblot of *M. hyopneumoniae* (Figure 4.1). N-terminal sequence data determined from the protein spot and the degenerate oligonucleotide probe generated is given below in Table 4.1.

Table 4.1. N-terminal sequence data and degenerate oligonucleotide probe generated for cloning the gene encoding the antigen of interest.

N-terminal Sequence	**Degenerate Oligonucleotide**
MDKFRYVKPGQIMAKDEEMI	5'- TAG ATA TGT TAA ACC TGG WCA AAT TAT GGC -3'

Where W is T or A

4.3.2. Southern Hybridisation Analysis Using the Degenerate Oligonucleotide

Before cloning was attempted, the ability of the degenerate oligonucleotide to identify the gene of interest was assessed by Southern hybridisation analysis. Genomic DNA from *M. hyopneumoniae* strains J and Beaufort, *M. hyorhinis* strain GDL, and *M. flocculare* strain MS42 was digested with *Eco*RI, electrophoresed, and transferred to a

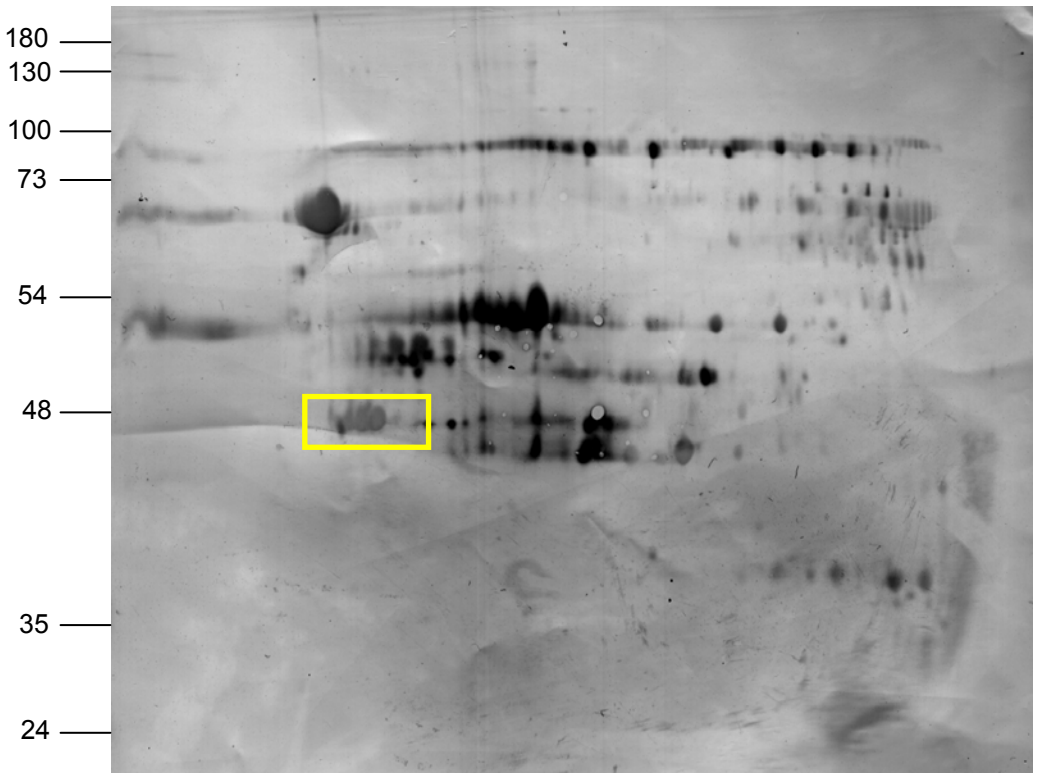


Figure 4.1. Selection of antigen for cloning. Immunoblot of Triton-X 114 extracted *M. hyopneumoniae* strain J aqueous phase proteins separated by two-dimensional gel electrophoresis (12.5% polyacrylamide) using a non-linear pH gradient of 3 - 10 and reacted with convalescent sera pooled from pigs infected with *M. hyopneumoniae*. Several immunoreactive proteins were subject to N-termainl sequencing as a start point for further characterisation. The yellow box identifies the protein cloned for characterisation in this chapter.

nylon membrane for Southern analysis. An image of the agarose gel used for transfer is given as Figure 4.2 (A) and shows the relative DNA loading on the gel. Figure 4.2 (B) shows the Southern result when probed with 3' end labelled oligonucleotide. The oligonucleotide identified a DNA fragment of approximately 2.5 kb in *M. hyopneumoniae* strains J and Beaufort but failed to hybridise to digested DNA from the related porcine mycoplasma species. Therefore the gene appears to be in single copy in the *M. hyopneumoniae* genome and at the level of stringency used is specific to *M. hyopneumoniae*.

In addition to providing information about the presence and number of copies of the gene of interest in the *M. hyopneumoniae* genome, hybridisation of the degenerate oligonucleotide to chromosomal DNA provided a start point for cloning and a means of identifying positive clones.

4.3.3. Cloning and Preliminary Characterisation

M. hyopneumoniae DNA was digested with *Eco*RI and fractionated on a Biorad Model 491 Prep Cell agarose column. The fractionated DNA was screened by Southern hybridisation analysis and the fractions containing the fragment of interest were pooled and concentrated for cloning. Cloning was achieved using the pPCR-Script kit (Stratagene) after making the fragments blunt-ended using Klenow fragment. Colony hybridisation was used for preliminary screening of clones and positives were confirmed through restriction digestion and Southern hybridisation analysis.

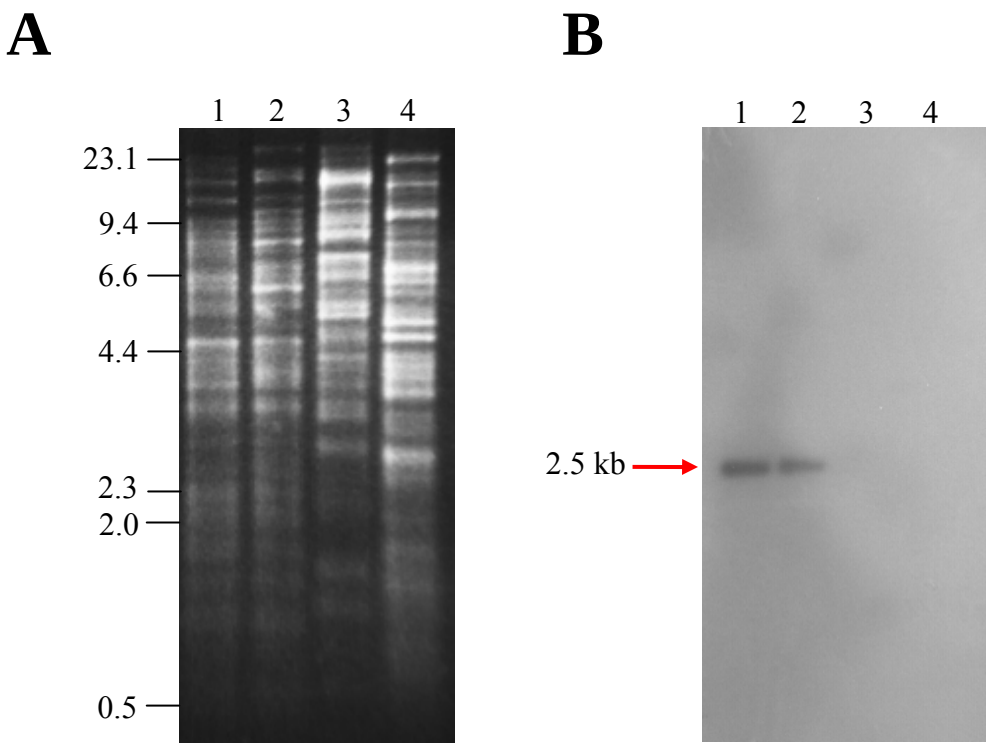


Figure 4.2. Southern hybridisation analysis using the degenerate oligonucleotide against *Eco*RI digested genomic DNA from *M. hyopneumoniae* strains J and Beaufort (lanes 1 and 2 respectively), *M. hyorhinis* strain GDL (lane 3), and *M. flocculare* strain MS42 (lane 4). An image of the agarose gel (1%) used for transfer to a nylon membrane is shown stained with ethidium bromide in (A). Southern hybridisation analysis against the digested genomic DNA using the degenerate oligonucleotide probe is given in (B). Molecular markers are shown to the left in kb. A red arrow indicates the fragment identified by the probe.

4.3.3.1. DNA Fractionation and Southern Hybridisation Analysis

Column fractions (every 5th) from the preparative electrophoresis were initially run on an agarose gel for visualisation. Figure 4.3 (A) shows the agarose gel used for Southern transfer of fractions covering the *Eco*RI fragments in the 2 to 3 kb size range. The resulting Southern blot as probed with the degenerate oligonucleotide is displayed in Figure 4.3 (B). The 2.5 kb fragment of interest was identified in digested genomic DNA from *M. hyopneumoniae* strain Beaufort (lane 1) as expected, and was also clearly contained within several fractions eluted from the column (lanes 6-11). The identified fractions (shown boxed) were pooled and concentrated for cloning.

4.3.3.2. Cloning the Gene Encoding the Immunoreactive Antigen

The pooled and concentrated fragments were blunt-ended for cloning into pPCR-Script. Positive clones were initially identified by colony hybridisation analysis and confirmed by Southern hybridisation analysis. A colony blot containing a positive clone (top left) and positive control DNA (bottom right) is given as Figure 4.4 (A). Potential positive clones, like the one in Figure 4.4 (A), were cultured, and their plasmid DNA extracted and digested for confirmation of correct insert by Southern hybridisation analysis. The ethidium bromide stained agarose gel and corresponding Southern blot of a representative digested clone and corresponding controls is given in Figure 4.4 (B). The degenerate oligonucleotide probe failed to hybridise to vector DNA (lane 1), but successfully identified a positive clone by hybridising to undigested clone DNA and the 2.5 kb DNA insert of the digested clone (lanes 2 and 3 respectively). The clone was designated pJW3.

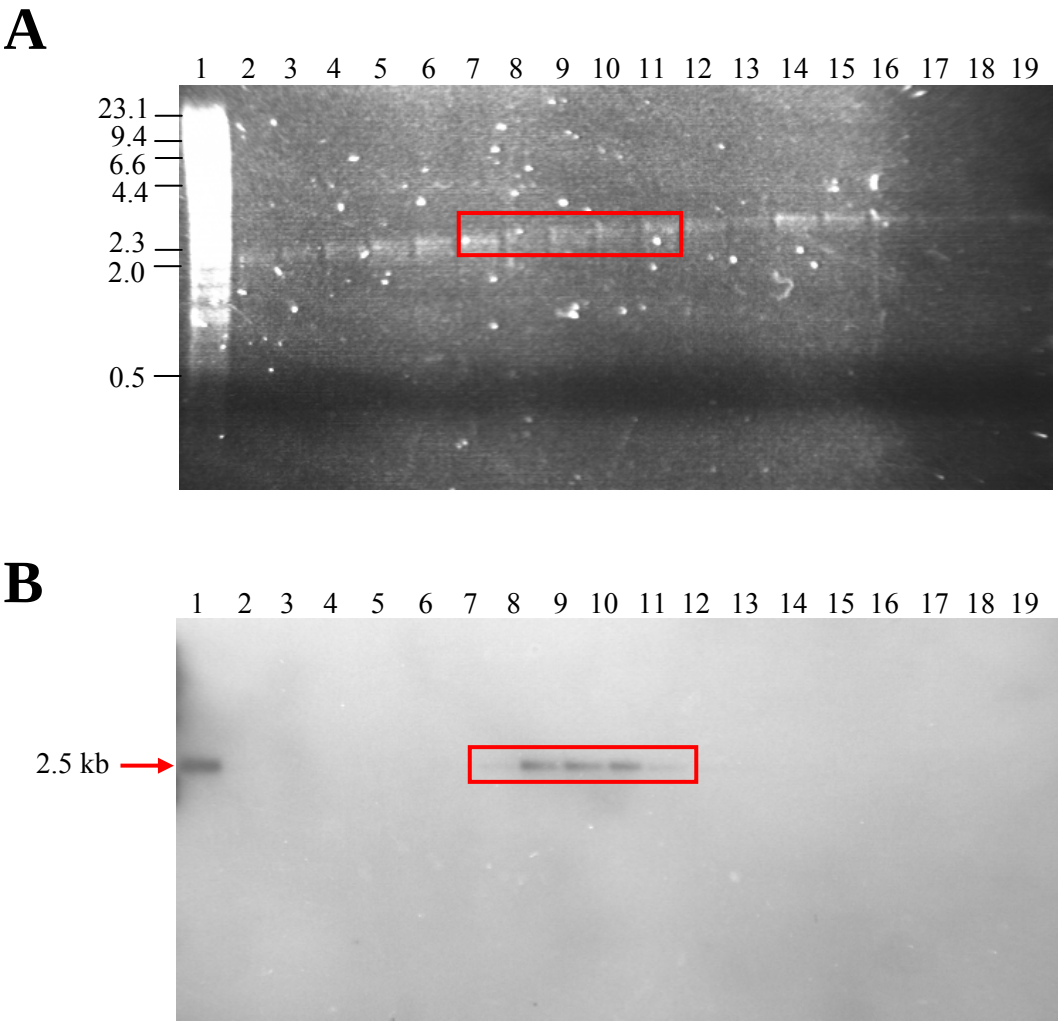


Figure 4.3. Agarose gel (1%) electrophoresis and Southern hybridisation analysis for identification of the fragment of interest in *Eco*RI digested and fractionated *M. hyopneumoniae* (strain Beaufort) DNA. Ethidium bromide stained agarose gel (A) and corresponding Southern blot as probed with the degenerate oligonucleotide (B), showing *Eco*RI digested *M. hyopneumoniae* DNA in lane 1, and fractions eluted from a preparative agarose column of *Eco*RI digested *M. hyopneumoniae* DNA in lanes 2-19. Fractions containing the 2.5 kb fragment are shown boxed in red. Molecular size markers are given in kb on the left of the agarose gel in (A).

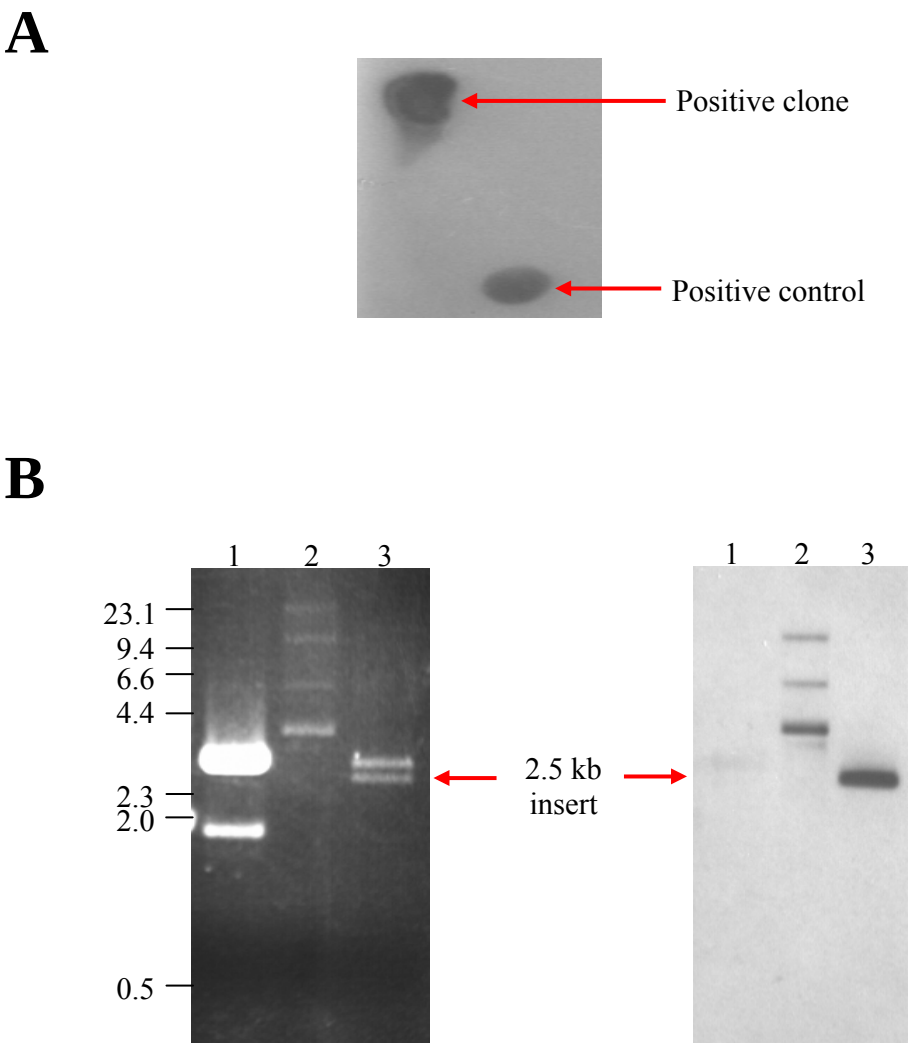


Figure 4.4. Colony and Southern hybridisation analysis to confirm cloning. Colonies from the PCR-Script™ cloning were lifted onto nitrocellulose for probing with the degenerate oligonucleotide in a colony blot (A). *M. hyopneumoniae* genomic DNA from strain Beaufort was spotted onto the membrane to act as a positive control (bottom right) and positive clones, like the one shown (top left), were subject to digestion and Southern hybridisation analysis (B). Undigested and digested vector and plasmid DNA were electrophoresed in 1% agarose (left) and transferred to a nitrocellulose membrane for Southern blotting (right). Lane 1 shows *HindIII* and *Bam*HI digested pPCR-Script vector and Lanes 2 and 3, undigested and *HindIII* and *Bam*HI digested plasmid DNA extracted from the positive clone identified in the colony blot. Molecular markers are given on the left of the agarose gel and the 2.5 kb insert is indicated by a red arrow.

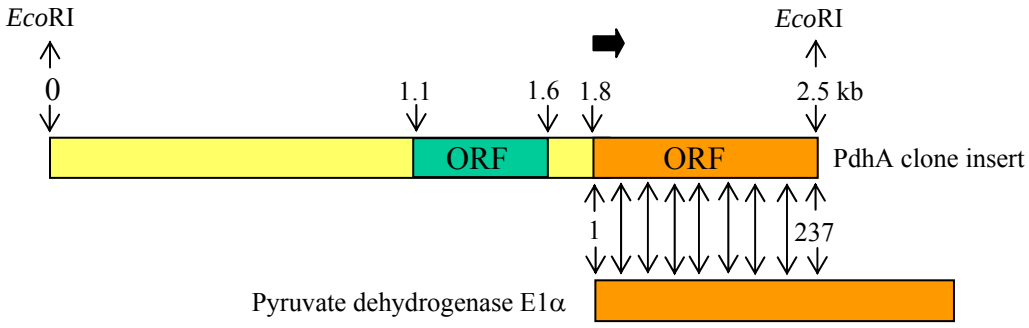
4.3.3.3. DNA Sequence Analysis of pJW3

Once confirmed as a positive clone, pJW3 was sequenced. Sequencing was initiated with vector primers and the degenerate oligonucleotide used to clone the fragment. Sequencing information obtained was used to design new sets of primers in order to "walk" along the cloned fragment, sequencing it in both directions. As sequence information was obtained it was translated and analysed for open reading frames. Upon sequencing the entire clone, two open reading frames were identified, ORF1 and ORF2 (Figure 4.5 (A)). When homology searches were conducted through ANGIS, ORF1 translated to what was believed to be the N-terminal 237 amino acids of the *M. hyopneumoniae* pyruvate dehydrogenase E1α (PdhA) protein. It showed homology to PdhA sequences from a number of bacterial species, and in particular with sequences from multiple mycoplasma species (Figure 4.5 (B)). When ORF2 was translated it showed significant homology to adenine phosphoribosyl transferase, a protein of 172 amino acids in length. The entire gene encoding the adenine phosphoribosyl transferase protein was contained within the clone but it was not the gene of interest. The translated ORF1 sequence showed identity with the N-terminal amino acid sequence used to clone the fragment and was confirmed as the sequence of interest. Therefore approximately two-thirds of the gene encoding PdhA had been cloned as the gene encoding the immunoreactive antigen of interest.

4.3.3.4. Alignment of Translated PdhA Clone Sequence with Homologous Proteins

When the cloned PdhA sequence was translated and subjected to searches through ANGIS, it was shown to have significant homology with a number of PdhA proteins in

A



B

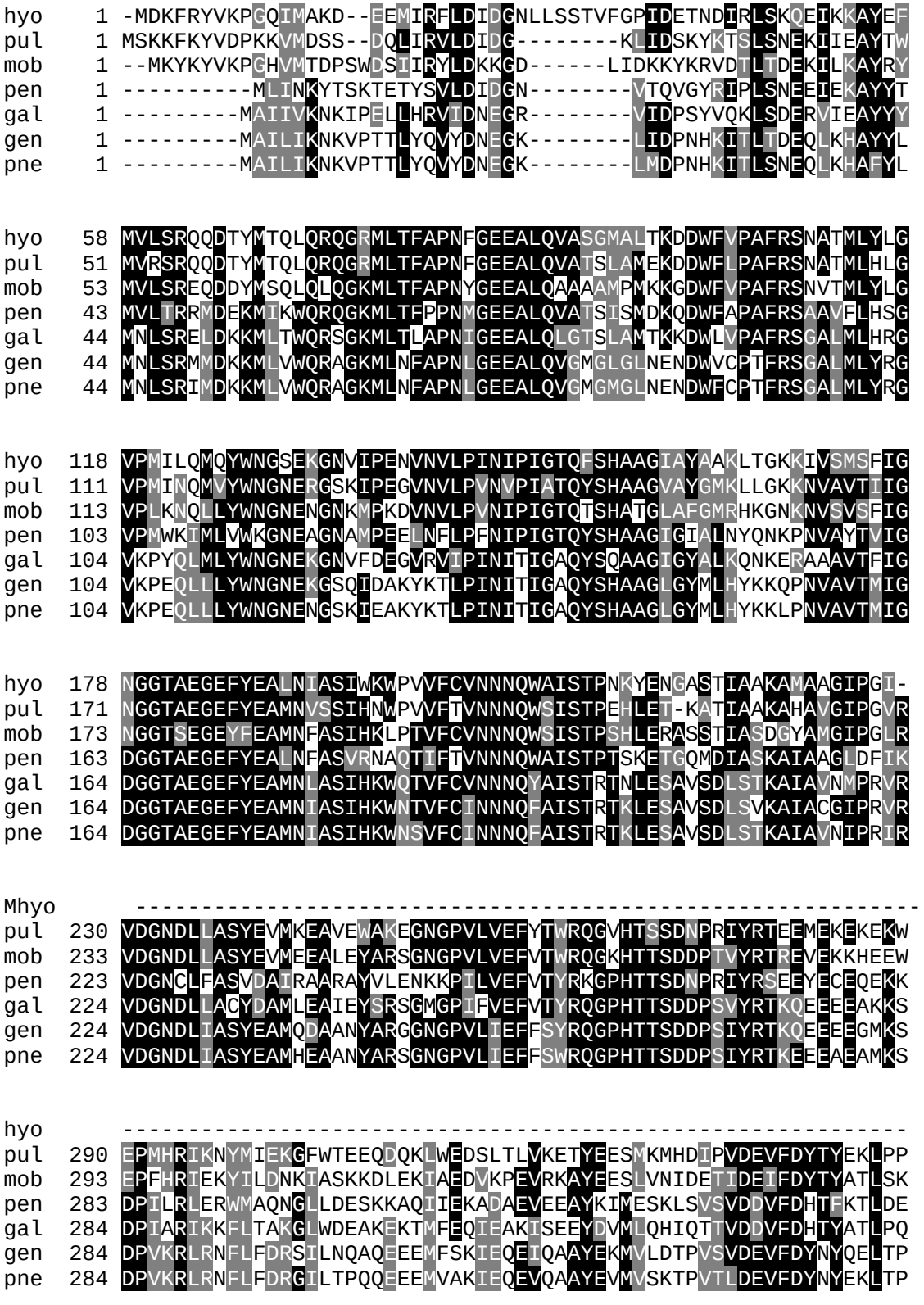
Species	**Description**	**% Identity**	**% similarity**
Mycoplasma. pulmonis	Pyruvate Dehydrogenase E1 component, alpha subunit	67 (156/234)	79 (186/234)
Mycoplasma mobile	Pyruvate Dehydrogenase E1 component, alpha subunit	59 (139/236)	75 (178/236)
Mycoplasma penetrans	Pyruvate Dehydrogenase E1 component, alpha subunit	56 (121/216)	67 (145/216)
Mycoplasma gallisepticum	Acyl-coenzyme A oxidase	55 (109/199)	70 (140/199)
Mycoplasma. genitalium	Pyruvate Dehydrogenase E1 component, alpha subunit	53 (106/199)	67 (133/199)
Mycoplasma. pneumoniae	Pyruvate Dehydrogenase E1 component, alpha subunit	53 (106/199)	66 (131/199)

Figure 4.5. Sequence analysis of the 2.5 kb clone insert. The gene map (A) shows the sequenced region of the *Eco*RI insert of the PdhA clone, the location of the oligonucleotide probe used for the cloning (indicated by a black arrow), and the protein with which it showed homology. The table (B) shows the results of the homology search conducted through ANGIS. It gives the species to which homology was detected, a description of the protein with which the translated 237 amino acids of the clone sequence had homology, and the percent identity and similarity between the two sequences.

the database. An alignment of the N-terminal 237 translated amino acids of PdhA from *M. hyopneumoniae* with PdhA sequences from *M. pulmonis*, *M. mobile*, *M. penetrans*, *M. gallisepticum*, *M. genitalium* and *M. pneumoniae* is given in Figure 4.6. All have greater than 50% identity to the translated sequence of the cloned *M. hyopneumoniae* PdhA, differing primarily at the N-terminus of the protein. A second region of significant difference towards the C-terminus is also indicated by the alignment of the six homologous sequences.

4.3.3.5. Sequence Analysis of the Genes Encoding PdhA and PdhB in *M. hyopneumoniae*

PdhA is a single sub-unit of the pyruvate dehydrogenase enzyme complex, which is also composed of sub-units PdhB, PdhC and PdhD. The genes encoding PdhA and PdhB are often found within an operon, as are the genes encoding sub-units C and D. Inverse PCR was attempted in order to obtain the remainder of the PdhA gene sequence and also potentially determine if the genes encoding the A and B sub-units of the Pdh complex were contained within an operon. Inverse PCR failed, but access to the *M. hyopneumoniae* strain 232 genome sequence was granted by an American colleague before its official release. The database was searched using the *M. hyopneumoniae* strain Beaufort *pdhA* gene sequence of the clone, and the contig showing alignment was analysed for open reading frames. The open reading frames were submitted to ANGIS and in addition to detecting the *pdhB* gene directly downstream from *pdhA*, the adenine phosphoribosyl transferase gene was detected upstream.


hyo 1 -MDKFRYVKPGQIMAKD--EEMIRFLDIDGNLLSSTVFGPIDETNDIRLSKQEIKKAYEF
pul 1 MSKKFKYVDPKKVMDSS--DQLIRVLDIDG--------KLIDSKYKTSLSNEKIIEAYTW
mob 1 --MKYKYVKPGHVMTDPSWDSIIRYLDKKGD------LIDKKYKRVDTLTDEKILKAYRY
pen 1 ----------MLINKYTSKTETYSVLDIDGN--------VTQVGYRIPLSNEEIEKAYYT
gal 1 ---------MAIIVKNKIPELLHRVIDNEGR--------VIDPSYVQKLSDERVIEAYYY
gen 1 ---------MAILIKNKVPTTLYQVYDNEGK--------LIDPNHKITLTDEQLKHAYYL
pne 1 ---------MAILIKNKVPTTLYQVYDNEGK--------LMDPNHKITLSNEQLKHAFYL
hyo 58 MVLSRQQDTYMTQLQRQGRMLTFAPNFGEEALQVASGMALTKDDWFVPAFRSNATMLYLG
pul 51 MVRSRQQDTYMTQLQRQGRMLTFAPNFGEEALQVATSLAMEKDDWFLPAFRSNATMLHLG
mob 53 MVLSREQDDYMSQLQLQGKMLTFAPNYGEEALQAAAAMPMKKGDWFVPAFRSNVTMLYLG
pen 43 MVLTRRMDEKMIKWQRQGKMLTFPPNMGEEALQVATSISMDKQDWFAPAFRSAAVFLHSG
gal 44 MNLSRELDKKMLTWQRSGKMLTLAPNIGEEALQLGTSLAMTKKDWLVPAFRSGALMLHRG
gen 44 MNLSRMMDKKMLVWQRAGKMLNFAPNLGEEALQVGMGLGLNENDWVCPTFRSGALMLYRG
pne 44 MNLSRIMDKKMLVWQRAGKMLNFAPNLGEEALQVGMGMGLNENDWFCPTFRSGALMLYRG
hyo 118 VPMILQMQYWNGSEKGNVIPENVNVLPINIPIGTQFSHAAGIAYAAKLTGKKIVSMSFIG
pul 111 VPMINQMVYWNGNERGSKIPEGVNVLPVNVPIATQYSHAAGVAYGMKLLGKKNVAVTIIG
mob 113 VPLKNQLLYWNGNENGNKMPKDVNVLPVNIPIGTQTSHATGLAFGMRHKGNKNVSVSFIG
pen 103 VPMWKIMLVWKGNEAGNAMPEELNFLPFNIPIGTQYSHAAGIGIALNYQNKPNVAYTVIG
gal 104 VKPYQLMLYWNGNEKGNVFDEGVRVIPINITIGAQYSQAAGIGYALKQNKERAAAVTFIG
gen 104 VKPEQLLLYWNGNEKGSQIDAKYKTLPINITIGAQYSHAAGLGYMLHYKKQPNVAVTMIG
pne 104 VKPEQLLLYWNGNENGSKIEAKYKTLPINITIGAQYSHAAGLGYMLHYKKLPNVAVTMIG
hyo 178 NGGTAEGEFYEALNIASIWKWPVVFCVNNNQWAISTPNKYENGASTIAAKAMAAGIPGI-
pul 171 NGGTAEGEFYEAMNVSSIHNWPVVFIVNNNQWSISTPEHLET-KATIAAKAHAVGIPGVR
mob 173 NGGTSEGEYFEAMNFASIHKLPTVFCVNNNQWSISTPSHLERASSTIASDGYAMGIPGLR
pen 163 DGGTAEGEFYEALNFASVRNAQTIFTVNNNQWAISTPTSKETGQMDIASKAIAAGLDFIK
gal 164 DGGTAEGEFYEAMNLASIHKWQTVFCVNNNQYAISTRTNLESAVSDLSTKAIAVNMPRVR
gen 164 DGGTAEGEFYEAMNIASIHKWNTVFCINNNQFAISTRTKLESAVSDLSVKAIACGIPRVR
pne 164 DGGTAEGEFYEAMNIASIHKWNSVFCINNNQFAISTRTKLESAVSDLSTKAIAVNIPRIR
Mhyo ----------
pul 230 VDGNDLLASYEVMKEAVEWAKEGNGPVLVEFYTWRQGVHTSSDNPRIYRTEEMEKEKEKKW
mob 233 VDGNDLLASYEVMEEALEYARSGNGPVLVEFVTWRQGKHTTSDDPTVYRTREVEKKHEEW
pen 223 VDGNCLFASVDAIRAARAYVLENKKPILVEFVTYRKGPHTTSDNPRIYRSEEYECEQEKK
gal 224 VDGNDLLACYDAMLEAIEYSRSGMGPIFVEFVTYRQGPHTTSDDPSVYRTKQEEEEAKKS
gen 224 VDGNDLIASYEAMQDAANYARGGNGPVLIEFFSYRQGPHTTSDDPSIYRTKQEEEEGMKS
pne 224 VDGNDLIASYEAMHEAANYARSGNGPVLIEFFSWRQGPHTTSDDPSIYRTKEEEAEAMKS
hyo ----------
pul 290 EPMHRIKNYMIEKGFWTEEQDQKLWEDSLTLVKETYEESMKMHDIPVDEVFDYTYEKLPP
mob 293 EPFHRIEKYILDNKIASKKDLEKIAEDVKPEVRKAYEESLVNIDETIDEIFDYTYATLSK
pen 283 DPILRLERWMAQNGLLDESKKAQIIEKADAEVEEAYKIMESKLSVSVDDVFDHTFKTLDE
gal 284 DPIARIKKFLTAKGLWDEAKEKTMFEQIEAKISEEYDVMLQHIQTTVDDVFDHTYATLPQ
gen 284 DPVKRLRNFLFDRSILNQAQEEEMFSKIEQEIQAAYEKMVLDTPVSVDEVFDYNYQELTP
pne 284 DPVKRLRNFLFDRGILTPQQEEEMVAKIEQEVQAAYEVMVSKTPVTLDEVFDYNYEKLTP

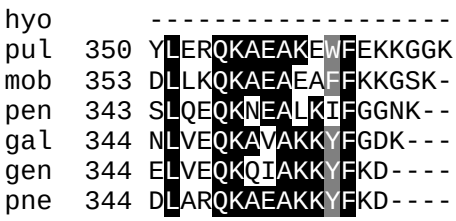


Figure 4.6. ClustalW alignment of translated PdhA clone sequence with homologous mycoplasma proteins. The 237 translated amino acids of the PdhA clone from *M. hyopneumoniae* (hyo) were aligned with PdhA proteins from *Mycoplasma pulmonis* (pul), *Mycoplasma mobile* (mob), *Mycoplasma penetrans* (pen), *Mycoplasma gallisepticum* (gal), *Mycoplasma genitalium* (gen) and *Mycoplasma pneumoniae* (pne) Black shading representing identical amino acids and grey shading similar amino acids.

4.3.3.6. Southern Hybridisation analysis of *pdhA* and *pdhB* in Strains of *M. hyopneumoniae* and Related Porcine Mycoplasmas *M. hyorhinis* and *M. flocculare*

Using the *pdhA* sequence generated from the Beaufort clone, and the *pdhB* sequence obtained from the 232 database, forward and reverse primers (See Appendix C) were designed for the amplification of respective fragments. The 0.4 kb *pdhA* fragment (generated from primers and PdhAF and PdhAR) and the 0.85 kb *pdhB* (generated from primers PdhBF and PdhBR) fragment were extracted from agarose and labelled with Digoxigenin-11-dUTP by random priming. The labelled PCR products were used in Southern hybridisation analysis against *Eco*RI digested genomic DNA from seven strains of *M. hyopneumoniae*, four strains of *M. hyorhinis*, and a single type strain of *M. flocculare*. Figure 4.7 (A) shows the relative loadings of digested chromosomal DNA used for Southern transfer, and (B) and (C) show the Southern blot as probed with the labelled *pdhA* and *pdhB* PCR products respectively.

The *pdhA* probe identified a single hybridisation fragment of approximately 2.6 kb in DNA from the seven strains of *M. hyopneumoniae* tested. This suggested that a homologous *pdhA* gene could be found in single copy in various strains of *M. hyopneumoniae*. The labelled PCR product was unable to hybridise to DNA from either of the related porcine mycoplasmas, suggesting the gene is specific to *M. hyopneumoniae*, at the level of stringency used. This was reflected in its homology with other PdhA proteins in the database. The *pdhB* probe showed a similar profile to that given by the *pdhA* probe, identifying a fragment of approximately 1.2 kb in six of the seven *M. hyopneumoniae* strains with a slightly larger fragment identified in strain YZ (lane 4). A secondary hybridisation fragment can be seen for strain Sue (lane 5) at

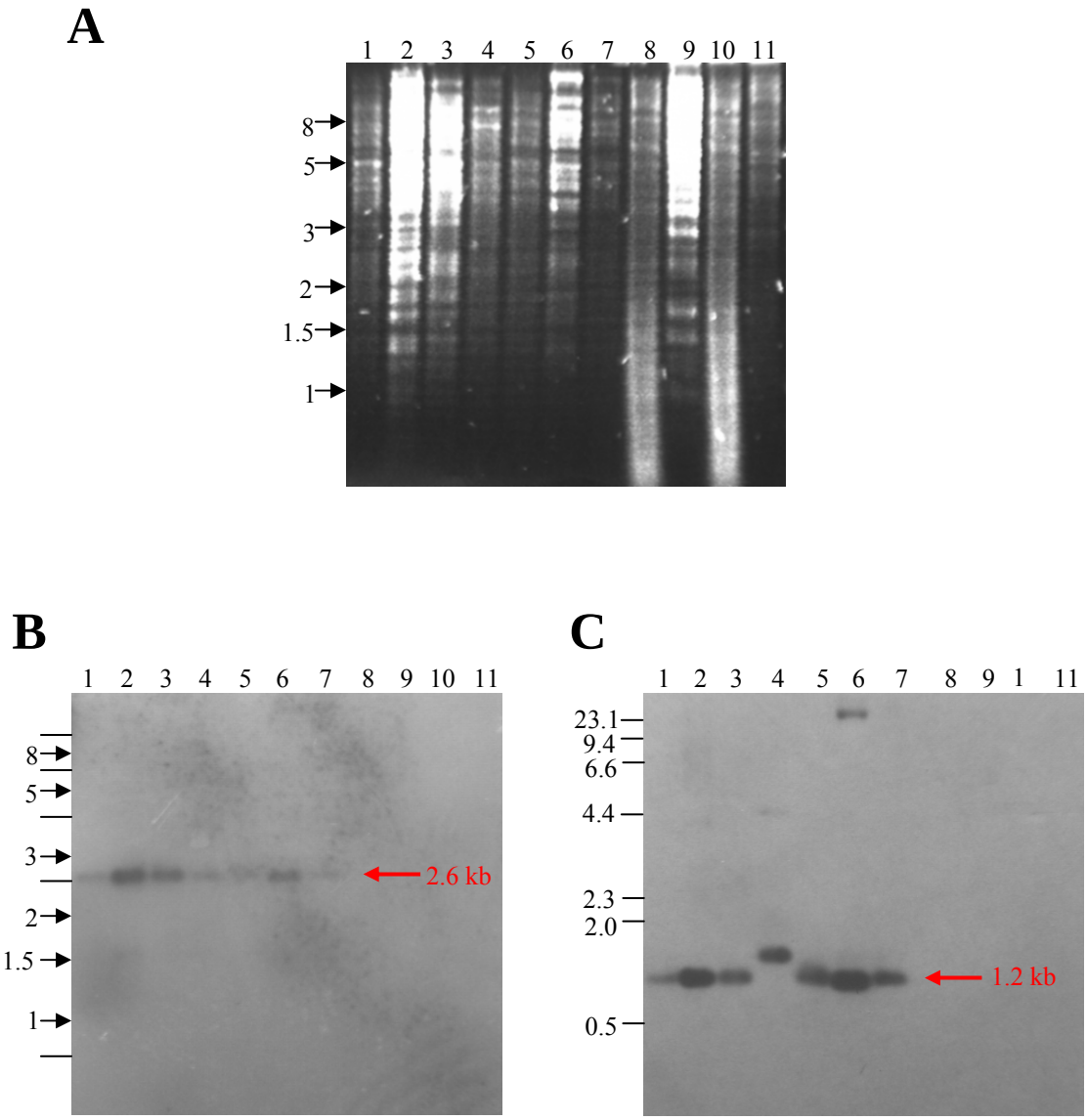


Figure 4.7. Southern hybridisation analysis of *pdhA* and *pdhB* in strains of *M. hyopneumoniae* and related porcine mycoplasmas *M. hyorhinis* and *M. flocculare*. Ethidium bromide stained agarose gel (A) and Southern blots reacted with the Dig-labelled *pdhA* (B) and *pdhB* (C) probes with *Eco*RI digested chromosomal DNA from *M. hyopneumoniae* strains J, Beaufort, 232, YZ, Sue, C1735/2 and OMZ407 in lanes 1-7 respectively, *M. hyorhinis* strains field 1, BTS7 and field 2 in lanes 8-10 respectively, and *M. flocculare* strain Ms42 in lane 11. Molecular size markers are given in kb on the left and the hybridisation fragments are indicated by a red arrow.

approximately 20 kb and being so large, may be the result of incomplete digestion of chromosomal DNA rather than the presence of a second copy of the gene in the chromosome. The probe was again unable to hybridise to DNA from the related porcine mycoplasmas.

4.3.4. Further Characterisation of PdhA

In order to further characterise PdhA it was cloned for expression. The recombinant antigen expressed was used to raise antiserum in rabbits and the antiserum was used in a series of immunoblots to aid characterisation.

4.3.4.1. Cloning for Expression of a Recombinant PdhA Antigen

Expression of full-length PdhA was prevented by the presence of eight UGA codons within the *M. hyopneumoniae* PdhA sequence at amino acids 102, 127, 196, 198, 209, 271, 297 and 322 as indicated in Figure 4.8 (A). Therefore the largest region that could be cloned for expression in *E. coli* was the N-terminal 101 amino acids. Primers were designed just inside the start codon and several UGA stop codons outside this particular N-terminal region (as indicated in figure 4.8 (A)) for the purpose of expressing the clone in the *E. coli* opal suppressor strain (able to bypass several UGA's) if required. The amplification using primers PdhAHisF and PdhAHisR (Appendix C) was performed using *Pfu* polymerase and gave a PCR product of approximately 700 bp as shown in Figure 4.8 (B).

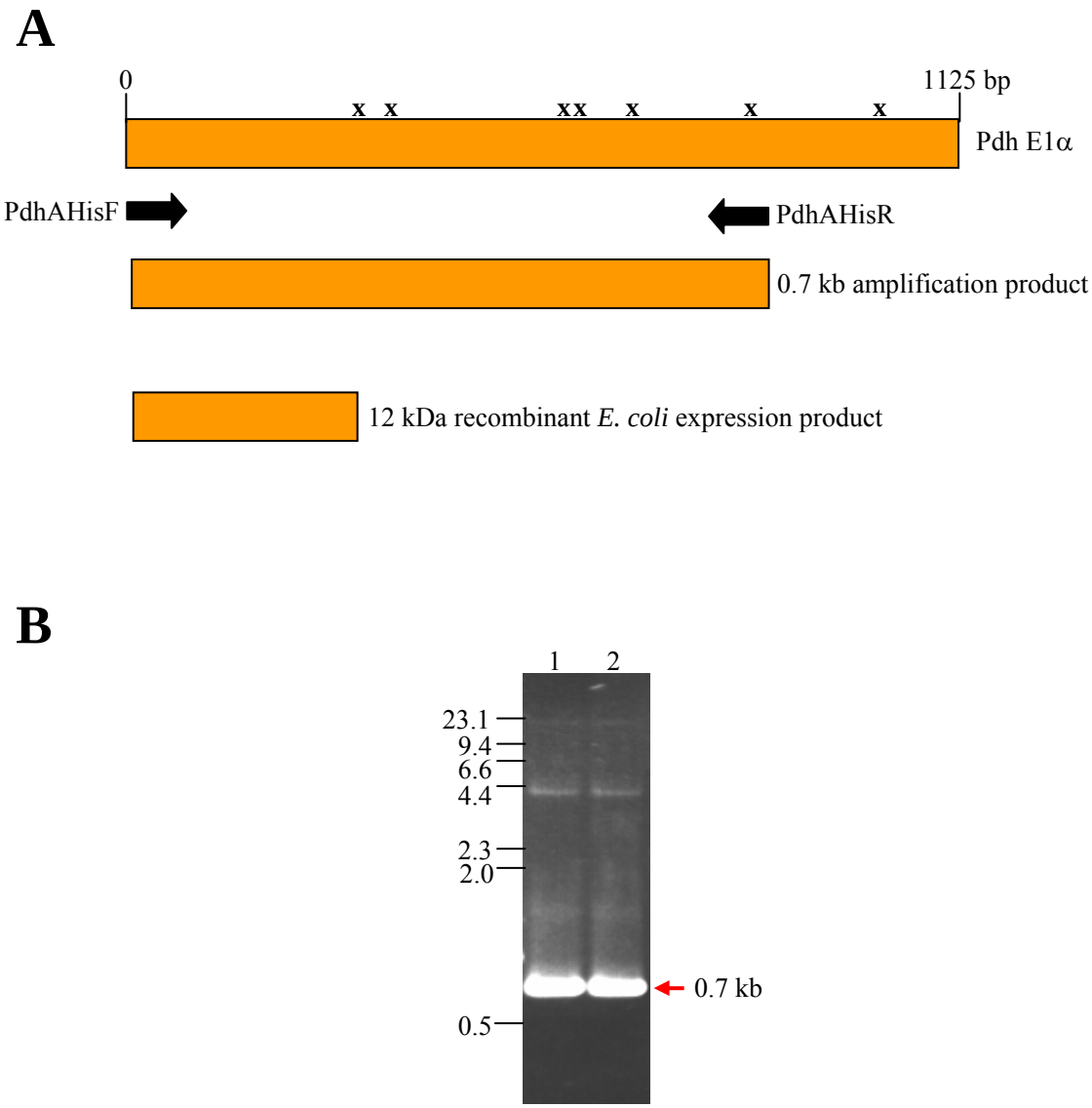


Figure 4.8. Amplification of a *pdh*A gene fragment for expression. The gene map in (A) shows the location of TGA codons in the 1125 bp pyruvate dehydrogenase E1a gene. The cloned portion of the *pdhA* gene is also shown in (A), as well as the primers (PdhAHisF and PdhAHisR) used for amplification of the expression fragment. The amplification product is found at 0.7 kb (lanes 1 and 2) in the ethidium bromide stained agarose gel (1%) of (B). Molecular size markers are given in kb on the left of the agarose gel.

The amplification product was cloned initially into pPCRScript, producing pJW4, before being sub-cloned into pQE9 for expression. The *M. hyopneumoniae pdhA* insert of pJW4 was purified from agarose and ligated with similarly digested and gel purified pQE9. The ligation mix was used to transform *E. coli* M15[pREP4] cells (QIAGEN) and selected colonies were subject to plasmid extraction and digestion to confirm the presence of insert DNA. Two positive clone representatives are shown (Figure 4.9). The pQE9 vector is seen at 3.4 kb (green arrow), the *pdhA* insert at 0.7 kb (red arrow), and the two pREP4 fragments at 3.25 kb and 0.45 kb (yellow arrows).

4.3.4.2. Induction and Purification of the PdhA antigen

A PdhA clone was selected and confirmed by DNA sequence analysis to be correct (data not shown). The clone, designated pJW5, was induced for expression and compared in SDS PAGE analysis to a non-induced control. The recombinant protein could not be detected in the induced culture by Coomassie staining and so denaturing nickel affinity purification was performed on a 4 h induction culture. Fractions were collected throughout the procedure and each run on a SDS PAGE gel for visualisation by Coomassie staining. An image of the Coomassie stained purification gel is given as Figure 4.10 (A). Lanes 1 and 2 show the lysate and flow-through respectively and have similar profiles. The lysate contains all *E. coli* and recombinant proteins passed over the column and the flow-through contains the bulk of those proteins that are unable to bind to the column. Lane 3 represents the wash fraction and contains a number of non-specifically bound proteins released by the decrease in pH. Lanes 4 to 8 represent the elution fractions and although contaminated by several larger proteins clearly show the presence of the recombinant PdhA protein at approximately 12 kDa.

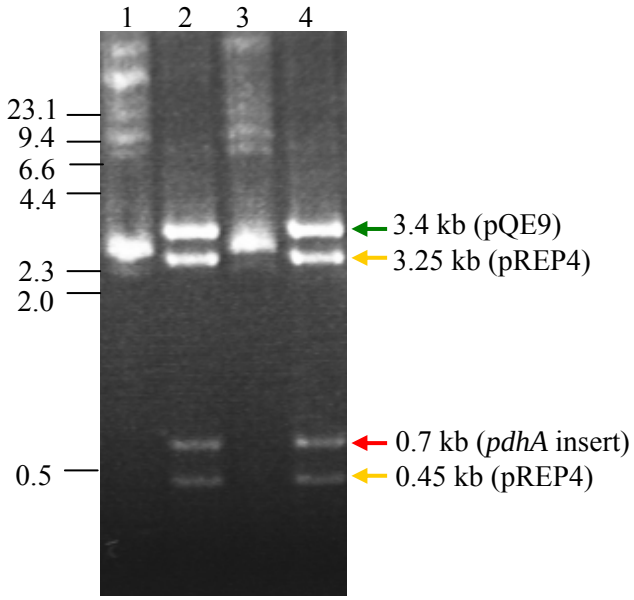


Figure 4.9. Cloning for expression of a PdhA antigen. Ethidium bromide stained agarose (1%) gel of two sub-clones in the expression vector pQE9. Lanes 1 and 3 represent undigested sub-clone DNA and lanes 2 and 4 represent *BamHI/PstI* digested sub-clone DNA. The green arrow at 3.4 kb indicates the pQE9 vector, the yellow arrows at 3.25 kb and 0.45 kb show the pREP4 plasmid, and the red arrow at 0.7 kb again shows the *M. hyopneumoniae pdhA* insert. Molecular size markers are given in kb on the left.

4.3.4.3. Production of Rabbit Antiserum Against the PdhA Antigen

The presence of *E. coli* contaminant proteins in the purified PdhA preparation dictated the method used for the generation of rabbit antiserum against PdhA. The recombinant protein was sliced from a Coomassie stained gel, ground in liquid nitrogen and emulsified in Freund's incomplete adjuvant for immunisation. The rabbit received two immunisations, several weeks apart and was monitored by ear-vein bleeds for a response. A response was detected when the rabbit sera reacted in a Western blot with purified recombinant protein as well as native PdhA in a whole cell lysate of *M. hyopneumoniae* as shown in Figure 4.10 (B). The antiserum is unable to detect the recombinant protein at low concentration in the induced *E. coli* whole cell lysate (lane 2) but reacts with the purified recombinant protein (lane 3) and the native 44 kDa PdhA protein in the whole cell lysate of *M. hyopneumoniae* (lane 4). The antiserum shows some cross-reactivity with a high molecular weight *E. coli* protein as seen in the *E. coli* whole cell lysates of lanes 1 and 2.

4.3.4.4. Cellular location of PdhA

As a metabolic enzyme, the proposed location of PdhA was intracellular. This was confirmed in two experiments using the antiserum generated against recombinant PdhA. The first was a Western blot of trypsin treated *M. hyopneumoniae* whole cells reacted with PdhA antiserum (Figure 4.11 (A)). PdhA was unaffected by trypsin treatment for the concentration range of trypsin used on the cells which was from 0 to 1000 ug/ml. The bulk of PdhA in *M. hyopneumoniae* was therefore almost certainly not located on the surface of *M. hyopneumoniae* and assumed to be intracellularly located.

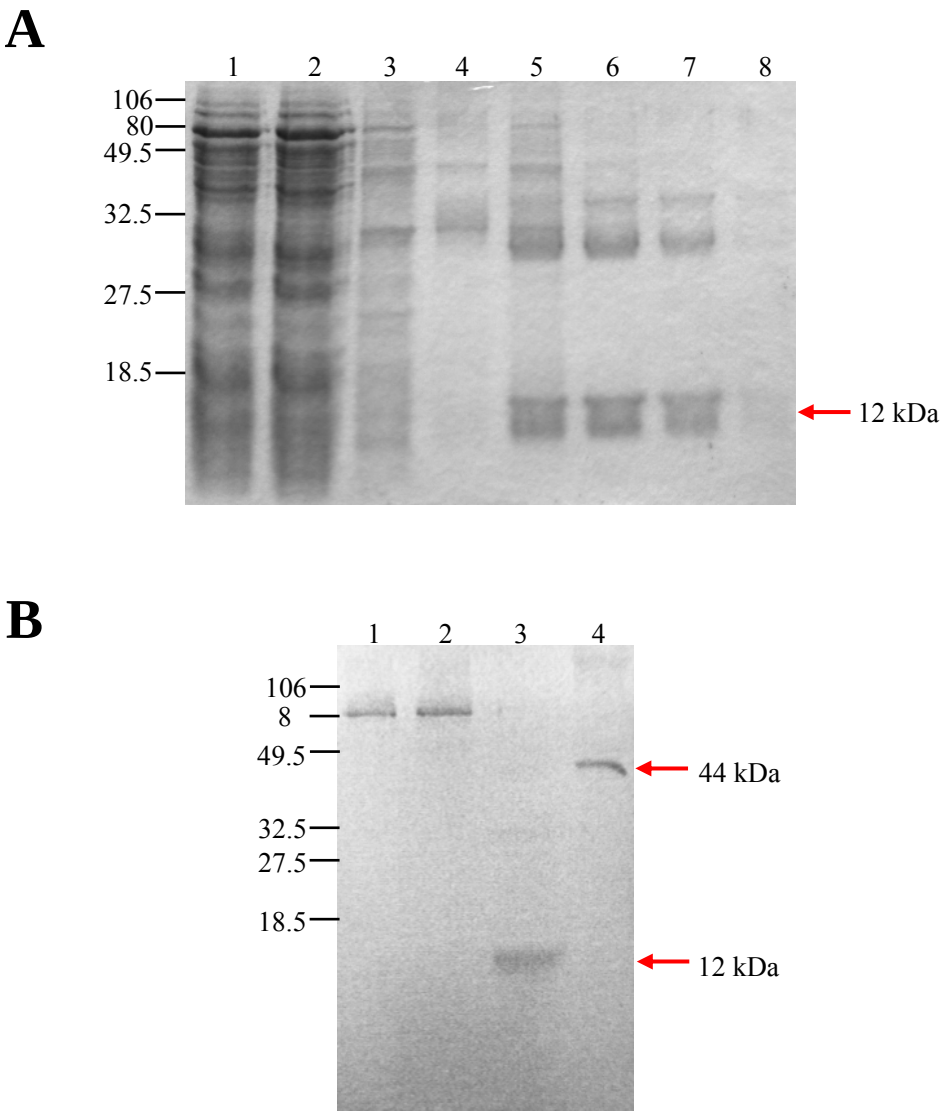


Figure 4.10. Purification of the PdhA antigen from M15[pREP4]pJW5 cells and generation of antiserum in rabbits. (A) A 4-hour induction culture (200 ml) was lysed in 8 M urea and the recombinant protein purified from the lysate by nickel affinity chromatography. Coomassie stained SDS PAGE analysis of the purification is shown with the whole cell lysate in lane 1, the column flow-through in lane 2, the wash fraction in lane 3 and the eluent fractions in lanes 4 to 8. The expected 12 kDa recombinant antigen is indicated by a red arrow. The recombinant protein was excised from a Coomassie stained polyacrylamide and used to immunise a rabbit for the generation of anti-PdhA serum. The serum collected was used in a Western blot (B) against non-induced and induced M15[pREP4]pJW5 whole cell lysates (lanes 1 and 2 respectively), purified recombinant protein (lane 3), and a M. hyopneumoniae whole cell lysate (lane 4). Red arrows indicate the purified recombinant (12 kDa) and native PdhA (44 kDa) proteins. Molecular size markers are given in kDa on the left.

Phases resulting from Triton X-100 and X-114 extraction of *M. hyopneumoniae* cells were reacted with the same antiserum in Western blots and supported this finding. As can be seen in Figure 4.11 (B), the antiserum primarily identified the 44 kDa PdhA protein in the aqueous phases of both extractions. There was also some reaction with the insoluble fraction indicating its potential distribution as a component of a membrane associated structure or complex.

4.3.4.5. Presence of PdhA Among Geographically Diverse Strains of *M. hyopneumoniae* and Related Porcine Mycoplasmas

With its ability to detect native PdhA in whole cell lysates of *M. hyopneumoniae*, the rabbit antiserum generated against the recombinant PdhA antigen was used in a Western blot against whole cell lysates of 5 strains of *M. hyopneumoniae*, 2 strains of *M. hyorhinis*, and a single type strain of *M. flocculare*. As can be seen in Figure 4.12 (A), a protein of approximately 44 kDa was identified in all six strains of *M. hyopneumoniae* as well as the three *M. hyorhinis* strains, and to a lesser degree the *M. flocculare* strain. This indicates that the PdhA protein is conserved amongst the porcine mycoplasmas as indicated by the alignment given in Figure 4.6.

4.3.4.6. Inability of Naturally Infected Pigs to Recognise Recombinant PdhA

Before being disregarded as a potential vaccine antigen, recombinant PdhA was reacted in a Western blot with sera from pigs naturally infected with *M. hyopneumoniae*. No reaction was observed with sera from any of the six *M. hyopneumoniae* ELISA-positive pigs or any of the three *M. hyopneumoniae* ELISA-negative pigs (Figure 4.12 (B)),

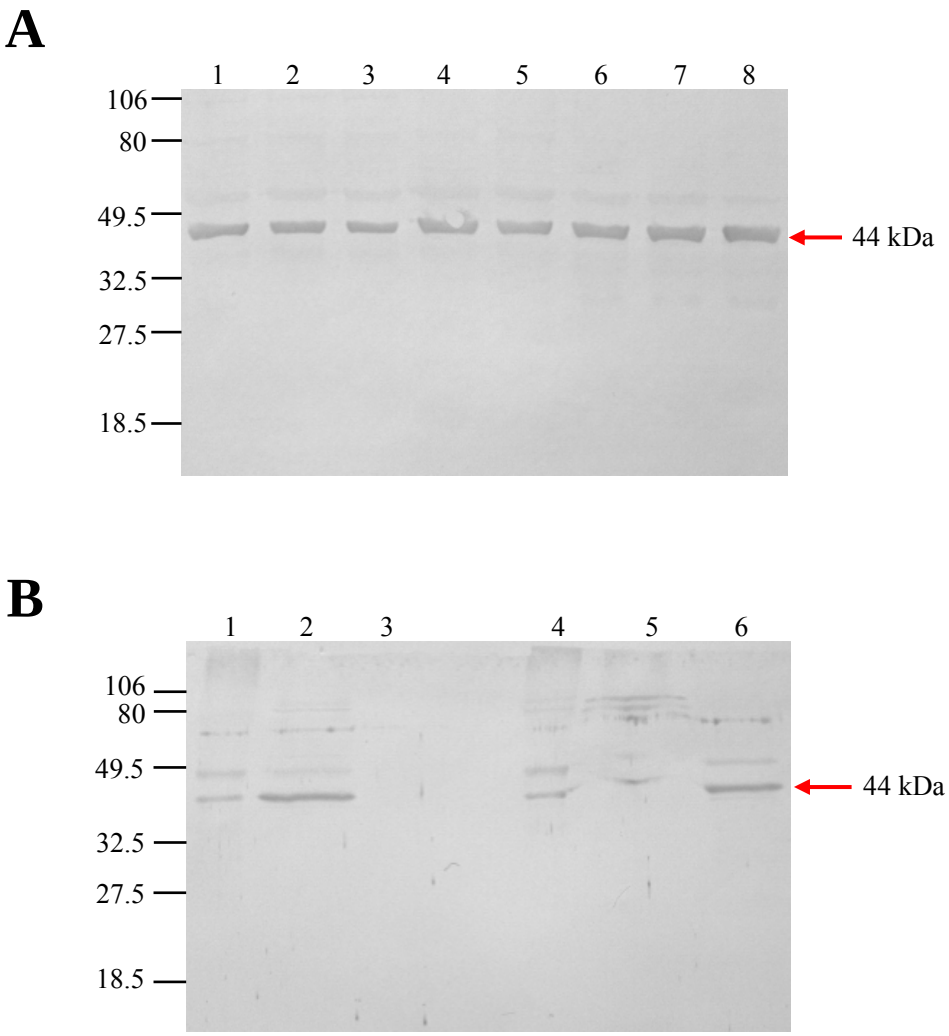


Figure 4.11. Cellular location of PdhA in *M. hyopneumoniae*. The cellular location of PdhA was determined through the treatment of whole *M. hyopneumoniae* (strain J) cells with trypsin and Triton extraction. The immunoblot in (A) shows cell lysates of *M. hyopneumoniae* whole cells treated with 0, 0.5, 1, 5, 10, 50, 100 and 500 µg of trypsin per ml (lanes 1-8 respectively) as separated in 12% polyacrylamide, transferred to PVDF, and reacted with anti-PdhA sera. The immunoblot in (B) shows the resultant phases of Triton extracted *M. hyopneumoniae*, separated in 12% polyacrylamide, transferred to PVDF, and reacted with anti-PdhA serum. Lane 1 is the insoluble phase and lanes 2 and 3 the aqueous phases produced from a Triton X-100 extraction. Lane 4 is the insoluble phase, Lane 5 the detergent phase and lane 6 the aqueous phase of a Triton X-114 extraction. Molecular size markers are given in kDa on the left and the 44 kDa PdhA antigen is indicated by a red arrow.

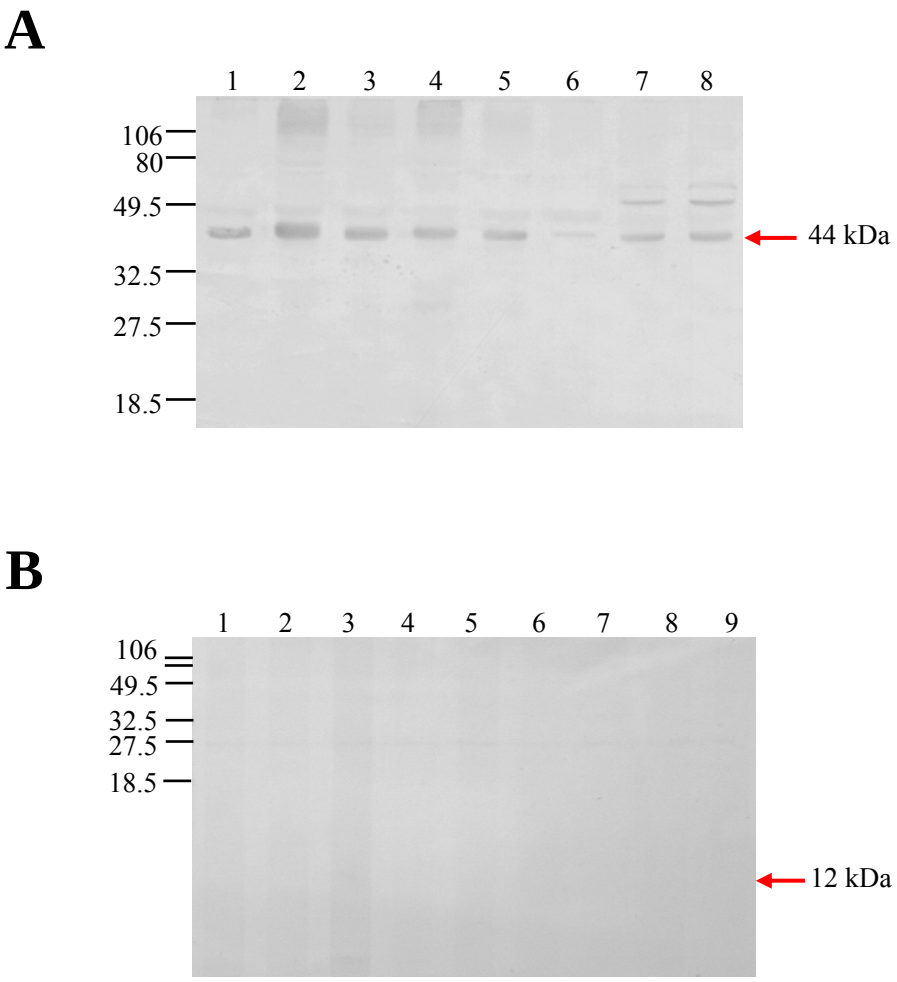


Figure 4.12. Presence of PdhA among geographically diverse strains of *M. hyopneumoniae* and related porcine mycoplasmas (A), and the inability of naturally infected pigs to recognise purified recombinant PdhA (B). Whole cell lysates of *M. hyopneumoniae* strains J, 232, Beaufort, C1735/2, and Sue (lanes 1 – 5 respectively), *M. flocculare* type strain MS42 (lane 6), and *M. hyorhinis* strains BTS-7 and GDL (lanes 7 and 8 respectively), were electrophoresed in 12% polyacrylamide, transferred to PVDF and reacted with PdhA antiserum in (A). Recombinant PdhA was electrophoresed in 15% polyacrylamide, transferred to PVDF and reacted with sera from six ELISA positive *M. hyopneumoniae* pigs (lanes 1 to 6) and 3 ELISA negative *M. hyopneumoniae* pigs (lanes 7 to 9). Molecular size markers are given in kDa on the left of the immunoblots and the 44 kDa and 12 kDa antigens are indicated by red arrows.

which suggests no IgG response is generated against the expressed portion of PdhA upon natural infection with *M. hyopneumoniae*.

4.4. Discussion

The protein cloned and characterised in this chapter was one of several chosen for further characterisation based on its size and immunoreactivity with *M. hyopneumoniae* convalescent sera pooled from pigs infected with *M. hyopneumoniae*. N-terminal amino acid sequence information generated from a 2-D immunoblot was used to clone the gene encoding the protein of interest using a novel technique involving degenerate oligonucleotide probes and the fractionation of *M. hyopneumoniae* chromosomal DNA. The degenerate oligonucleotide was designed from N-terminal amino acid sequence information and used in Southern blots against *M. hyopneumoniae* chromosomal DNA digested with an array of enzymes. Hybridising fragments between 2 and 6 kb were required for two primary reasons, firstly to potentially encode the entire protein of interest, and secondly for ease of cloning. An enzyme which gave a fragment within the desired size range was selected and used to digest a large quantity of chromosomal DNA for fractionation. The digested DNA was separated in an agarose column using a BioRad Prep Cell and aliquots of fractionated DNA were collected for analysis.

Agarose gel electrophoresis of the aliquots was performed initially to confirm successful fractionation, and determine the range of aliquots that would need to be analysed by Southern hybridisation. The degenerate oligonucleotide probe was then used in Southern analysis of fractions within the desired size range. The fractions identified as containing the fragments of interest were pooled, concentrated and cloned into pPCR-Script (Stratagene). The procedure was successful but unfortunately the positive clone (pJW3) did not encode the entire protein of interest. Plasmid pJW3 contained a 2.5 kb *M. hyopneumoniae* insert within which two open reading frames

were identified. The first ORF, encoding the protein of interest, showed significant homology (>50% identity) with several pyruvate dehydrogenase E1α (PdhA) proteins of other mycoplasmas. When translated, it was found that the clone encoded the N-terminal 237 amino acids of what was believed to be the *M. hyopneumoniae* PdhA protein. PdhA is part of the pyruvate dehydrogenase enzyme complex, which catalyses the first step in the "pyruvate roundhouse" of *M. hyopneumoniae*. The use of the pyruvate roundhouse by *M. hyopneumoniae* is a mechanism that has evolved to conserve ATP and counter the lack of a citric acid cycle in the organism (Pollack, 1992). The second open reading frame, 195 bp upstream from the first, showed significant homology to adenine phosphoribosyl transferase protein. The gene encoding adenine phosphoribosyl transferase was also found upstream of the *pdh*A gene in *Mycoplasma genitalium* and *Mycoplasma pneumoniae* (Bork *et al*., 1995; Fraser *et al*., 1995; Himmelreich *et al*., 1996) and was found downstream from *Pdh*D but still in close proximity in *M. capricolum* (Bork *et al*., 1995). In other mycoplasma species, PdhA was significantly distant from the adenosine phosphoribosyl transferase gene (Vasconceles *et al*., 2005; Jaffe *et al*., 2006; Westberg *et al*., 2006; Sirand-Pugnet *et al*., 2007).

The pyruvate dehydrogenase complex is composed of four distinct enzyme sub-units, pyruvate dehydrogenase E1α (PdhA), pyruvate dehydrogenase E1β (PdhB), dihydrolipoamide acetyltransferase (PdhC), and dihydrolipoamide dehydrogenase (PdhD). In mycoplasmas, the genes encoding the Pdh proteins are most commonly arranged in two operons; the *pdh*A and *pdh*B genes in the *pdh*AB operon, and the *pdh*C and *pdh*D genes in the *pdh*CD operon (Matic *et al*., 2003). Pre-publication access to the *M. hyopneumoniae* strain 232 genome database was granted by an American colleague

(Minion *et al*., 2004) and when the cloned PdhA sequence (strain Beaufort) was used to search the genome sequence of *M. hyopneumoniae*, it aligned with 100% homology to the first 237 amino acids of PdhA from strain 232. Immediately downstream of *pdh*A was an open reading frame with homology to the *pdh*B gene. Further analysis showed *pdh*B to be arranged in an operon with *pdh*A, and it was hypothesised that the two genes may be arranged as a two cistron system when the start codon of the *pdh*B gene was found to overlap the stop codon of *pdh*A. The genes encoding *pdh*C and *pdh*D were found in a second operon in another region of the genome (Matic *et al*., 2003).

Although a metabolic enzyme believed to be intracellularly located, PdhA has been found complexed with the P1 adhesin on the surface of *M. pneumoniae* where it has a proposed structural role in the attachment organelle (Layh-Schmitt *et al*., 2000). The glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase and enolase are bi-functional enzymes of group A streptococci. In addition to their role in metabolism, both display plasminogen binding activity when localised on the cell surface (Pancholi and Fischetti, 1992; Pancholi and Fischetti, 1998; Bergman *et al*., 2001; Chhatwal *et al*., 2002). The glycolytic enzymes phosphoglycerate kinase, fructose bisphosphate aldolase, and enolase were also found on the surface of *Streptococcus oralis* (Wilkins *et al*., 2003). The R2 subunit of the cytosolic enzyme ribonucleotide reductase has been found on the surface of *M. hyopneumoniae* (Fagan *et al*., 1996) offering promise of a dual function for PdhA in this organism. Characterision of PdhA proceeded for this reason.

Further characterisation required the production of specific antisera. The cloned portion of the gene encoding PdhA contained six TGA codons making the largest region able to

be expressed in *E. coli* only 101 amino acids long. This region of the PdhA protein, which equated to a recombinant antigen of approximately 12 kDa, was successfully expressed in *E. coli* and used to raise antiserum in rabbits. Characterisation experiments assessed the presence of PdhA among geographically diverse strains of *M. hyopneumoniae* and related porcine mycoplasmas; determined the number of copies of the gene encoding PdhA in the genome; and examined the cellular location of PdhA.

PdhA antiserum was used to probe western blots of whole cell lysates of *M. hyopneumoniae* and related porcine mycoplasmas *M. hyorhinis* and *M. flocculare.* A protein of approximately 42 kDa was identified in all five strains of *M. hyopneumoniae* as well as the two *M. hyorhinis* strains and to a lesser degree the *M. flocculare* strain examined. This indicates that the protein is conserved amongst the porcine mycoplasmas. Southern hybridisation analysis of similar strains identified a single fragment of 2.6 kb in seven strains of *M. hyopneumoniae* but was unable to hybridise to the three *M. hyorhinis* or single *M. flocculare* strain examined. This may have been because the hybridising probe was based on the N-terminus of the PdhA gene which displays the greatest variation in sequence as evidenced by the PdhA alignment. While *Alcaligenes eutrophus* contains a second copy of the PdhA encoding gene (Hein and Steinbuchel., 1996), the Southern experiment also confirmed the presence of only a single copy of the gene encoding PdhA in *M. hyopneumoniae*.

The surface accessibility of PdhA was assessed through the treatment of whole *M. hyopneumoniae* cells with increasing concentrations of trypsin. Lysates of the trypsin treated cells were analysed by immunoblotting with anti-PdhA serum. The 44 kDa PdhA protein of *M. hyopneumoniae* identified by the antiserum appeared unaffected by

the trypsin treatment and was therefore believed to be located intracellularly. However, if a small portion of total cellular PdhA was located on the surface with the majority residing within the cell, the same result would be obtained and hence the experiment is not ultimately conclusive. Triton X-100 and X-114 extractions were also performed on *M. hyopneumoniae* cells and the resultant phases were analysed by immunoblotting for the presence of PdhA. The aqueous phases of both extractions contained the majority of PdhA also indicating its intracellular location and supporting the trypsin experimental result. A small amount of PdhA was retained in the insoluble pellet of both extractions suggesting that it may in addition be part of some kind of complex, insoluble, membrane associated structure.

A relationship between immunoreactive antigens and surface proteins of *M. hyopneumonia*e has been previously demonstrated (Djordjevic *et al*., unpublished results). The antigen characterised in this chapter was selected for its reactivity with hyperimmune serum in the hope that it would be surface located and enhance the existing knowledge of surface topography and pathogenesis of *M. hyopneumoniae*. Although this does not seem to be the case, it is interesting that intracellular enzymes such as these are immunoreactive. Immunoreactive antigens have the potential to be immunogens and evoke an immune response in animals when used as vaccinating antigens. A very good example of this is NrdF. NrdF or ribonucleotide reductase, is a metabolic enzyme that catalyses the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, an essential step in DNA replication. When a recombinant *M. hyopneumoniae* NrdF antigen that was strongly recognised by swine hyperimmune sera was expressed as a fusion protein with beta-galactosidase and delivered intramuscularly to animals in a vaccine trial, it resulted in a significant

reduction in PEP in vaccinated animals compared to unvaccinated controls (Fagan *et al*., 1996).

Unlike NrdF, the recombinant PdhA antigen was not recognised by sera from naturally *M. hyopneumoniae* infected animals or by porcine hyperimmune serum in Western blots. The capacity of the 12 kDa antigen characterised in this work to be protective can only be properly assessed by conducting a vaccine trial but this lack of immunoreactivity renders it a less likely candidate for use in the development of a recombinant vaccine. Hyperimmune serum is able to recognise the native full-length PdhA protein however, and so the C-terminal portion of the protein may be contributing to its observed immunoreactivity. Further work is required to map the immunoreactive region of the molecule. If identified, that particular region could be assessed as a vaccine candidate.

Chapter Five

Cloning and Characterisation of a 216 kDa Adhesin-like Protein of *M. hyopneumoniae*

5.1. Introduction

Information on the surface architecture of *M. hyopneumoniae* remains limited. P97, has been well characterised as a cilial adhesin (Zhang *et al*., 1995; Hsu *et al*., 1997; Hsu and Minion 1998; Wilton *et al*., 1998; Minion *et al*., 2000; Djordjevic *et al*., 2004) but there is evidence to suggest that other proteins are involved in adherence of *M. hyopneumoniae* to cilia, and consequently pathogenesis (Zhang *et al*., 1995). With a demonstrated correlation between immunoreactive antigens and membrane proteins (Wise and Kim, 1987; Scarman *et al*., 1997; Djordjevic *et al*., unpublished data), the third antigen characterised in this research was also chosen for its immunoreactivity with convalescent sera pooled from pigs infected with *M. hyopneumoniae*.

Differentiating the antigen described in this chapter from the previous two antigens described in this thesis is that it was chosen as an immunoreactive internal tryptic peptide of a Fraction 2 antigen (M. Hahn, Honours thesis). Fraction 2 antigens were described in section 3.1 and encompass a group of immunoreactive proteins of M. hyopneumoniae between 85 and 150 kDa. N-terminal amino acid sequence information generated from the tryptic peptide of the Fraction 2 antigen suggested it as novel and enabled cloning using the same procedure described in the previous chapter for PdhA. As a Fraction 2 antigen in the size range 85 to 150 kDa, this particular protein is potentially surface located. It also had the potential to be protective as part of a pool of antigens that had previously afforded some protection against disease in pigs in an experimental trial (Djordjevic *et al*., unpublished data; Djordjevic *et al*., 1997).

The 216 kDa adhesin-like protein described in this chapter was extensively characterised using a range of molecular techniques. Cloning and expression of several recombinant antigens allowed investigation of its presence amongst geographically diverse strains of *M. hyopneumoniae*, processing events, cellular location and heparin binding activity.

5.2. Methods

Unless otherwise specified in the methods section below, all experimental procedures relating to this chapter were performed as described in Chapter 2 (General Methods).

5.2.1. Tryptic Digestion and Selection of Fraction 2 Antigens

This research was performed by M. Hahn (Honours thesis, 1995). Briefly, Fraction 2 proteins (identified From ELISA profiles) were pooled separately and concentrated by ultrafiltration. The protein preparations were dialysed against 50 mM ammonium hydrogen carbonate before being subject to tryptic digestion. Optimal conditions for tryptic digestion were determined and the procedure was carried out at 37°C for 30 min using 1 µl of a 50 mM ammonium hydrogen carbonate solution containing 1 mg/ml of trypsin for every 200 µL of concentrated protein sample. Digested proteins were resolved using tricine gel (10-20%) electrophoresis and transferred to PVDF membranes for immunoblotting. The most immunoreactive bands were selected for N-terminal microsequencing.

5.2.2. PCR

5.2.2.1. Inverse PCR

PCR can be used to amplify sequences that lie outside the boundary of known sequences. A restriction enzyme is used to digest chromosomal DNA and generate a 2 to 3 kb fragment containing the target DNA. The fragment is circularised by ligation

and a PCR reaction performed on the circularised DNA using primers complimentary to the target DNA sequence. Digested *M. hyopneumoniae* strain Beaufort DNA (see section 2.2.2.3) was diluted 1/2, 1/5, 1/10, 1/20, 1/50, 1/100 and 1/200 for ligation, to obtain conditions that favoured the formation of monomeric circles. Diluted DNA (5 μL), made up to a volume of 17 μL with water, was incubated at 65°C for 10 min, 37°C for 10 min, room temperature for 10 min, and finally 10°C for 10 min, before T4 DNA ligase (4 units) and 10X buffer was added and gently mixed prior to overnight incubation at 10°C. All eight ligation mixtures were subjected to PCR using *Taq* DNA polymerase (see section 2.2.2.6) with primers designed from the target sequence.

5.2.2.2. Gradient PCR

Gradient amplification with *Taq* polymerase and subsequent temperature optimised repeat amplification with *Pwo* polymerase were performed in a 50 μL reaction mixture containing 35 ng of plasmid DNA (pCR2.1ORF545A or pCR2.1ORF545B), 20 pmol of each oligonucleotide primer (see Appendix C), 200 μM deoxyribonucleotide triphosphates, 2 units of polymerase (*Taq* or *Pwo*) and 10X PCR buffer (as supplied with the polymerase). Reaction mixtures were incubated at 94°C for 3 min for 1 cycle, followed by 35-40 cycles of denaturation at 94°C for 30 sec, primer annealing at 50-60°C for 30 (*Taq*) or 60 (*Pwo*) sec, and extension at 72 °C (*Taq*) or 68°C (*Pwo*) for 1 min. A final extension at 72°C (*Taq*) or 68°C (*Pwo*) for 10 min was also included in the cycle parameters. The products were visualised on agarose slab gels stained with ethidium bromide. PCR products generated with *Pwo* polymerase were purified using QIAQUICK PCR purification prior to visualisation and cloning.

5.2.3. Cloning into pET161

Fragments amplified from ORF545 plamids were cloned into pET161 using a Champion™ pET TOPO directional cloning kit (Invitrogen) offering rapid and efficient directional cloning of blunt-end PCR products. Procedures were performed largely as described in the manufacturer's instructions. The concentration of purified PCR fragments was estimated by an absorbance reading at 280 nm. Approximately 6-7 ng of PCR product was incubated at room temperature for 5 min in a reaction mix containing a salt solution, water and 1 μL TOPO vector solution (approximately 15-20 ng vector). The ligation mix was stored on ice until transformation. If transformation was being performed with chemically competent *E. coli* Top 10 cells then 3 μL of ligation mix is added to a 50 μL aliquot of competent cells and mixed gently prior to incubation on ice for 30 min. Cells were then heat-shocked at 42°C in a block heater for 30 sec before being immediately transferred back to ice. RT SOC medium (250 μL) was then added and the cells cultured at 37°C with shaking for 1 h. Plating of two different volumes on selective media followed with an overnight incubation at 37°C. Colonies were selected and cultured for plasmid extraction and digestion analysis (described in section 2.2.2.2 and 2.2.2.4). All recombinant clones were sequenced to confirm their integrity with the *mhp493* gene sequence (Minion *et al*., 2004). Recombinant $F1_{p216}$–$F3_{p216}$ plasmids were transformed into *E. coli* BL21 cells. Expression and purification of the recombinant antigens was performed as described in General Methods section 2.2.3.6. except that cultures were induced with IPTG to a final concentration of 0.5 mM.

5.2.4. Post Two-Dimensional Gel Separation Analysis

Proteins spots were manually excised from Coomassie stained acrylamide gels and placed in 96-well V-bottom microtitre plates (Greiner). Conditions used to digest proteins with trypsin and for the generation of peptide mass fingerprints have been described previously (Djordjevic *et al*., 2003 and 2004). Briefly, gel pieces were washed with 50 mM ammonium bicarbonate–100% acetonitrile (60:40 [vol/vol]) by shaking for 1 h at RT and then dried in vacuum dessicator for 90 min. Gel pieces were then hydrated for 1 h at 4°C in 8 μL of a 17 ng/μL concentration of sequencing-grade modified trypsin (Promega). Excess trypsin solution was removed, and the gel pieces incubated overnight in 50 mM ammonium bicarbonate (25 μL) at 37°C. If in low concentration or producing poor spectra, eluted peptides were concentrated and desalted using C18 Zip-Tips (Millipore). All samples were spotted onto a target plate in matrix solution (10 mg of α-cyano-4-hydroxycinnamic acid [Sigma]/mL in 70% acetonitrile). Matrix-assisted laser desorption ionization–time-of-flight mass spectrometry was performed as described by Wilton *et al*. (2006). Mass spectra were acquired from an Omniflex II (Bruker) using internal calibration standard Pep Mix 1 (Laser Bio Laboratories). Protein identifications were assigned using Mascot software (Matrix Science) by comparing the peak lists generated from peptide mass fingerprinting data to a database containing theoretical tryptic digests of *M. hyopneumoniae* strain 232. Successful identifications were made on the basis of the number of matching peptide masses and the percentage of sequence coverage from at least 3 independent samples giving peptide matches within 20 ppm.

5.2.5. Heparin binding

5.2.5.1. Heparin blots

Recombinant proteins were electrophoresed in acrylamide and transferred to PVDF membranes as described in section 2.2.3.1 and 2.2.3.3. Membranes to be probed with biotinylated heparin were blocked by gentle shaking in PBS containing 1% BSA for 1 h at RT. Blots were washed for 3 x 10 min at RT with more vigorous shaking in PBS containing 0.1% BSA. Incubation with biotinylated heparin at 10 μg/mL in wash buffer followed for 1.5 h at RT with gentle shaking. Blots were washed as previously prior to the addition of SA-POD diluted 1/3000 in wash buffer for 1 h at RT with gentle shaking. After a final wash development was achieved with freshly prepared DAB substrate solution.

5.2.5.2. Heparin binding assays

Microtitre plate heparin binding assays were performed as described by Wilton *et. al*. (2009). Briefly, 96-well plates (Linbro/Titertek; ICN Biomedicals Inc., Aurora, Ohio) were coated overnight in a humidified chamber with 20 nM of purified recombinant P216 proteins ($F1_{P216}$ – $F3_{P216}$) per well diluted in 100 μL carbonate coating buffer. Proteins were heated to 100°C prior to their dilution in coating buffer containing 10 mM DTT to prevent the possible creation of artifactual multimeric forms of $F1_{P216}$, $F2_{P216}$, and $F3_{P216}$. These may have been produced by the formation of disulfide bridges between cysteine residues on the lumio tag present in these constructs as P216 does not contain any cysteine residues. The wells were blocked in PBS containing 2% skim milk for 1 h at RT. Biotinylated heparin was then added in PBS containing 1% skim milk for

1.5 h at RT. The specificity of heparin binding for $F1_{P216}$, $F2_{P216}$, and $F3_{P216}$ was determined with the addition of serial 2-fold dilutions of biotinylated heparin, beginning at a saturating concentration of 20 μg/mL which were pre-mixed with a 50-fold excess of unlabelled heparin prior to addition to the plates. Bound biotinylated heparin was detected using peroxidase conjugated streptavidin (Roche) diluted 1/3000 in PBS containing 1% skim milk for 1 h at RT. The addition of ABTS substrate solution followed prior to the absorbances being measured at 414 nm using a Multiskan Ascent ELISA plate reader. Plates were washed 5 times between each step in PBS containing 0.05% Tween 20 using an automated plate washer (SLT Lab Instruments).

Competitive binding assays were also performed as above but with the addition of a 1, 5, 10, 20, 30, 40 and 50-fold excess of a range of sulfated polysaccharides including unlabelled heparin, fucoidan, mucin and chondroitin sulfate B (Sigma). The polysaccharides were pre-mixed with biotinylated heparin immediately prior to addition to the wells. In all microtitre plate assays, controls were performed in uncoated wells, wells where no heparin was added, or wells where no streptavidin-peroxidase was added. Controls which employed protein-specific antiserum and horseradish peroxidase (HRP)-labelled anti-rabbit IgG were also conducted to ensure that proteins were adhering to the microtitre plate wells. All experiments were performed in triplicate. Results were graphed with GraphPad Prism version 4.02 for Windows (GraphPad Software) using non-linear regression with one-site binding and one-site competition analysis.

5.2.6. Growth Assay

The processing of P216 into cleavage fragments P120 and P85 was examined over the entire growth cycle of *M. hyopneumoniae* by immunoblotting samples of synchronous *M. hyopneumoniae* cultures over a time period of 56 h. Numerous *M. hyopneumoniae* (strain J) cultures (6 mL) were established (as described in section 2.2.1.2) and harvested at 10 h (in early-log phase), 18 and 24 h (in mid-log phase), 42 and 46 h (in late-log phase) and at 50, 55 and 59 h (in stationary phase). Cells were harvested at 13,000 g for 6 min and washed twice with PBS prior to analysis by SDS PAGE and immunoblotting with anti-$F2_{P216}$, and anti-rP27 serum as described in sections 2.2.3.1 and 2.2.3.3.

5.3. Results

5.3.1. Cloning and Preliminary Analysis

The gene encoding the 3rd antigen of interest was cloned in a similar way to that described in the previous chapter for the gene encoding PdhA. The N-terminal sequence data used to clone the gene encoding the protein of interest was generated from an immunoreactive internal tryptic peptide of a *M. hyopneumoniae* Fraction 2 antigen. See Table 5.1 for the N-terminal peptide sequence and the degenerate oligonucleotide sequence designed for cloning.

Table 5.1. N-terminal sequence data and degenerate oligonucleotide probe designed to clone the antigen of interest.

N-terminal Sequence	Degenerate Oligonucleotide
ELEDNTKLIAPNIRQ	5'- GAA YTW GAA GAT AAT ACH AAA TTA ATT GCW CCT AAT ATT -3'

* Where Y is T or C, W is T or A, and H is C, A or T

5.3.1.1. Southern Hybridisation Analysis with the Degenerate Oligonucleotide

The degenerate oligonucleotide was initially used to probe a Southern blot of *Eco*RI digested DNA from *M. hyopneumoniae* strains J and Beaufort, *M. hyorhinis* strain GDL and *M. flocculare* strain MS42. Figure 5.1 (A) shows the agarose gel from which the digested DNA was transferred for Southern analysis. The Southern blot result is shown

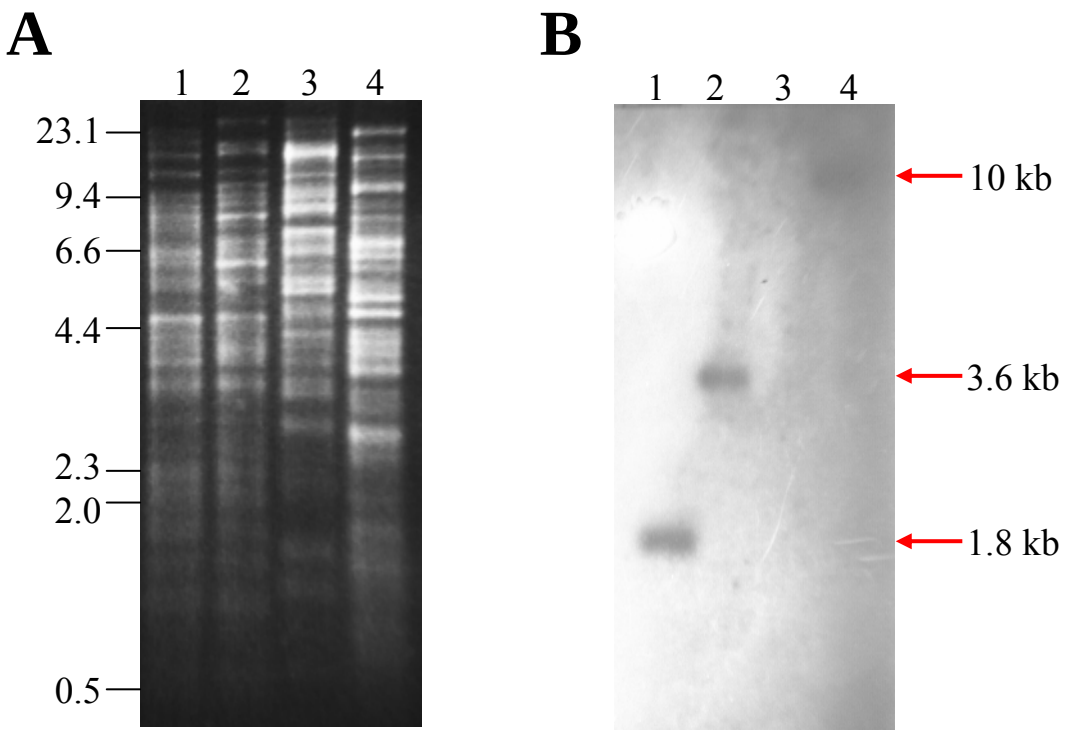


Figure 5.1. Southern hybridisation analysis of the degenerate oligonucleotide against *Eco*RI digested genomic DNA from *M. hyopneumoniae* strains J and Beaufort (lanes 1 and 2 respectively), *M. hyorhinis* strain GDL (lane 3) and *M. flocculare* strain MS42 (lane 4). Molecular size markers are given to the left of the agarose gel (top) in kb. An image of the agarose gel (1%) used for transfer is shown stained with ethidium bromide in (A). Southern hybridisation analysis against the digested genomic DNA using probe 3 is given in (B). The fragments identified by the probe are indicated with an arrow.

as Figure 5.1 (B). The probe identified a fragment of 1.8 kb in *M. hyopneumoniae* strain J DNA, and a fragment of 3.6 kb in *M. hyopneumoniae* strain Beaufort DNA. The degenerate oligonucleotide probe also hybridised weakly to a fragment of approximately 10 kb in DNA from the type strain of *M. flocculare* suggesting that *M. flocculare* may possesses a gene similar to that of the target gene of *M. hyopneumoniae*. The probe did not hybridise to digested DNA from the *M. hyorhinis* strain used. The gene appeared to be in single copy in the *M. hyopneumoniae* genome.

5.3.1.2. DNA Fractionation and Southern Hybridisation Analysis Identifying the Fragment for Cloning

The 3.6 kb *Eco*RI fragment of *M. hyopneumoniae* (strain Beaufort) was identified in preparative agarose fractionated DNA. Figure 5.2 shows the agarose gel (A) and corresponding Southern blot (B) of the *Eco*RI fractions in the 3 to 4 kb size range. A size increase of fragments is evident in the gel and the boxed region corresponds to those fractions identified in the Southern blot as containing the fragment to be cloned. The intensity of the signal in the Southern is weak, nevertheless fractions containing the fragment could be determined (shown boxed). The identified fragments were pooled, concentrated and blunt-ended for cloning into pPCR Script (Stratagene).

5.3.1.3. Cloning the Fragment into pPCR-Script™

Positive pPCR-Script clones were initially identified by colony hybridisation with the degenerate oligonucleotide probe, as shown in Figure 5.3 (A). A positive clone is shown in the top left of the blot and chromosomal DNA acting as a positive control on the

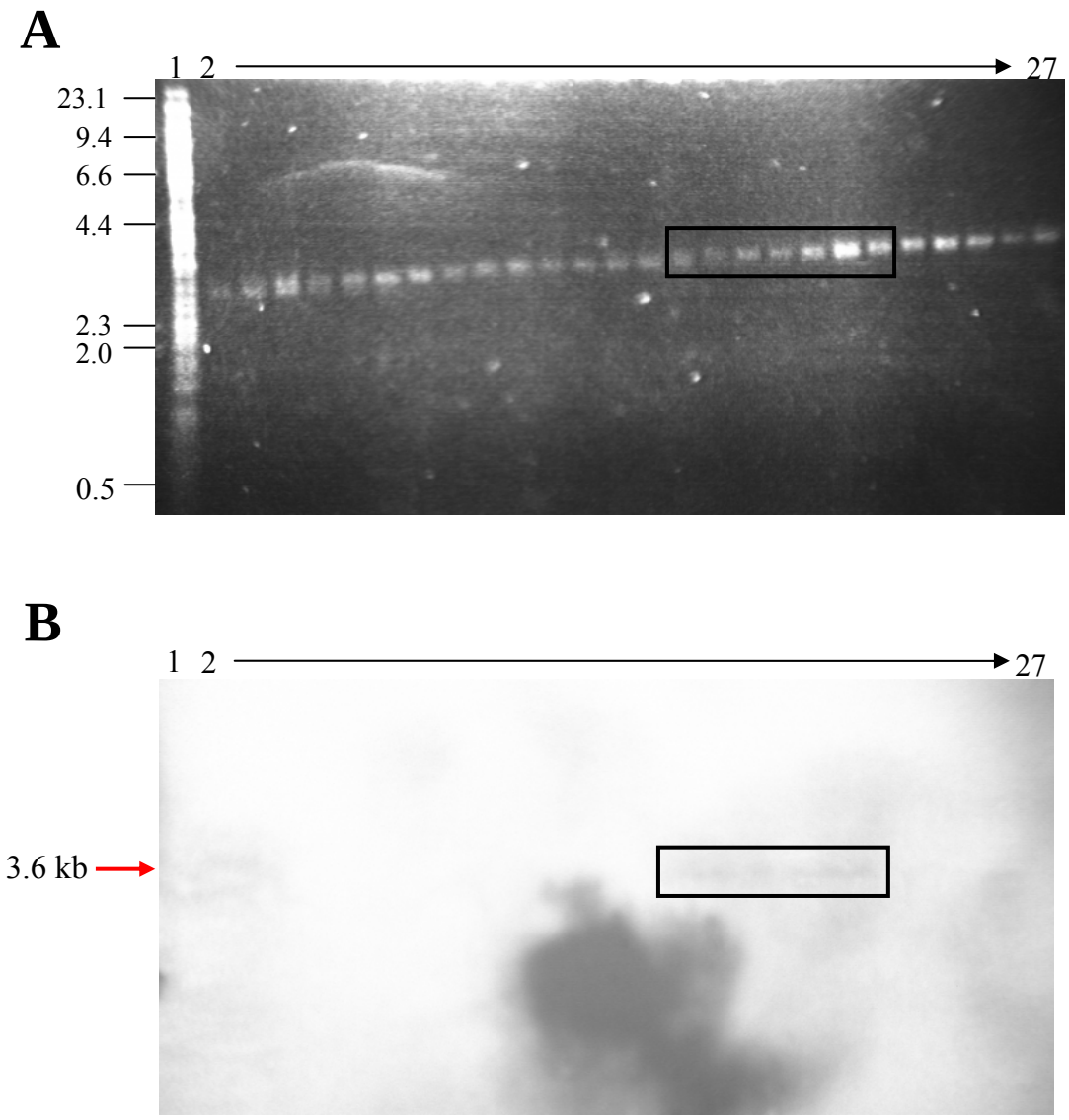


Figure 5.2. Agarose gel (1%) electrophoresis and Southern hybridisation analysis for identification of the fragment of interest in *Eco*RI digested and fractionated *M. hyopneumoniae* (strain Beaufort) DNA. Agarose gel stained with ethidium bromide (A) and corresponding Southern blot probed with oligonucleotide 3 (B), with *Eco*RI digested *M. hyopneumoniae* DNA in lane 1, and fractions eluted from the preparative agarose column of *Eco*RI digested *M. hyopneumoniae* DNA in lanes 2-27. Fractions containing fragment 3 are shown boxed. Size markers are given in kb on the left of the agarose gel in (A).

bottom right. Clones identified as positive by colony blotting were cultured for further analysis. Plasmid DNA was extracted from the cultures using a miniprep procedure and digested with *Bam*HI and *Sac*I for the release of insert DNA. Digested clones were confirmed positive by Southern hybridisation analysis.

Figure 5.3 (B) shows the digested plasmid DNA as electrophoresed in agarose, and the resulting Southern blot after transfer and probing. The agarose gel shows *Eco*RI digested chromosomal DNA of *M. hyopneumoniae*, *Bam*HI/*Sac*I digested vector to act as a negative control, and undigested as well as *Bam*HI/*Sac*I digested plasmid DNA of the positive clone. When transferred and probed with the degenerate oligonucleotide in Southern analysis, hybridisation was evident in the lanes containing plasmid DNA of the positive clone. The probe was unable to hybridise to vector DNA (lane 2), but hybridised to undigested plasmid (lane 3) and the released insert (lane 4) of the clone, as expected.

5.3.1.4. Sequence Analysis of the Cloned Fragment

Once the clone (designated pJW6) was confirmed as positive, it was sequenced, translated, and analysed for open reading frames. Vector primers as well as the degenerate oligonucleotide were used as a sequencing start point. Sequence information generated was used to sequence along the cloned fragment, in both directions, by primer "walking". Sequence analysis revealed that the clone insert was 3.7 kb in length, and that the gene encoding the protein of interest had only been partially cloned. The carboxy-terminal 744 amino acids of the protein of interest (ORF1) were encoded by the clone as indicated in the gene map of Figure 5.4. The length of the native protein

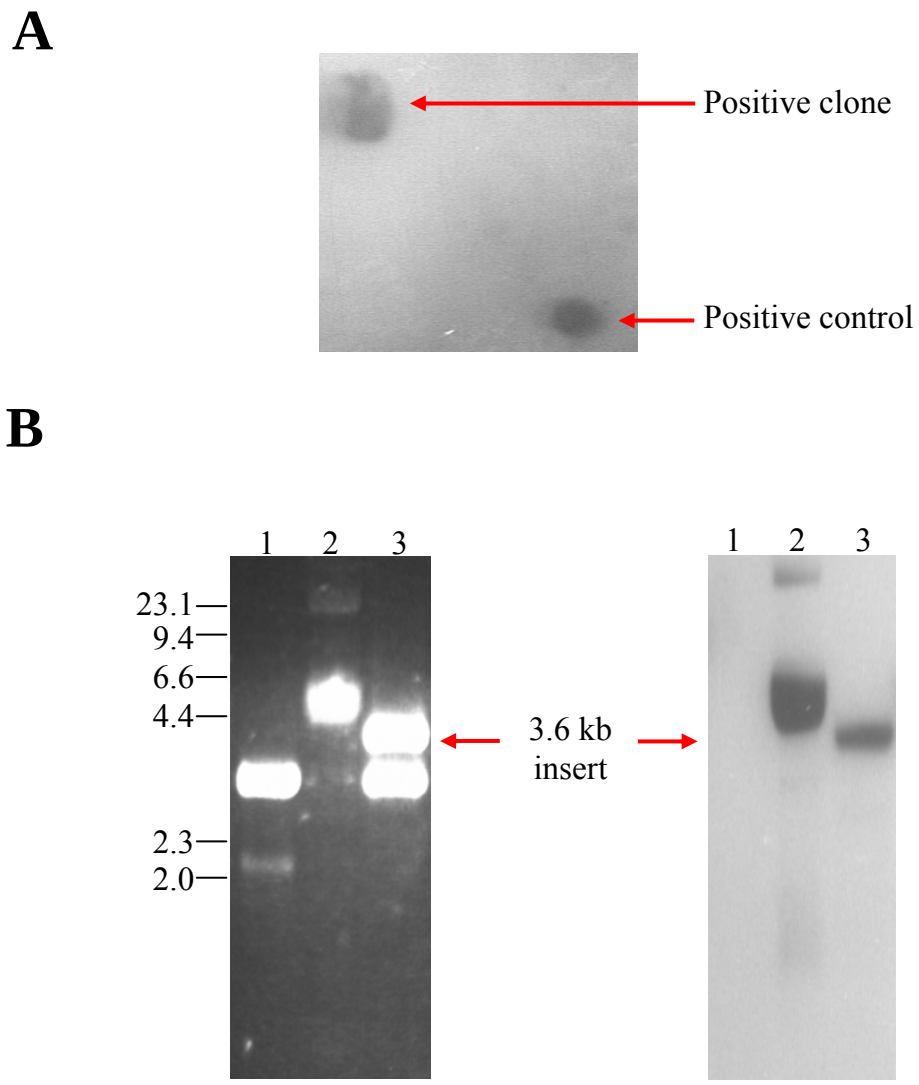


Figure 5.3. Colony and Southern hybridisation analysis to confirm cloning. Colonies from the PCR-Script™ cloning were lifted onto nitrocellulose for probing with the oligonucleotide 3 in a colony blot (A). *M. hyopneumoniae* genomic DNA from strain Beaufort was spotted onto the membrane to act as a positive control. The positive clone and the positive control are indicated. Undigested and digested vector and plasmid DNA was electrophoresed in agarose (1%) for Southern hybridisation analysis with the degenerate oligonucleotide (B). Lane 1, *Sac*I and *Bam*HI digested pPCR-Script vector; and Lanes 2 and 3, undigested and *Sac*I and *Bam*HI digested plasmid DNA extracted from the positive clone identified in the colony blot. Molecular markers are given on the left of the agarose gel and the 3.6 kb insert is indicated by a red arrow.

was unknown, as the translated sequence showed no homology to any gene in the database when submitted for searching through ANGIS. A second open reading frame (ORF2) of approximately 900 bp translated to a protein with strong homology to mannose-6-phosphate isomerase from several sequenced mycoplasma species. However, it was the novel sequence of ORF1 that encoded the protein of interest.

5.3.2. Inverse PCR to Obtain the Full Gene Sequence

Inverse PCR was used in an attempt to obtain the entire sequence of the gene of interest as a start codon was not identified in the open reading frame (2.24 kb) of the original clone.

5.3.2.1. Digestion of Chromosomal DNA and Southern Hybridisation

A total of 15 restriction enzymes were used to digest chromosomal DNA from strain Beaufort in order to identify a fragment of approximately 2-3 kb containing the target sequence. As can be seen in Figure 5.5 (A), showing an ethidium bromide stained gel of the restriction digestions, the enzymes digested the DNA to differing degrees creating an extensive and varied range of fragment sizes. The digested DNA was transferred to a nylon membrane for Southern hybridisation analysis. The Southern blot was probed with a PCR product of approximately 1 kb amplified from the cloned target DNA. The resulting blot is shown in Figure 5.5 (B). As can be seen in the figure, the majority of fragments identified by the PCR product as containing the target sequence, are greater

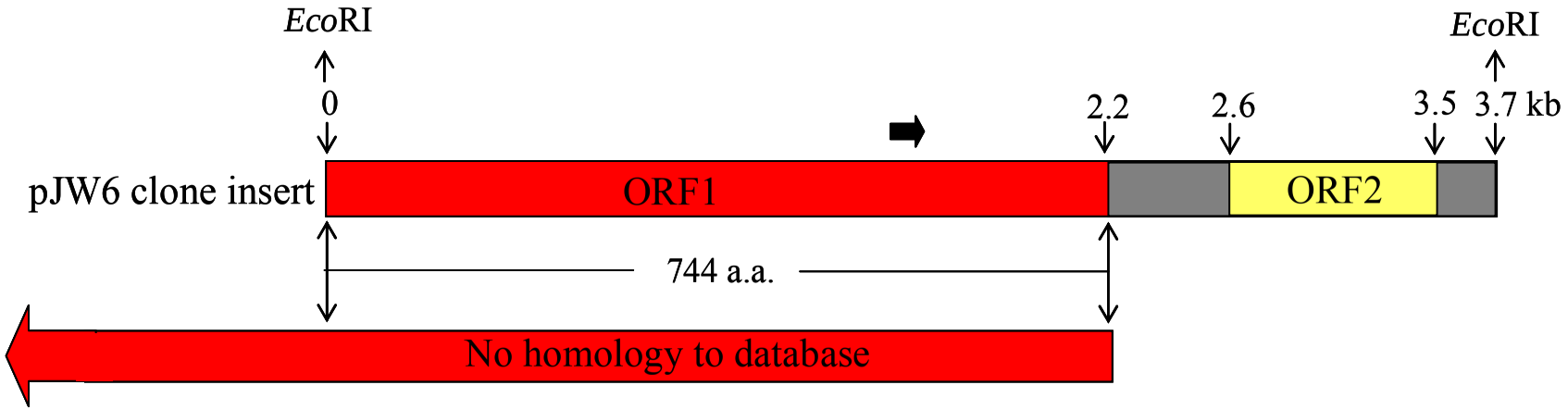


Figure 5.4. Sequence analysis of the 3.7 kb *Eco*RI insert of pJW6. The gene map shows the open reading frames (ORF1 and ORF2) identified when the sequence was translated and their respective sizes. A black arrow indicates the location of the probe sequence used to clone the gene of interest. The translated sequence of ORF1 had no homology with any gene in the ANGIS database and so the gene size is unknown.

than 5 kb. One fragment however, of approximately 4 kb, a product of digestion with *Nru*I (lane 10), was chosen for use in inverse PCR.

5.3.2.2. Inverse PCR

*Nru*I restricted chromosomal DNA was diluted 1/2, 1/5, 1/10, 1/20, 1/50, 1/100 and 1/200 for ligation with T4 DNA ligase overnight. The seven ligation reactions of circularised *Nru*I fragments were used separately as template for the seven inverse PCR reactions performed. The inverse PCR samples were electrophoresed in agarose and stained with ethidium bromide for visualisation of any amplified product/s. A fragment of approximately 1.4 kb was successfully amplified from all seven ligation reactions (Figure 5.6). The amplified product quite visibly decreases in concentration with the increase in dilution of *Nru*I digested DNA used in the ligation reaction.

5.3.2.3. DNA Sequence Analysis of the Amplified Inverse PCR Product

For sequencing, the three most concentrated amplification products from the inverse PCR were electrophoresed in agarose and purified by extraction using the Bresa-Clean method. Sequencing was initiated using the same primers that were used for amplification of the 1.4 kb product and completed by primer walking along the fragment. When sequenced completely in both directions, the information generated was assembled and analysed for open reading frames. Unexpectedly, a start codon was not found within the 0.86 kb of target DNA sequenced, and when translated still showed no homology to any proteins in the ANGIS database. Therefore, the length of the gene

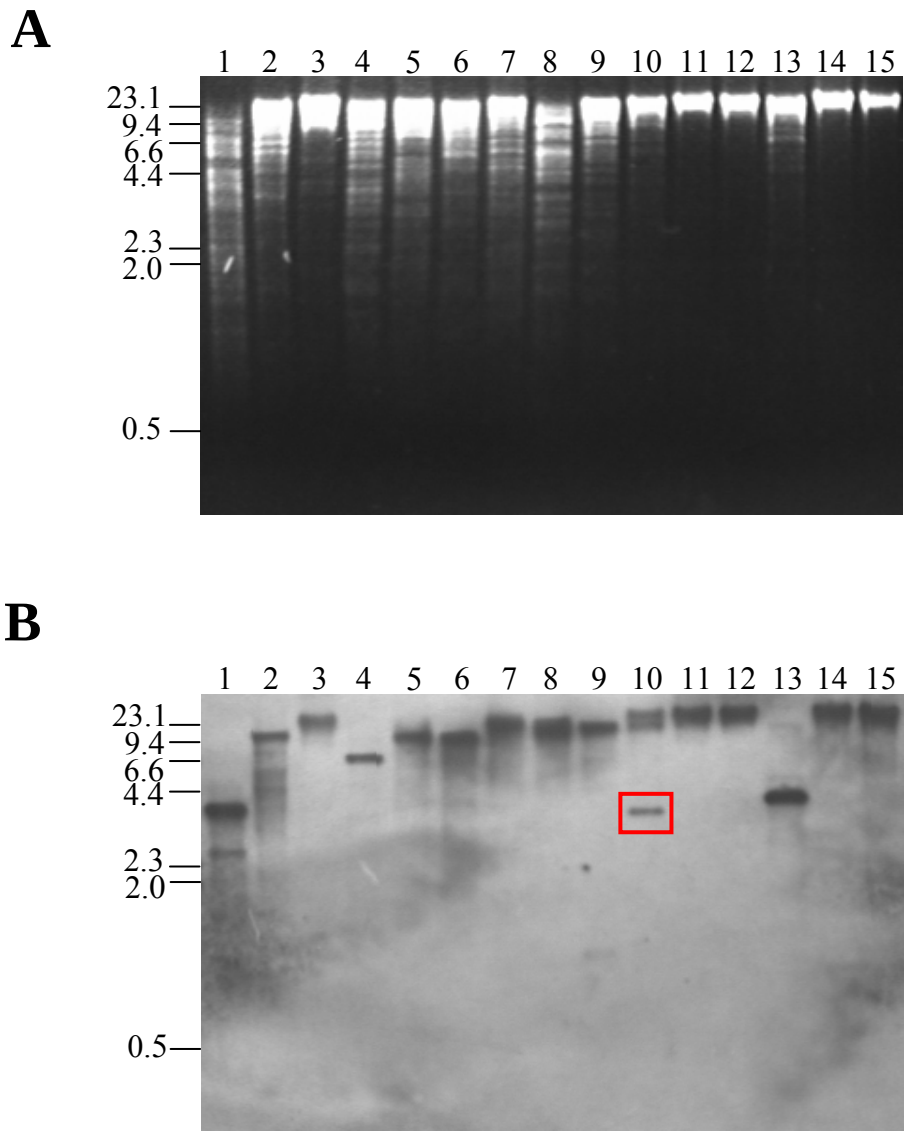


Figure 5.5. Restriction of Beaufort DNA for Inverse PCR. Genomic DNA from *M. hyopneumoniae* strain Beaufort was digested with *Eco*RI, *Acc*I, *Bam*HI, *Bgl*II, *Cla*I, *Dra*II, *Eco*RV, *HindIII*, *Nde*I, *Nru*I, *Pst*I, *Sal*I, *Sca*I, *Sma*I, and *Xho*I (lanes 1 to 15 respectively). Digested DNA was electrophoresed in agarose (A) and transferred to a nylon membrane for Southern hybridisation analysis (B). A 1 kb fragment amplified from the cloned portion of the gene of interest was labelled and used as the Southern probe. Molecular size markers are given on the left in kb and the red box indicates the fragment chosen for inverse PCR.

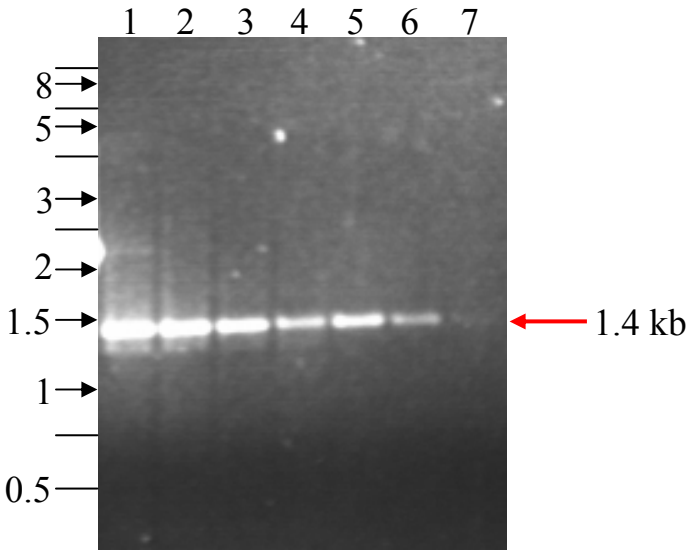


Figure 5.6. Inverse PCR to obtain the entire gene sequence encoding the novel antigen. Ethidium bromide stained agarose gel (1%) of the eight inverse PCR reactions. Lanes 1 to 8 represent the amplification products with the ligation reactions using restricted DNA diluted 1/2, 1/5, 1/10, 1/20, 1/50, 1/100, 1/200, and 1/500 respectively. Molecular size markers are given on the left in kb and the 1.4 kb amplification product is indicated.

of interest remained unknown, as did the sequence of the 5' end. Nonetheless, an additional 0.86 kb of the gene was successfully sequenced.

5.3.3. Access to *M. hyopneumoniae* Genome Database to Sequence the 5' End of the Novel Gene

Access to the *M. hyopneumoniae* strain 232 genome database was granted by Minion *et al*. (2000) when an affiliation was made between our research groups. The sequence already obtained was used in an alignment with the database and the gene of interest was identified as the Beaufort homologue of a strain 232 gene (Mhp 493) encoding a 216 kDa protein (P216). The Mhp493 gene sequence from strain 232 was used to design primers for PCR and sequence analysis of the 5' end of the target gene from strain Beaufort. Access to the database also allowed rapid sequencing of the avirulent *M. hyopneumoniae* strain J equivalent.

5.3.3.1. Amplification and DNA Sequence Analysis of the 5' End of the *p216* Gene from Strain Beaufort

A forward primer (5'F) designed upstream of the Mhp493 sequence, and a reverse primer (5'R) designed from the sequence that had been generated for strain Beaufort (Figure 5.7 (A)) were used in a gradient PCR for the amplification of a product that was expected be approximately 2.5 kb in length. The PCR result is shown in Figure 5.7 (B). A 2.5 kb product was successfully amplified at all gradient temperatures (60°C to 53°C) but a temperature of 53°C was determined optimal as it gave the greatest concentration of product without non-specific amplification. The PCR product generated from primers

5'F and 5'R at an annealing temperature of 53°C was purified from agarose (Bresa-Clean) for sequencing. The primers used for sequencing were designed from the Mhp493 sequence.

5.3.3.2. DNA Sequence Assembly and Analysis of the *p216* Gene from Strain Beaufort

The sequence information obtained for the 5' end of the gene of interest was assembled and aligned with the already established 3' sequence. The entire open reading frame encoding the *p216* gene from *M. hyopneumoniae* strain Beaufort was 5613 base pairs in length (5637 for strain 232) which translated to a protein of 1871 amino acids (1,879 for strain 232), and was calculated as having a mass of 215 kDa (216 kDa for strain 232). The G+C content was calculated at 31%, which is typical for mycoplasmas (Maniloff, 1996). Although it is now believed that the mycoplasmal –10 region and to a lesser degree the –35 region do resemble eubacterial promotor consensus sequences (Razin *et al*., 1998), neither were identified upstream from the ATG start codon. A Shine-Dalgarno like sequence was also not located, which is not particularly unusual (Razin *et al*., 1998). A large number of TGA codons (17 in total) were identified throughout the sequence (these specify a tryptophan residue in mycoplasma but a stop in *E. coli*). The complete DNA sequence was deposited in the Genbank database under accession number AF541877.

The primers used to sequence *p216* from strain Beaufort were also used to sequence the equivalent gene from the avirulent *M. hyopneumoniae* strain J. The assembled sequence

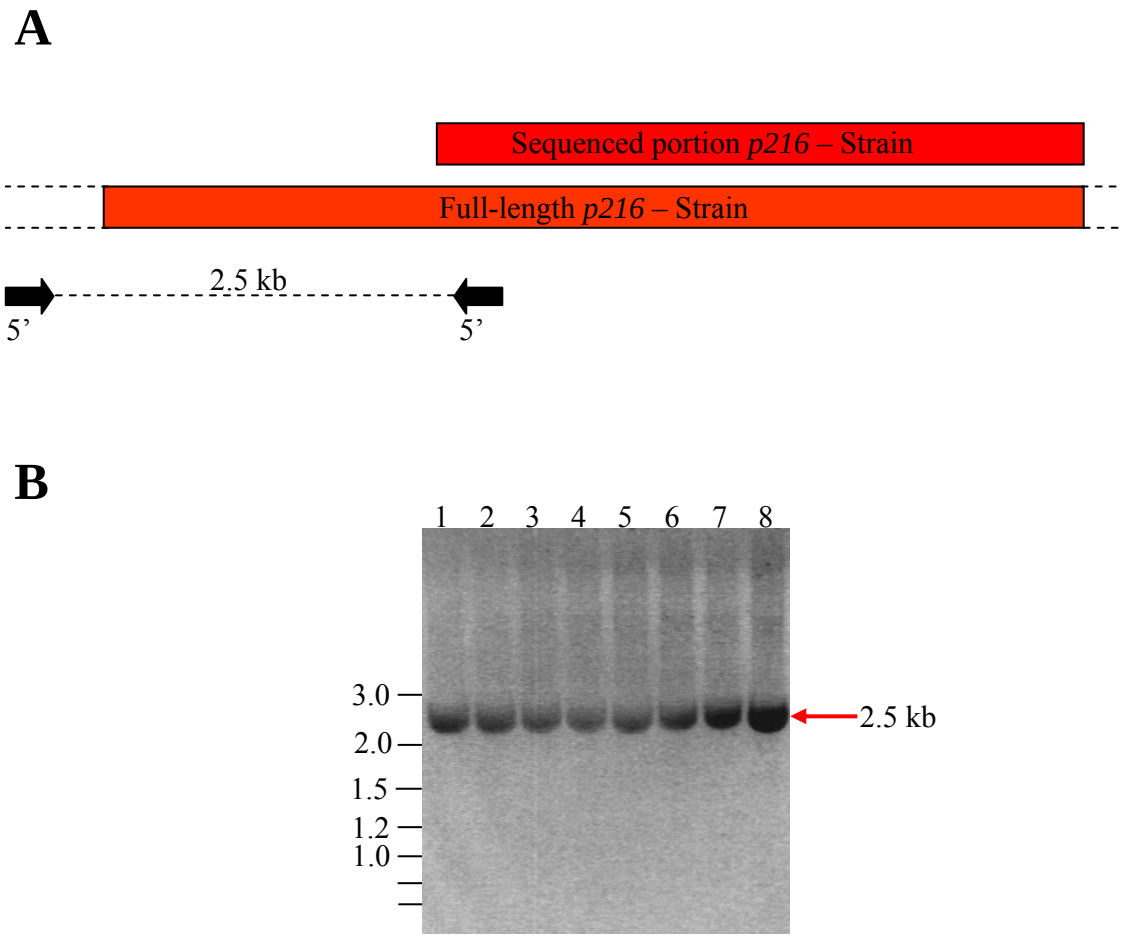


Figure 5.7. PCR of the 5' end of the novel gene from strain Beaufort, using the strain 232 sequence. (A) Gene map showing full-length p216 (strain 232) and the sequenced portion of the same gene from strain Beaufort. The forward primer (5'F) was designed from the 232 sequence and the reverse primer (5'R) from the Beaufort sequence. The primers were used in a gradient PCR to amplify the unsequenced 5' portion of the novel gene from strain Beaufort (B). Lanes 1 to 8 represent the temperatures of the gradient PCR, decreasing from 60°C to 53°C respectively. Molecular markers are given in kb on the left and the 2.5 kb amplification product is indicated.

was 5634 nucleotides in length and translated to a protein of 1878 amino acids. It was deposited in the Genbank database under accession number AF540380.

5.3.3.3. Protein Analysis of P216 from strain Beaufort

A series of tools available on the ExPASy Proteomics Server were used for protein analysis of P216 from strain Beaufort. The amino acid composition was high in lysine (10.26%), leucine (10.20%) and asparagine (8.23%), low in methionine (0.59%) and histidine (0.69%), absent of cysteine and was determined to have a pI of 8.26 (8.51 in strain 232). Computer analysis using ProtScale indicated a largely hydrophilic protein (Figure 5.8 (A)) with a potential transmembrane domain predicted from the fact that there is a small but hydrophobic region at the N-terminus of the molecule (while the rest of the molecule is reasonably hydrophilic). Computer analysis using TMpred predicted with significance (score of 2029) a transmembrane domain from amino acid 7 to 30 (Figure 5.8 (B)).

The prediction of a potential bipartite nuclear binding domain in the centre of the protein at amino acid position 1012 to 1029, was determined using ProfileScan. Also identified were a number of potential glycosylation, phosphorylation, and myristoylation sites. A search of KEKE motifs using an algorithm kindly supplied by Prof. Rechsteiner (University of Utah, Salt Lake City, Utah, USA) identified one region with the sequence KIKDASNKKGEEK at amino acid position 1497 to 1509.

BLAST analysis with Genbank identifies P216 as a *M. hyopneumoniae*-specific protein of unknown function with no significant related sequences found in other Mycoplasma

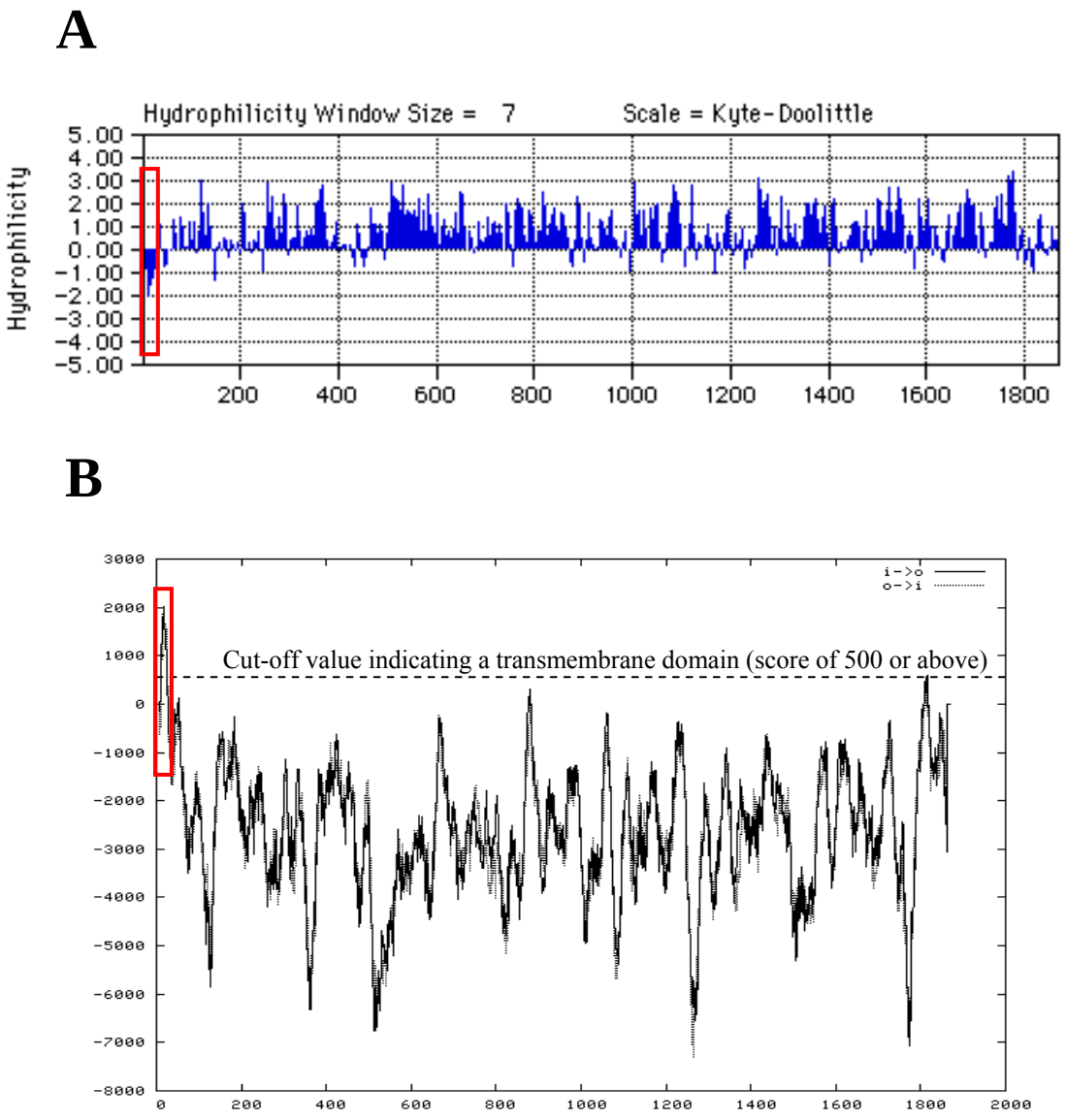


Figure 5.8. Prediction of a transmembrane domain in P216. ProtScale hydrophilicity plot (A) and TMpred output (B) produced through the ExPASy Proteomic Server (http://au.expasy.org/tools/). The predicted transmembrane domain is shown boxed in red with a TMpred score of 2029, well above the cut-off value of 500 (broken line).

or prokaryote genomes. It did align with equivalent proteins from *M. hyopneumoniae* strains J and 7448 (Vasconcelos *et al*., 2005). Interestingly, BLASTP analysis of P216 against the *M. hyopneumoniae* genome revealed 21% sequence identity (38% similarity with 19% gaps) with the P97 cilium adhesin (Expect = 4e-18), identifying P216 as a P97 paralog. The most closely related protein outside of the *M. hyopneumoniae* genome is the LppS adhesin (Expect = 2e-21) from *M. conjunctivae* with 27% identical amino acids (48% similarity with 7% gaps).

5.3.4. Alignment of P216 from Strains Beaufort, 232, J and 7448

The amino acid sequence of P216 from *M. hyopneumoniae* strains Beaufort, 232, J and 7448 were aligned using ClustalW from the Embnet website (Figure 5.9). The 232, J and 7448 sequences were all longer than the Beaufort sequence at 1879, 1878 and 1885 amino acids in length respectively. The four sequences showed 95.8% sequence identity with the majority of differences localised to two regions of glutamine repeats (shown in orange). These glutamine repeats varied in length from 5 (strain Beaufort) to 15 (strain 7448) between amino acids 1257 – 1272, and 3 (strain 232) to 11 (strain 7448) between amino acids 1379 – 1390. The BNBD motif shown in green and the KEKE motif shown in red are both 100% conserved among the four sequences. A single J strain sequence was shown in the alignment as the sequence described by Vasconcelos *et al*. (2005) differed from our J strain sequence by only one amino acid at position 76 despite the strains being maintained in different laboratories and undergoing different degrees of *in vitro* passage.

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Beaufort        MKNKKSTLLLATAAAIIGSTVFGTVVGLASKVKYRGVNPTQGVISQLGLIDSVAFKPSIA
232             MKNKKSTLLLATAAAIIGSTVFGTVVGLASKVKYRGVNPTQGVISQLGLIDSVAFKPSIA
J               MKNKKSTLLLATAAAIIGSTVFGTVVGLASKVKYRGVNPTQGVISQLGLIDSVAFKPSIA
7448            MKNKKSTLLLATAAAIIGSTVFGTVVGLASKVKYRGVNPTQGVISQLGLIDSVAFKPSIA
                ************************************************************

Beaufort        NFTSDYQSVKKALLNGKTFDPKSSEFTDFVSKFDFLTNNGRTVLEIPKKYQVVISEFSPE
232             NFTSDYQSVKKALLNGKTFDPKSSEFTDFVSKFDFLTNNGRTVLEIPKKYQVVISEFSPE
J               NFTSDYQSVKKALLNWKTFDPKSSEFTDFVSKFDFLTNNGRTVLEIPKKYQVVISEFSPE
7448            NFTSDYQSVKKALLNGKTFDPKSSEFTDFVSKFDFLTNNGRTVLEIPKKYQVVISEFSPE
                *************** ********************************************

Beaufort        DDKERFRLGFHLKEKLEDGNIAQSATKFIYLLPLDMPKAALGQYSYIVDKNFNNLIIHPL
232             DDKERFRLGFHLKEKLEDGNIAQSATKFIYLLPLDMPKAALGQYSYIVDKNFNNLIIHPL
J               DDKERFRLGFHLKEKLEDGNIAQSATKFIYLLPLDMPKAALGQYSYIVDKNFNNLIIHPL
7448            DDKERFRLGFHLKEKLEDGNIAQSATKFIYLLPLDMPKAALGQYSYIVDKNFNNLIIHPL
                ************************************************************

Beaufort        SNFSAQSIKPLALTRSSDFIAKLNQFKNQDELWVYLEKFFDLEALKANIRLQTADFSFEK
232             SNFSAQSIKPLALTRSSDFIAKLNQFNNQDELWVYLEKFFDLEALKANIRLQTADFSFEK
J               SNFSAQSIKPLALTRSSDFIAKLNQFKNQDELWVYLEKFFDLEALKANIRLQTADFSFEK
7448            SNFSAQSIKPLALTRSSDFIAKLNQFKNQDELWVYLEKFFDLEALKANIRLQTADFSFEK
                **************************:*********************************

Beaufort        GNLVDPFVYSFIRNPQNQKEWASDLNQDQKTVRLYLRTEFSPQAKTILKDYKYKDETFLS
232             GNLVDPFVYSFIRNPQNQKEWASDLNQDQKTVRLYLRTEFSPQAKTILKDYKYKDETFLS
J               GNLVDPFVYSFIRNPQNGKEWASDLNQDQKTVRLYLRTEFSPQAKTILKDYKYKDETFLS
7448            GNLVDPFVYSFIRNPQNEKEWASDLNQDQKTVRLYLRTEFSPQAKTILKDYKYKDETFLS
                ***************** ****************************************

Beaufort        SIDLKASNGTSLFANEDDLKDQLDVDLLDVSDYFGGQSETITSNSQVKPVPASERSLKDR
232             SIDLKASNGTSLFANENDLKDQLDVDLLDVSDYFGGQSETITSNSQVKPVPASERSLKDR
J               SIDLKASNGTSLFANENDLKDQLDVDLLDVSDYFGGQSETITSNSQVKPVPASERSLKDR
7448            SIDLKASNGTSLFANEDDLKDQLDVDLLDVSDYFGGQSETITSNSQVKPVPASERSLKDR
                ****************:*******************************************

Beaufort        VKFKKDQQKPRIEKFSLYEYDALSFYSQLQELVSKPNSIKDLVNATLARNLRFSLGKYNF
232             VKFKKDQQKPRIEKFSLYEYDALSFYSQLQELVSKPNSIKDLVNATLARNLRFSLGKYNF
J               VKFKKDQQKPRIEKFSLYEYDALSFYSQLQELVSKPNSIKDLVNATLARNLRFSLGKYNF
7448            VKFKKDQQKPRIEKFSLYEYDALSFYSQLQELVSKPNSIKDLVNATLARNLRFSLGKYNF
                ************************************************************

Beaufort        LFDDLASHLDYTFLVSKAKIKQSSITKKLFIELPIKISLKSSILGDQEPNIKTLFEKEVT
232             LFDDLASHLDYTFLVSKAKIKQSSITKKLFIELPIKISLKSSILGDQEPNIKTLFEKEVT
J               LFDDLASHLDYTFLVSKAKIKQSSITKKLFIELPIKISLKSSILGDQEPNIKTLFEKEVT
7448            LFDDLASHLDYTFLVSKAKIKQSSITKKLFIELPIKISLKSSILGDQEPNIKTLFEKEVT
                ************************************************************

Beaufort        FKLDNFRDVEIEKAFGLLYPGVNEELEQARRDQRASLEKEKAKKGLKEFSQQKDENLKAI
232             FKLDNFRDVEIEKAFGLLYPGVNEELEQARKAQRASFEKEKSKKGLKEFSQQKEENSKAI
J               FKLDNFRDVEIEKAFGLLYPGVNEELEQARREQRASLEKEKAKKGLKEFSQQKDENLKAI
7448            FKLDNFRDVEIEKAFGLLYPGVNEELEQARRDQRASLEKEKAKKGLKEFSQQKDENSKAI
                *****************************: ****:****:***********:** ***

Beaufort        NNQDGLEEDDNITERLPENSPIQYQQEKAGLGSSPDKPYMIKDVQNQRYYLAKSQIQELI
232             NNQEGLEEDDNITERLPENSPIQYQQENAGLGASPDKPYMIKDVQNQRYYLAKSQIQELI
J               NNQDGLEEDDNITERLPENSPIQYQQEKAGLGSSPDKPYMIKDVQNQRYYLAKSQIQELI
7448            NNQDGLEEDDNITERLPENSPIQYQQENAGLGSSPDKPYMIKDVQNQRYYLAKSQIQELI
                ***:**********************:****:**************************
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Beaufort        KAKDYTKLAKLLSNRHTYNISLRLKEQLFDVNPRIPSSRDIENAKFVLDKTEKNKYWQIY
232             KAKDYTKLAKLLSNRHTYNISLRLKEQLFDVNPRIPSSRDIEKAKFVLDKTEKNKYWQIY
J               KAKDYTKLAKLLSNRHTYNISLRLKEQLFEVNPRIPSSRDIENAKFVLDKTEKNKYWQIY
7448            KAKDYTKLAKLLSNRHTYNISLRLKEQLFDVNPRIPSSRDIENAKFVLDKTEKNKYWQIY
                ****************************:************:*****************

Beaufort        SSASPVFQNKWSLFGYYRYLLGLDPKQTIHELVKLGQKAGLQFEGYENLPSDFNLEDLKN
232             SSASPVFQNKWSLFGYYRYLLGLDPKQTIHELVKLGQKAGLQFEGYENLPSDFNLEDLKN
J               SSASPAFQNKWSLFGYYRYLLGLDPKQTIHELVKLGQKAGLQFEGYENLPSDFNLEDLKN
7448            SSASPVFQNKWSLFGYYRYLLGLDPKQTIHELVKLGQKAGLQFEGYENLPSDFNLEDLKN
                *****.******************************************************

Beaufort        IRIKTPLFSQKDNFKLSLLDFNNYYDGEIKAPEFGLPLFLPKELRKNSSNSGNSQNSNSL
232             IRIKTPLFSQKDNFKLSLLDFNNYYDGEIKAPEFGLPLFLPKELRRNSSNSGGSQNSNSP
J               IRIKTPLFSQKDNFKLSLLDFNNYYDGEIKAPEFGLPLFLPKELRKNSSNIGSSQNSNSP
7448            IRIKTPLFSQKDNFKLSLLDFNNYYDGEIKAPEFGLPLFLPKELRKNSSNSGNSQNSNSP
                *******************************************:**** *.******

Beaufort        WEQEIISQFKDQNLSNQDQLAQFSTKIWEKIIGDENEFDQNNRLQYKLLKDLQESWINKT
232             WEQEIISQFKDQNLSNQDQLAQFSTKIWEKIIGDENEFDQNNRLQYKLLKDLQESWINKT
J               WEQEIISQFKDQNLSNQDQLAQFSTKIWEKIIGDENEFDQNNRLQYKLLKDLQESWINKT
7448            WEQEIISQFKDQNLSNQDQLAQFSTKIWEKIIGDENEFDQNNRLQYKLLKDLQESWINKT
                ************************************************************

Beaufort        RDNLYWTYLGDKLKVKPKNNLDAKFRQISNLQELLTAFYTSAALSNNWNYYQDSGAKSTI
232             RDNLYWTYLGDKLKVKPKNNLEAKFRQISNLQELLTAFYTSAALSNNWNYYQDSGAKSTI
J               RDNLYWTYLGDKLKVKPKNNLDAKFRQISNLQELLTAFYTSAALSNNWNYYQDSGAKSTI
7448            RDNLYWTYLGDKLKVKPKNNLDAKFRQISNLQELLTAFYTSAALSNNWNYYQDSGAKSTI
                *********************:**************************************

Beaufort        IFEEIAELDPKVKEKVGADVYQLKFHYAIGFDDNAGKFNQEVIRSSSRTIYLKTSGKSKL
232             IFEEIAELDPKVKEKVGADVYQLKFHYAIGFDDNAGKFNQEVIRSSSRTIYLKTSGKSKL
J               IFEEIAELDPKVKEKVGADVYQLKFHYAIGFDDNAGKFNQEVIRSSSRTIYLKTSGKSKL
7448            IFEEIAELDPKVKEKVGADVYQLKFHYAIGFDDNAGKFNQEVIRSSSRTIYLKTSGKSKL
                ************************************************************

Beaufort        EADAIDQLNQAVENAPLGLQSFYLDTERFGVFQKLATSLAVQHKQKEKTLPKKLNNDGYT
232             EADTIDQLNQAVKNAPLGLQSFYLDTERFGVFQKLATSLAVQHKQKEKTLPKKLNNDGYT
J               EADTIDQLNQAVENAPLGLQSFYLDTERFGVFQKLATSLAVQHKQKEKPLPKKLNNDGYT
7448            EADTIDQLNQAVENAPLGLQSFYLDTERFGVFQKLATSLAVQHKQKEKTLPKKLNNDGYT
                ***:********:*********************************.***********

Beaufort        LIHDKLKKPVIPQISSSPEKDWFEGKLNQNGQSQNVNVSTFGSIIESPYFSTNFQEESDL
232             LIHDKLKKPVIPQISSSPEKDWFEGKLNQNGQSQNVNVSTFGSIIESPYFSTNFQEDADL
J               LIHDKLKKPVIPQISSSPEKDWFEGKLNQNGQSQNVNVSTFGSIIESPYFSTNFQEEADL
7448            LIHDKLKKPVIPQISSRREKDWFEGKLNQNGQSQNVNVSIFGSIIESPYFSTNFQEEADL
                ****************  ********************** ***************::**

Beaufort        DQEGQDDSKQGNNSLDNQEAGLLKQKLAIXLGNQFIQYYQQNDKEIEFEIINVEKVSELS
232             DQDGQDDSRQGNNSLDNQEAGLLKQKLAILLGNQFIQYYQQNDKEIEFEIINVEKVSELS
J               DQEGQDDSKQGNKSLDNQEAGLLKQKLAILLGNQFIQYYQQNDKEIEFEIINVEKVSELS
7448            DQDGQDDSRQGNNSLDNQEAGLLKQKLAILLGNQFIQYYQQNDKEIEFEIINVEKVSELS
                **:*****:***:*************** ******************************

Beaufort        FRVEFKLAKILEDNGKTIRVLSDETMSLIVNTTIEKAPEMSAVPEVFDTKWVEQYDPRTP
232             FRVEFKLAKTLEDNGKTIRVLSDETMSLIVNTTIEKTPEMSAVPEVFDTKWVEQYDPRTP
J               FRVEFKLAKTLEDNGKTIRVLSDETMSLIVNTTIEKAPEMSAAPEVFDTKWVEQYDPRTP
7448            FRVEFKLAKILEDNGKTIRVLSDETMSLIVNTTIEKAPEMSAVPEVFDTKWVEQYDPRTP
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Beaufort        LAAKTKFVLKFKDQIPVDASGNISDKWLASIPLVIHQQMLRLSPVVKTIRELGLKTEEQQ
232             LAAKTKFVLKFKDQIPVDGSGNISDKWLASIPLVIHQQMLRLSPVVKTIRELGLKTEQQQ
J               LAAKTKFVLKFKDQIPVDASGNISDKWLASIPLVIHQQMLRLSPVVKTIRELGLKTEQQQ
7448            LAAKTKFVLKFKDQIPVDASGNISDKWLASIPLVIHQQMLRLSPVVKTIRELGLKTEQQQ
                ******************.****************************************:**

Beaufort        QQQ---------KKAVRKEEELETYNPKDEFNILNPLTKAHRLTLSNLVNNDPNYKIEDL
232             QQQQQQQQQQPQKKAVRKEEELETYNPKDEFNILNPLTKAHRLTLSNLVNNDPNYKIEDL
J               QQQQQQQ-----KKAVRKEEELETYNPKDEFNILNPLTKAHRLTLSNLVNNDPNYKIEDL
7448            QQQQQQQQQQQQKKAVRKEEELETYNPKDEFNILNPLTKAHRLTLSNLVNNDPNYKIEDL
                ***         *********************************************

Beaufort        KVIKNEAGDHQLEFSLRANNIKRLMNTPIXFADYNPFFYFNEDWRNIDKYLNNKGNVSSQ
232             KVIKNEAGDHQLAFSLRANNIKRLMNTPITFADYNPFFYYNEDWRSIDKYLNNKGNVSSH
J               KVIKNEAGDHQLEFSLRANNIKRLMNTPITFADYNPFFYFNEDWRNIDKYLNNKGNVSSQ
7448            KVIKNEAGDHQLEFSLRANNIKRLMNTPITFADYNPFFYFNEDWRNIDKYLNNKGNVSSQ
                *********** *************** *********:*****.*************:

Beaufort        QQQQQ-----AGGGNQGSGLIQRLNKNIKPETFTPALIALKRDNNTNLSNYSDKIIMIKP
232             QQQ-------AAGGNQGSGLIQRLNKNIKPETFTPALIALKRDNNTNLSNYSDKIIMIKP
J               QQQQQQQQ--PGGGNQGSGLIQRLNKNIKPETFTPALIALKRDNNTNLSNYSDKIIMIKP
7448            QQQQQQQQQQPGGGNQGSGLIQRLNKNIKPETFTPALIALKRDNNTNLSNYSDKIIMIKP
                ***       ..************************************************

Beaufort        KYLVERSIGVPWSTGLDGYIGSEQLKGGTSSNSQMG-FKQDFIQALGLKNTEYHGKLGLS
232             KYLVERSIGVPWSTGLDGYIGSEQTKDGTSSSSQQKGFKQDFIQALGLKNTEYHGKLGLS
J               KYLVERSIGVPWSTGLDGYIGSEQLKGGTSSNGQKR-FKQDFIQALGLKNTEYHGKLGLS
7448            KYLVERSIGVPWSTGLDGYIGSEQLKGGTSSNSQNG-FKQDFIQALGLKNTEYHGKLGLS
                ********************** *.****..*   **********************

Beaufort        IRVFDPSNELAKIKDASNKKGEEKLLKSYDLFKNYLNEYEKKSPKIAKGWTNIHPDQKEY
232             IRIFDPGNELAKIKDASNKKGEEKLLKSYDLFKNYLNEYEKKSPKIAKGWTNIHPDQKEY
J               IRIFDPGNELAKIKDASNKKGEEKLLKSYDLFKNYLNEYEKKSPKIAKGWTNIHPDQKEY
7448            IRVFDPSNELAKIKDASNKKGEEKLLKSYDLFKNYLNEYEKKSPKIAKGWTNIHPDQKEY
                **:***.****************************************************

Beaufort        PNPNQKLPENYLNLVLNQPWKVTLYNSSDFITNLFVEPEGSDRGSGTKLKQVIQKQVNNN
232             PNPNQKLPENYLNLVLNQPWKVTLYNSSDFITNLFVEPEGSDRGSGTKLKQVIQKQVNNN
J               PNPNQKLPENYLNLVLNQPWKVTLYNSSDFITNLFVEPEGSDRGSGAKLKQVIQKQVNNN
7448            PNPNQKLPENYLNLVLNQPWKVTLYNSSDFITNLFVEPEGSDRGSGTKLKQVIQKQVNNN
                ********************************************:*************

Beaufort        YADWGSAYLTFWYDKNIITNQPNVITANIADVFIKDVKELEDNTKLIAPNITQWWPNING
232             YADWGSAYLTFWYDKNIITNQPNVITANIADVFIKDVKELEDNTKLIAPNITQWWPNISG
J               YADWGSAYLTFWYDKDIITNQPNVITANIADVFIKDVKELEDNTKLIAPNITQWWPNISG
7448            YADWGSAYLTFWYDKNIITNQPNVITANIADVFIKDVKELEDNTKLIAPNITQWWPNISG
                ***************:*****************************************.*

Beaufort        SKEKFYKPTVFFGNWENENSNSNSQGQTPTWEKIREGFALQALKSSFDQKTRTFVLTTNA
232             SKEKFYKPTVFFGNWENENSSMNSQAQTPTWEKIREGFALQALKSSFDQKTRTFVLTTNA
J               SKEKFYKPTVFFGNWENENSNMNSQGQTPTWEKIREGFALQALKSSFDQKTRTFVLTTNA
7448            SKEKFYKPTVFFGNWENGNSNGNSQVQTPTWEKIREGFALQALKSSFDQKTRTFVLTTNA
                ***************** **. *** **********************************

Beaufort        PLPLWKYGPIGFQNGPDFKKQDWRLVFQNDDNQIAALRVQEQDRPEKSSEDKDKQKWIKF
232             PLPLWKYGPLGFQNGPNFKTQDWRLVFQNDDNQIAALRVQEQDRPEKSSEDKDKQKWIKF
J               PLPLWKYGPLGFQNGPNFKTQDWRLVFQNDDNQIAALRVQEQDRPEKSSEDKDKQKWIKF
7448            PLPLWKYGPIGFQNGPDFKKQDWRLVFQNDDNQIAALRVQEQDRPEKSSEDKDKQKWIKF
                *********:******:**.****************************************
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Beaufort        KVVIPEEMFNSGNIRFVGVMQIQGPNTLWLPVINSSVIYDFYRGTGDSNDVANLNVAPWQ
232             KVVIPEEMFNSGNIRFVGVMQIQGPNTLWLPVINSSVIYDFYRGTGDSNDVANLNVAPWQ
J               KVVIPEEMFNSGNIRFVGVMQIQGPNTLWLPVINSSVIYDFYRGTGDSNDVANLNVAPWQ
7448            KVVIPEEMFNSGNIRFVGVMQIQGPNTLWLPVINSSVIYDFYRGTGDSNDVANLNVAPWQ
                ************************************************************

Beaufort        VKTIAFTNNAFNNVFKEFNISKKIVE
232             VKTIAFTNNAFNNVFKEFNISKKIVE
J               VKTIAFTNNAFNNVFKEFNISKKIVE
7448            VKTIAFTNNAFNNVFKEFNISKKIVE
                *************************
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Figure 5.9. Amino acid alignment of P216 from strain Beaufort, 232, J and 7448. Identical amino acids are indicated by an asterix (*), conserved amino acid changes by a double dot (**:**) and non-conserved amino acid changes by a single dot (**.**). The majority of differences localised to two regions of glutamine repeats shown in green. Computer analysis identified a transmembrane domain at the N-terminus shown in red, a bipartite nuclear binding domain in the middle shown in pink and a KEKE motif towards the C-terminus shown in blue, all 100% conserved among the four sequences.

5.3.5. Cloning and Expression of a C-terminal P216 Antigen from Strain Beaufort

Cloning for expression of P216 was initiated before access to the *M. hyopneumoniae* strain 232 database was granted and therefore before the entire *p216* gene from strain Beaufort had been sequenced. As previously mentioned, *p216* had a large number of TGA codons present within its gene sequence, particularly at the 3' end of the gene, which made expression difficult. An antigen of approximately 27 kDa was however successfully expressed in *E.coli* using the pQE9 expression system.

5.3.5.1. Primer Design and PCR Amplification

A region spanning approximately 190 amino acids was chosen for expression, as it was the largest sequence between TGA codons in the region that had been sequenced (Figure 5.10 (A)). A forward (P216-HisF) and reverse (P216-HisR) primer (Appendix C), both containing restriction sites, were designed for amplification and directional cloning of a *p216* fragment for expression of a P216 antigen. The forward primer was designed just inside the 5' TGA codon of the region to be expressed. The reverse primer however, was designed with several TGA codons upstream to enable a larger antigen to be expressed from the clone if it was ever to be expressed in a mutant *E.coli* host strain that was able to incorporate tryptophan at a TGA codon. Figure 5.10 (A) shows the relative positions of the TGA codons (X) in the *p216* gene as well as the relative positions of PCR primers. A single fragment of approximately 1.5 kb was successfully amplified from genomic DNA of *M. hyopneumoniae* strain Beaufort using primers P216-HisF and P216-HisR, as shown in Figure 5.10 (B).

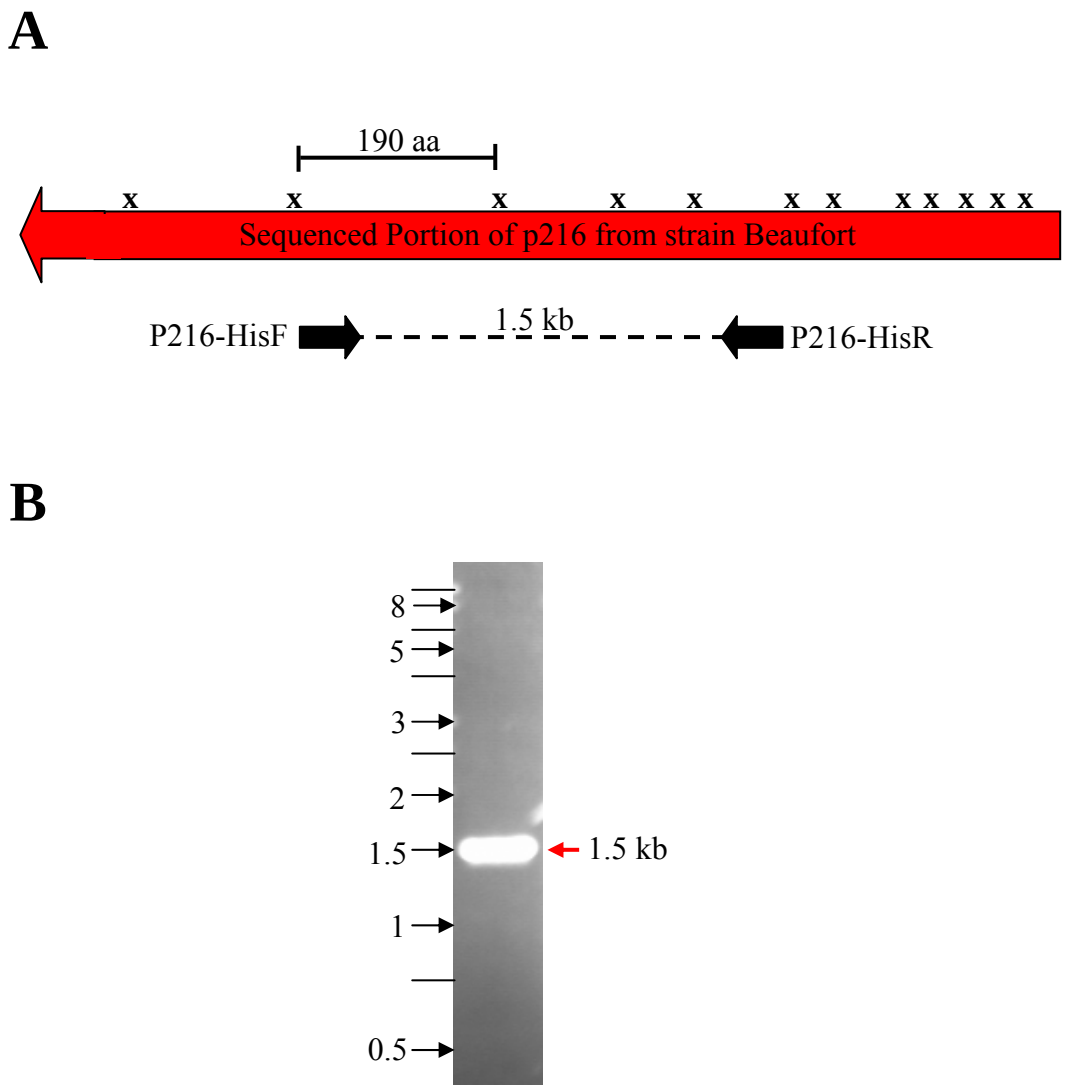


Figure 5.10. PCR of a p216 gene fragment for cloning and expression. (A) Gene map showing the location of UGA codons (**x**) within the sequenced portion of p216, the 190 amino acid (aa) region chosen for expression, and the location of primers P216-HisF and P216-HisR used to amplify a 1.5 kb gene fragment. The fragment amplified with *Pfu* is shown in the ethidium bromide stained agarose (1%) gel of (B). Molecular size markers are in kb.

5.3.5.2. Cloning for Expression

The fragment generated following amplification with primers P216-HisF and P216-HisR was blunt-end ligated into pPCR-Script and transformed into supercompetent *E. coli* MRF' kan cells. A single positive clone was identified by antibiotic selection and cultured for plasmid extraction and digestion to confirm the presence of the 1.5 kb *M. hyopneumoniae* insert (data not shown). A single positive pPCR-Script clone, designated pJW7, acted as the intermediate for directional cloning into pQE9.

For expression and purification using the His-tag system, the 1.5 kb *p216* fragment had to be released from pJW7 and cloned into pQE9. Both pQE9 and pJW7 were digested with *Bam*HI and *Pst*I and electrophoresed in agarose. Linearised pQE9 and the 1.5 kb *p216* fragment released from pJW7 were purified from agarose using the Bresa-Clean method. This allowed for efficient ligation of the *p216* gene fragment into pQE9 and transformation into the expression strain *E. coli* M15[pREP4]. The Bresa-Clean fragments used in the ligation are shown in Figure 5.11 (A) lanes 1 and 2. Positive clones were identified by antibiotic selection and confirmed by digestion of extracted plasmid as shown in Figure 5.11 (A) lanes 3 and 4. Extracted plasmid (lane 3) was digested with *Bam*HI and *Pst*I which released the 1.5 kb *p216* insert from pQE9 (lane 4). Also visible in the agarose gel is pREP4, which is simultaneously extracted with pQE9 (3.4 kb) and is observed as two fragments of approximate sizes 2.8 kb and 0.9 kb. The positive clone was designated pJW8.

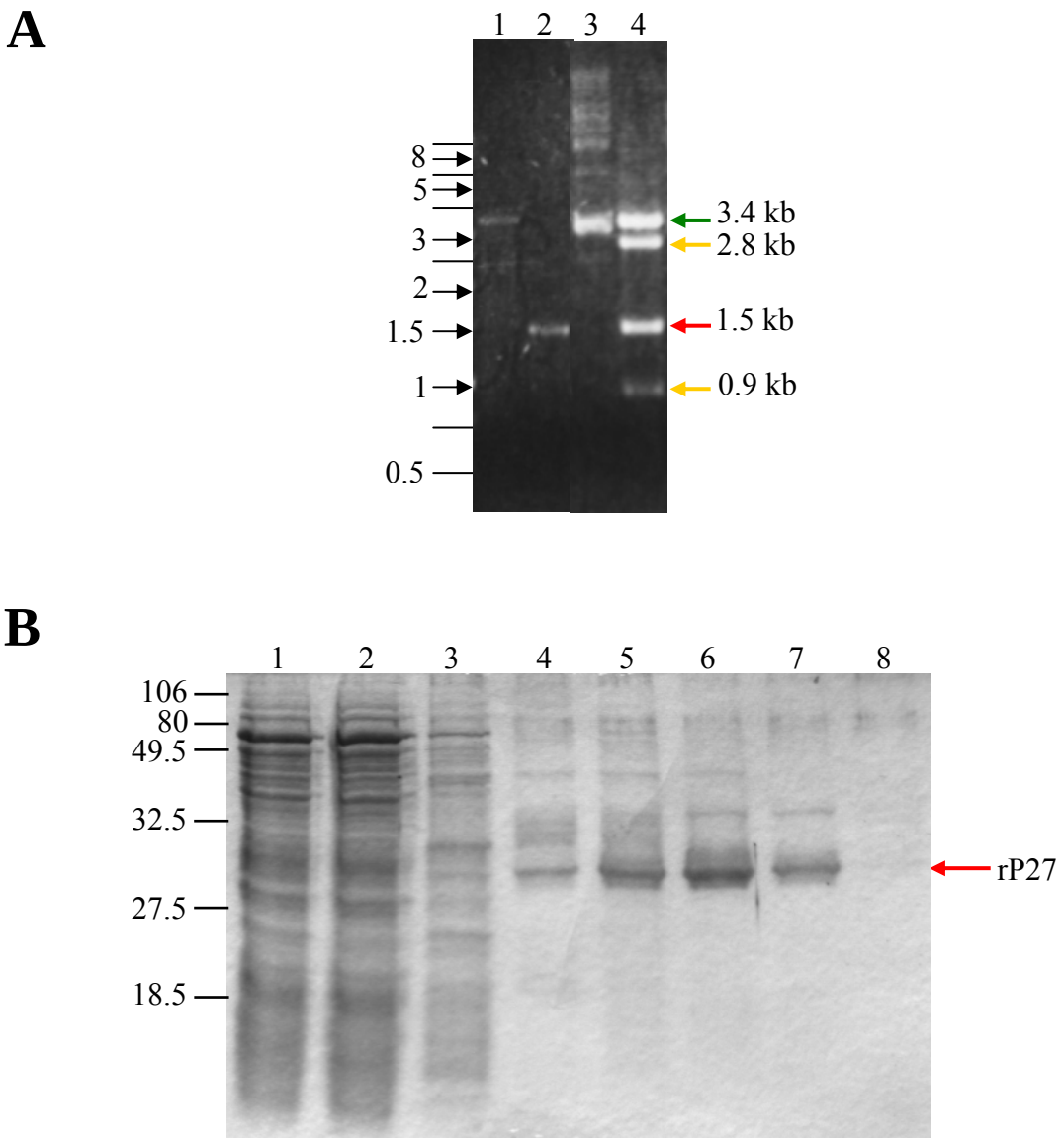


Figure 5.11. Cloning of a p216 fragment into pQE9 for expression in M15[pREP4] cells. (A) Ethidium bromide stained agarose (1%) gel showing the Bresa-clean fragments used for ligation: *Bam*HI/*Pst*I digested pQE9 in lane 1 and *Bam*HI/*Pst*I 1.5 kb *M. hyopneumoniae* insert released from pCR-Script in lane 2. Lane 3 and 4 contain undigested and *Bam*HI/*Pst*I digested plasmid respectively. Identified by coloured arrows are the 1.5 kb *M. hyopneumponoiae* insert (red arrow), pQE9 (3.4 kb) and pREP4 (2.8 kb and 0.9 kb fragments). Molecular size markers are given in kb. The positive clone was expressed and the recombinant antigen purified by nickel affinity chromatography. The Coomassie stained polyacrylamide gel showing this result is given in (B). Lanes 1 and 2 contain uninduced and induced whole cell lysates of M15[pREP4](pJW8) respectively, lane 3 shows the wash fraction and lanes 4 to 8 the elution fractions collected from the purification column. Molecular size markers are given in kDa and a red arrow indicates the recombinant antigen (rP27).

5.3.5.3. Expression of the P216 Antigen

Once the expression clone had been confirmed by plasmid digestion, it was induced for expression with IPTG, purified by Nickel affinity chromatography and analysed by SDS PAGE. The Coomassie stained acrylamide gel in Figure 5.11 (B) shows a simple small-scale purification of the P216 antigen to confirm for expression. The 27 kDa antigen (P27) expressed from the clone is not visible in the induced whole cell lysates (lane 2), does not appear in the wash fraction (lane 3), but can be seen in four of the five eluent fractions (lanes 4 to 8). There are a number of higher molecular weight proteins that appear to co-purify with P27 that may be *E.coli* contaminants that non-specifically bind nickel or could represent multimers of P27.

5.3.6. Cloning and Expression of P216 Antigens from Strain 232

Having only expressed 30 kDa from the C-terminus of a 216 kDa protein hindered its characterisation but even after access to the *M. hyopneumoniae* strain 232 genome sequence was granted the number and location of TGA's limited expression of larger antigens (Figure 5.12 (A)). Our colleagues from Iowa State University had however been working on the site-directed mutagenesis of Mhp493 to change some of the TGA codons to TGG codons for expression in *E.coli*. They had successfully mutagenised 8 of the N-terminal TGAs when they provided us with two clones (ORF545A and ORF545B shown in Figure 5.12 (B)) allowing expression of 1049 of the 1879 amino acids representing 75% of P216. Three slightly overlapping expression clones were generated using the Invitrogen Champion^{TM} pET161 TOPO cloning kit featuring directional cloning with a C-terminal His-tag and a Lumio-tag.

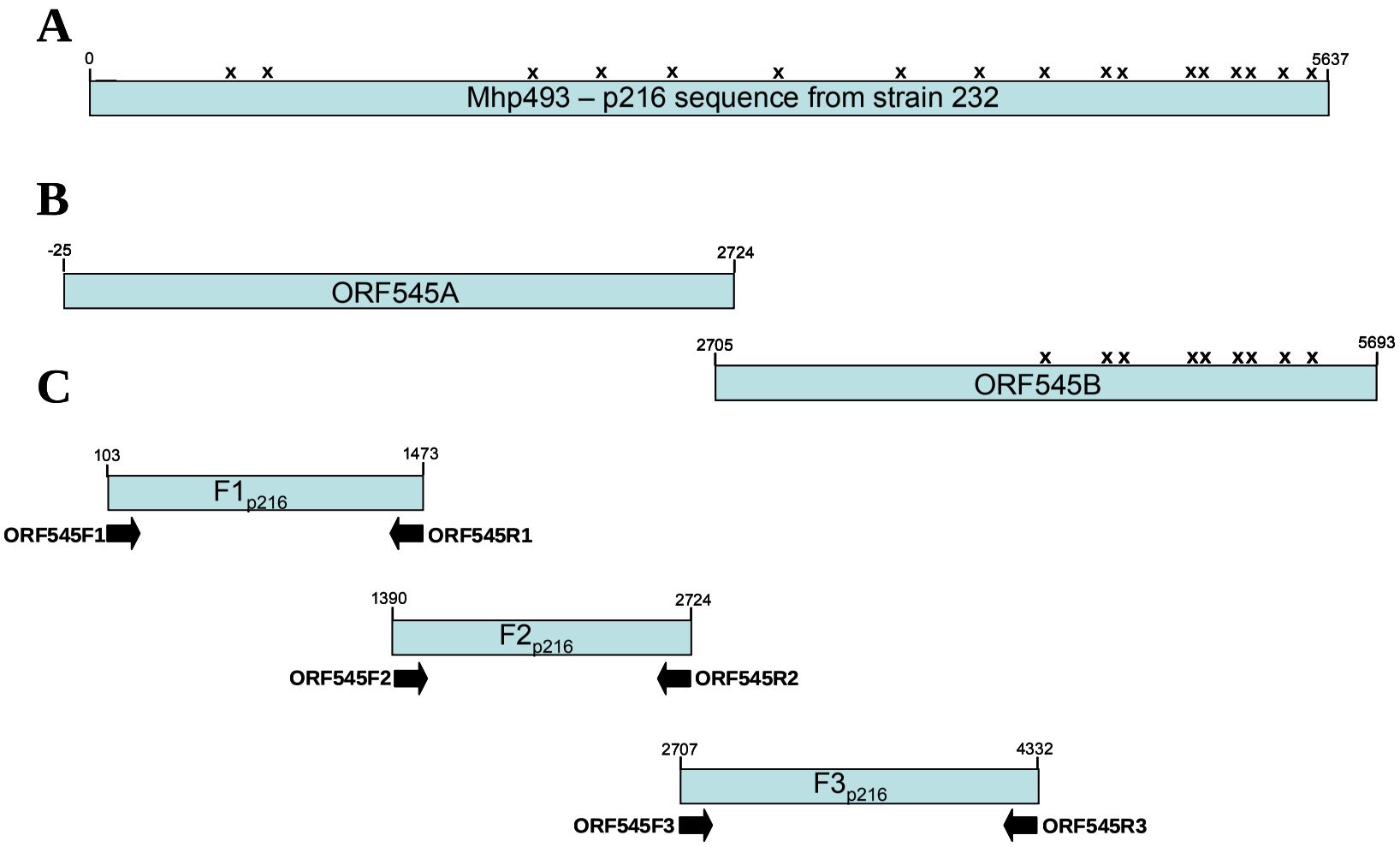


Figure 5.12. Cloning P216 antigens from strain *M. hyopneumoniae* 232. (A) Gene map of p216 showing location of UGA codons as indicated by an **x.** (B) Clones (ORF545A and ORF545B) provided by Minion and colleagues (Iowa State University, USA) that had a number of the UGA codons mutagenised to UGG for expression in *E. coli*. (C) Fragments $F1_{p216,}$ $F2_{p216,}$ $F3_{p216)}$ to be amplified (primers indicated by black arrows) for cloning and expression.

5.3.6.1. Primer Design and PCR Amplification

Antigens between 40 and 60 kDa were preferred for expression so three slightly overlapping sets of primers (Appendix C) were designed to cover the mutagenised region of *p216* (Mhp 493) which was equivalent to approximately 75% of the molecule (Figure 5.12 (C)). The C-terminal 25% of the gene contained multiple UGA codons preventing its expression. Directional cloning was achieved with the addition of a CACC overhang on the 5' end of the forward primers (see Appendix B for vector and map). As can be seen in Figure 5.12, primer set 1 (ORF545F1 and ORF545R1) spanned nucleotides 103 to 1473 (amino acids 35-491 avoiding the predicted transmembrane domain), primer set 2 (ORF545F2 and ORF545R2) from 1390 to 2724 (amino acids 464-908) and primer set 3 (ORF545F3 and ORF545R3) spanned from 2707-4332 (amino acids 903-1444).

A gradient PCR was performed initially with *Taq* polymerase over the temperature range of 52-59ºC for all 3 sets of primers (data not shown). A temperature of 52 ºC was chosen for a separate PCR using the polymerase *Pwo* which has proofreading capability. PCR products of approximately 1400 bp (F1*p216*), 1300 bp (F2*p216*) and 1600 bp (F3*p216*) were amplified for primer sets 1 (lane 1), 2 (lane 2) and 3 (lane 3) respectively (Figure 5.13 (A)).

5.3.6.2. Cloning for Expression

The three fragments amplified from mutagenised Mhp493 using *Pwo* polymerase were cloned into pET161 and transformed into *E. coli* Top10 cells for screening and

maintenance. Ampicillin resistant colonies selected for screening were cultured and their plasmid extracted for digestion. When digested with *Bgl*II, a positive $F1_{p216}$ clone was to release fragments of 6093 bp and 1139 bp and a positive $F2_{p216}$ clone was to release fragments of 5918 bp and 1278bp. A positive $F3_{p216}$ clone was to give fragments of 4233 bp and 3248 bp when digested with *Eco*RV. As can be seen in Figure 5.13 (B) these digestion profiles were obtained for all three fragments indicating a positive clone for each. The three plasmids were sequenced and designated pJW9, pJW10 and pJW11 respectively.

5.3.6.3. Expression of the P216 Antigens

The three plasmids were transformed into an *E. coli* BL21 expression strain for induction and purification of the recombinant antigens. Cultures were grown to mid-log phase (OD_{600nm} of 0.6-0.8) before being induced with IPTG to a final concentration of 0.5 mM and allowed to grow overnight. ODs were monitored over the culture period and samples taken at 0, 1, 2, 3 and 4 hours after induction as well as overnight for analysis by SDS PAGE. Induction did not have a significant affect on culture growth OD compared to a non-induced control (data not shown). Induced samples showed that $F1_{P216}$, $F2_{P216}$, and $F3_{P216}$ were successfully expressed in the presence of IPTG and their masses were consistent with theoretical calculations of 52.3, 51.9 and 61.9 kDa respectively. Figure 5.13 (C) shows the 0 h non-induced controls (lanes 1, 3 and 5) and 4 h (lanes 2, 4 and 6) induction samples for the three respective clones.

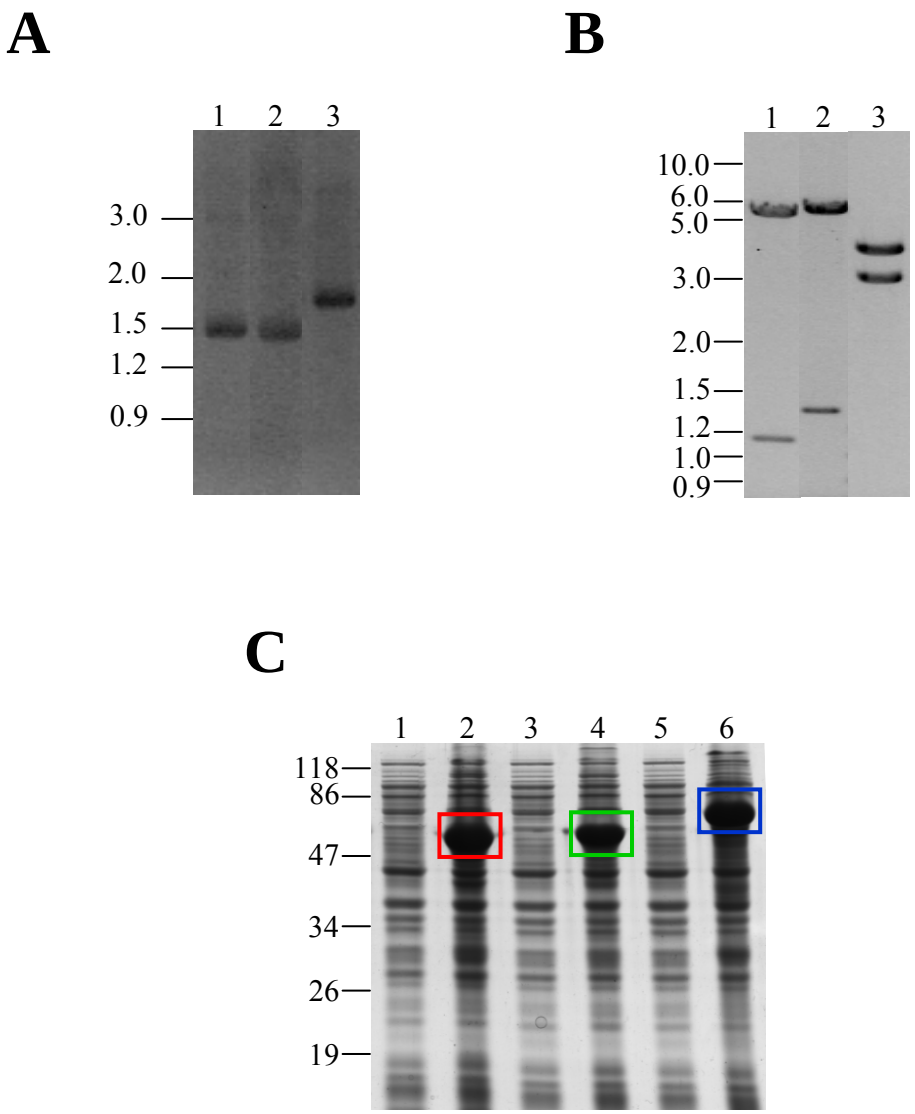


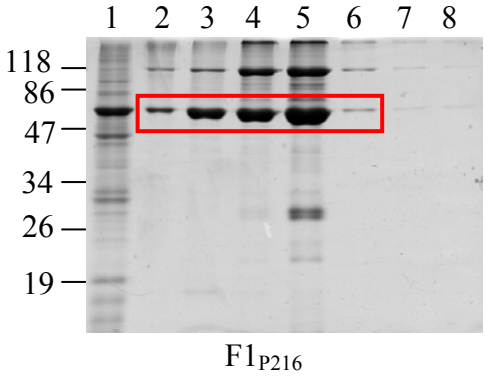
Figure 5.13. Cloning and expression of *p216* fragments from *M. hyopneumoniae* strain 232. The ethidium bromide stained agarose gel (1%) in (A) shows PCR products of approximately 1400 bp, 1300 bp and 1600 bp representing amplification fragments $F1_{p216}$, $F2_{p216}$ and $F3_{p216}$ in lanes 1-3 respectively. The ethidium bromide stained agarose gel (1%) in (B) shows the digested pET161 F1p216, F2p216 and F3p216 clones in lane 1-3 respectively confirming them as positive prior to expression. Size markers are given in kb. Positive clones were cultured and induced with IPTG to confirm expression. The Coomassie stained polyacrylamide gel showing this result is given in (C). Lanes 1, 3 and 5 show the non-induced controls and lanes 2, 4 and 6 the 4 hour induction samples of the 3 clones. Molecular size markers are given in kDa. Expressed $F1_{P216}$, $F2_{P216}$ and $F3_{P216}$ antigens are shown in red, green and blue boxes respectively.

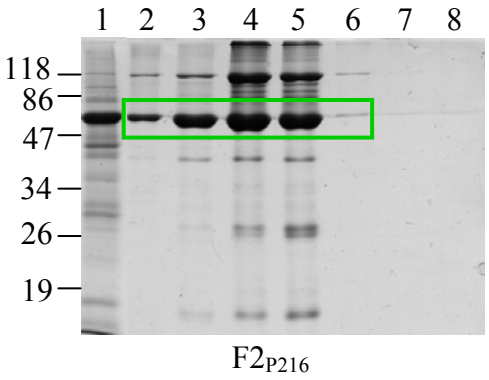
5.3.6.4. Purification of the P216 Antigens

Large-scale (1L) cultures of the three P216 clones were induced for expression with IPTG for 4 h and the *E.coli* pellets collected by centrifugation for purification by nickel-affinity chromatography. The wash (W) and elution (D1, D3 and E1-E5) fractions collected for each were analysed by SDS-PAGE and are shown in Figure 5.14. $F1_{P216}$ can be seen eluting at 52 kDa in the D1 and D3 fractions, primarily in the E1 and E2 fractions and minimally in fraction E3. $F2_{P216}$ and $F3_{P216}$ show an almost identical elution profile at 52 kDa and 62 kDa respectively. Proteins at approximately twice the molecular size of the recombinants are also clearly visible in all elutions and were confirmed as multimers when they disappeared with boiling in reducing solution (data not shown). All three recombinant proteins can also be seen in the wash fraction suggesting insufficient resin volume and purification conditions that require further optimisation. Those elution fractions containing protein were pooled and dialysed against PBS/0.1% SDS for the generation of antisera and further characterisation.

5.3.7. Generation of P216 Antiserum in Rabbits

All four P216 antigens ($F1_{P216}$, $F2_{P216}$, P27 and $F3_{P216}$ – Figure 5.15 (A)) were expressed and purified for production of antiserum in rabbits. Expressions were scaled up and purified proteins were pooled and dialysed against PBS containing 5% glycerol. Each was used separately to immunise a rabbit. An immune response was confirmed when sera from the rabbit reacted in Western analysis with the respective recombinant as well as a *M. hyopneumoniae* whole-cell lysate, as shown in Figure 5.15 (B) and (C). Anti-$F1_{P216}$ sera recognised a protein of approximately 120 kDa in the *M. hyopneumoniae*





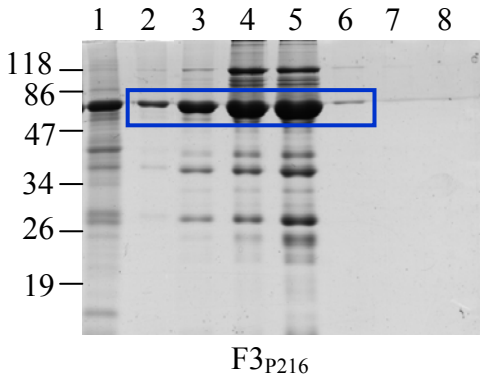


Figure 5.14. Purification of P216 fragments from strain 232. P216 antigens were expressed in *E. coli* for purification by nickel affinity chromatography. The Coomassie stained polyacrylamide gels show the wash (lane 1) and elution (lanes 2-8) fractions collected from the purification columns. Purified $F1_{P216}$, $F2_{P216}$ and $F3_{P216}$ antigens are shown in red, green and blue boxes respectively. Elution fractions were pooled and concentrated. Molecular size markers are given in kDa.

whole cell lysate in addition to the recombinant antigen as did the anti-$F2_{P216}$ serum. Anti-$F3_{P216}$ serum recognised proteins of approximately 85 kDa and 120 kDa in the *M. hyopneumoniae* whole cell lysate in addition to the recombinant antigen. The antiserum raised against rP27 identified a protein of approximately 85 kDa in the *M. hyopneumoniae* whole cell lysate. The 120 kDa and 85 kDa proteins identified by these sera are believed to be the N and C terminal cleavage products of the full-length protein. The immunoblot profiles suggest a cleavage event between amino acids 903 and 1049 (see Figure 5.15 (A)) with anti-$F1_{P216}$ and anti-$F2_{P216}$ sera recognising only the 130 kDa antigen, anti-$F3_{P216}$ serum both the 120 and 85 kDa antigens and anti-P27 recognising only the 85 kDa antigen.

5.3.8. Proteomic Analysis of P120 and P85

Two-dimensional gel electrophoresis and MALDI-TOF MS of *M. hyopneumoniae* strains J and 232 proteins led to the characterisation of two series of protein spots from 2D gels that mapped to P216 (Figure 5.16). The first was a series with an approximate mass of 85 kDa (P85) that were spaced along the pI gradient from pH 7.5 to 9.5 (A). Tryptic digestion and MALDI-TOF MS of the spots indicated that they all represented the C-terminal region of P216 (highest Mascot score 221 with 20 matching peptides). The second series of spots with an approximate mass of 120 kDa (P120) and stretching along the pH gradient from 8.5 to 9.5 (A) represented the N-terminal region of P216 (highest Mascot score 150 with 22 matching peptides). The peptides identified by MALDI-TOF MS for the two sets of spots did not overlap when mapped to Mhp493 suggesting that P216 is cleaved somewhere between amino acids 1040 and 1093 (B). N-

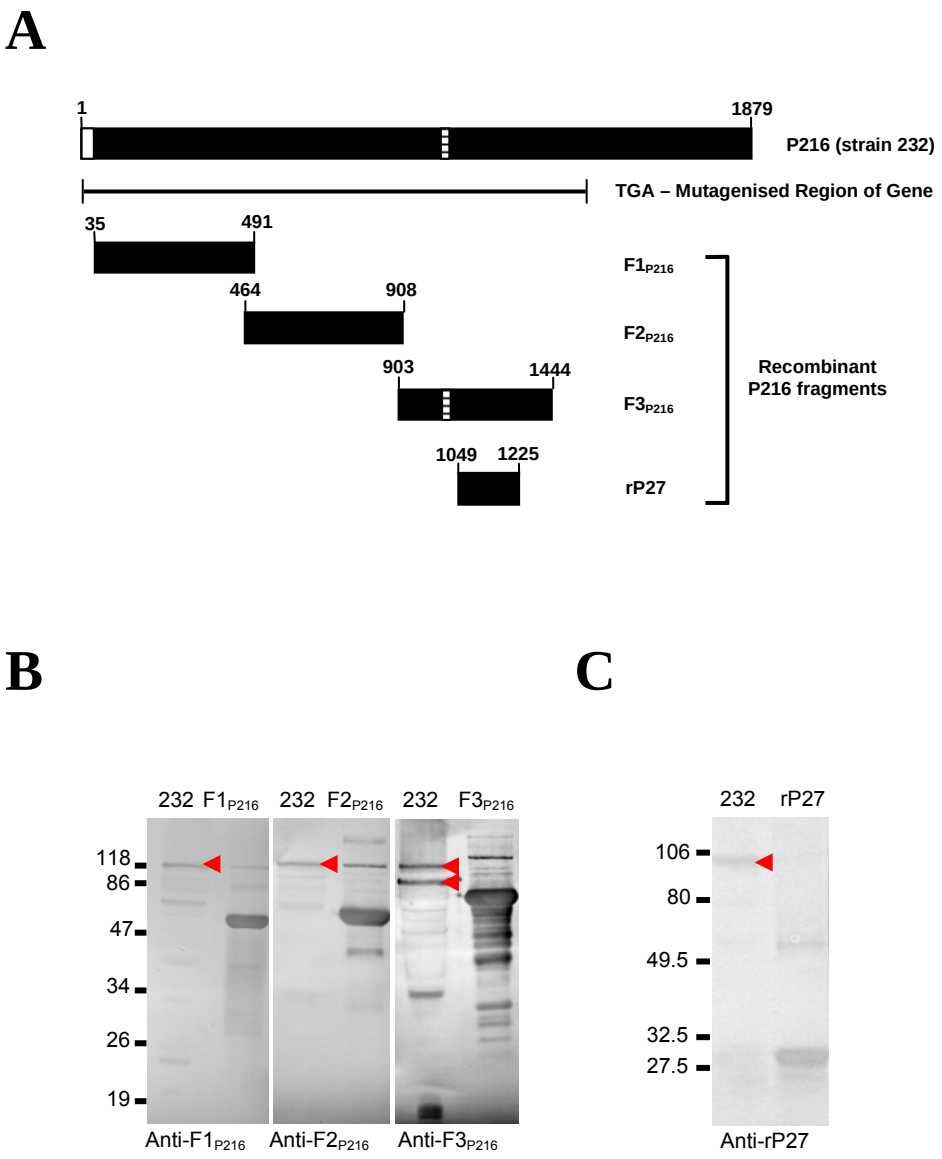


Figure 5.15. Generation of P216 antisera. (A) Schematic representation of P216. The white box depicts a putative transmembrane domain (Tm score 2090) and the hatched box identifies the position of a putative bipartite nuclear binding domain (BNBD). Cloning allowed expression of regions comprising ~75% of P216 represented by $F1_{P216}$, $F2_{P216}$, $F3_{P216}$ and rP27. (B) Western blots containing *M. hyopneumoniae* strain 232 whole cell lysate and affinity purified $F1_{P216}$, $F2_{P216}$ and $F3_{P216}$ exposed to anti-$F1_{P216}$, anti-$F2_{P216}$, and anti-$F3_{P216}$ sera respectively. (C) Western blot of *M. hyopneumoniae* strain 232 whole cell lysate and purified rP27 exposed to anti-rP27 serum. Molecular mass markers are indicated on the left in kDa.

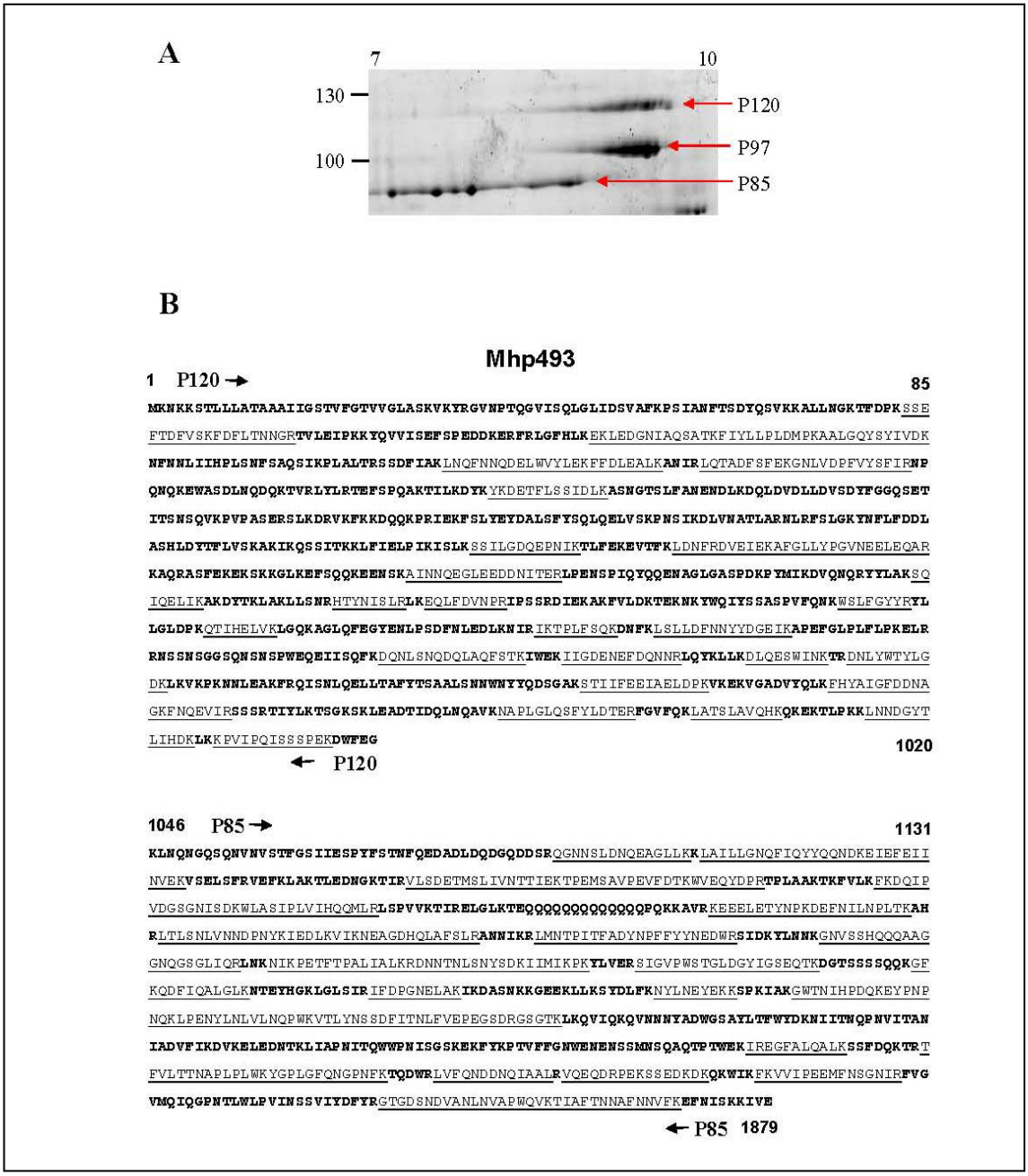


Figure 5.16. Peptide mass mapping of P216. (A). 2-D electrophoresis. *M. hyopneumoniae* strain 232 whole cell proteins separated by two-dimensional gel electrophoresis (8 - 18% polyacrylamide) using a pH gradient of 6 - 11 and stained with colloidal Coomassie Blue. Protein spots migrating with masses from 75 - 130 kDa and with a p*I* from 7 - 10 are shown. (B). Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDITOF-MS) was used to analyse tryptic digests of boxed spots shown in (A). Peptide mass matches of spots resolving between pH values of 7.0 to 9.0 and with a mass of ~ 85 kDa consistently mapped to the C-terminal region of P216. Similarly, peptide mass maps representing three regions of the large spot appearing with a mass of ~ 120 kDa within the pH range of 8.5 - 9.0 consistently mapped to the N-terminal region of P216. Matched peptides are denoted by underlined sequences in non-bold text.

terminal sequence from protein spots representing P85 failed to identify the precise cleavage site despite using several sample preparations.

5.3.9. Characterisation of P216

The anti-P27 serum recognising the C-terminal 85 kDa of P216 as well as that raised against the $F2_{P216}$ fragment (anti- $F2_{P216}$) recognising the 120 kDa N-terminal portion of P216, was used in a number of experiments in an effort to characterise the novel 216 kDa *M. hyopneumoniae* antigen.

5.3.9.1. Presence of P216 Among Geographically Diverse Strains of *M. hyopneumoniae* and Related Porcine Mycoplasmas

Expression of the 216 kDa antigen encoded by *p216* in geographically different strains of *M. hyopneumoniae* and related porcine mycoplasmas *M. hyorhinis* and *M. flocculare* was assessed by immunoblotting. Whole cell lysates of the mycoplasma strains were probed sequentially with anti-P27 and anti-$F2_{P216}$ sera raised in rabbits (Figure 5.17). Anti-P27 sera recognised an antigen of approximately 85 kDa in the five *M. hyopneumoniae* strains but not the *M. hyorhinis* or *M. flocculare* strains. A number of other proteins of various sizes were identified by the antisera but with reduced reactivity. The anti-$F2_{P216}$ sera showed a similar pattern, identifying a 120 kDa antigen in all five strains of *M. hyopneumoniae*. It also identified a protein of approximately 115 kDa in the *M. flocculare* type strain but failed to react with either strain of *M. hyorhinis* assessed. These results show that P216 undergoes a major cleavage event generating

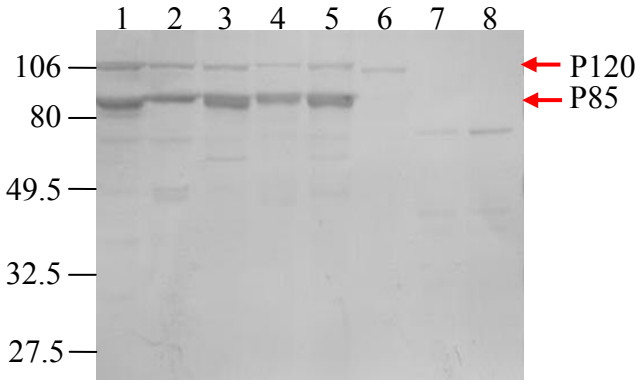


Figure 5.17. Immunoblot analysis of cell lysates of different strains of *M. hyopneumoniae* and type strains of *M. hyorhinis* and *M. flocculare*. An immunoblot containing whole cell lysates of different porcine mycoplasmas was separately exposed to anti-rP27 followed by anti-F1P216 sera. rP27 antiserum identified an 85-kDa protein (presumably P85) in all five *M. hyopneumoniae* strains but failed to identify an 85-kDa protein in cell lysates of *M. hyorhinis* or *M. flocculare*. Anti-$F1_{P216}$ serum identified a protein with a mass of approximately 120 kDa (presumably P120) in all five strains *of M. hyopneumoniae* but failed to recognise P120 in *M. hyorhinis* strains BTS7 and GDL. A protein with a mass of ~ 110 kDa reacted with this serum in *M. flocculare*.

P120 and P85 among strains of *M. hyopneumoniae* originating from different geographic locations

5.3.9.2. Cellular location of P216

Exposure of viable *M. hyopneumoniae* cells to trypsin was used to determine the cellular location of P85 and P120. *M. hyopneumoniae* cells were treated with 0, 0.1, 0.3, 0.5, 1, 3, 5, 10 and 30 ug/ml trypsin and subjected to immunoblotting with anti-P27 sera and anti-$F2_{P216}$ sera. The result is shown as Figure 5.18 (A). P85 was readily digested with trypsin at concentrations ranging from 0.1 to 1.0 ug/mL after which it became undetectable. P130 was more resilient to trypsin digestion and remained detectable to a concentration of 10 ug/mL after which it had completely degraded. Immunoblot analysis using PdhA (shown in the previous chapter to reside in the cytosol) as a cell lysis control protein was performed on the same samples and indicated the *M. hyopneumoniae* cells remained intact with the trypsin treatment (data not shown). These data suggest both P85 and P120 are surface accessible membrane associated proteins.

5.3.9.3. 2D Immunoblotting Studies of P120 and P85

Figure 5.19 (A) depicts a region of a 2D gel of *M. hyopneumoniae* strain J proteins displaying P120 and P85 as well as the cilium adhesin P94. 2D blots of the same region were reacted separately with anti-*M. hyopneumoniae* hyperimmune sera (Figure 5.19 (B)) and porcine convalescent swine sera from *M. hyopneumoniae* ELISA positive pigs (Figure 5.19 (C)). The blot reacted with hyperimmune sera identified both P120 and

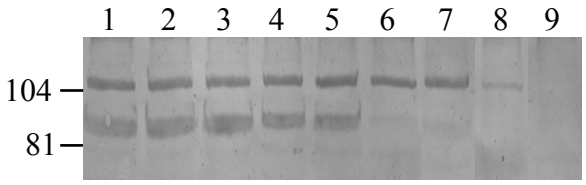


Figure 5.18. Localization of P216 cleavage products on the surface of *M. hyopneumoniae*. Immunoblot of whole cell preparations of freshly cultured *M. hyopneumoniae* strain J cells exposed to various concentrations of trypsin ranging from 0, 0.1, 0.3, 0.5, 1, 3, 5, 10, and 30 μg/ml for 15 minutes at 37°C (lanes 1-9 respectively) reacted sequentially with anti-rP27 sera followed by anti-F1P216 sera. Ribosomal protein PdhA was detectable at all trypsin concentrations depicted (data not shown). PdhA resides in the cytosol and acted as a control for cell lysis in these experiments.

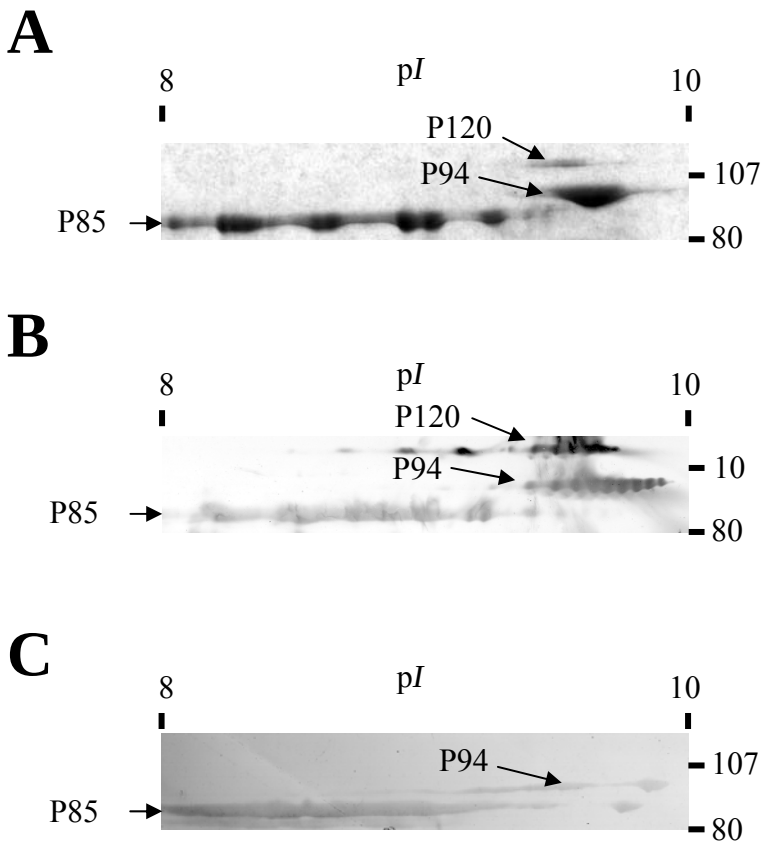


Figure 5.19. Two-dimensional gel electrophoresis and immunoblot analysis of P216 cleavage products P120 and P85. (A) Coomassie stained gel of *M. hyopneumoniae* proteins (strain J) showing P120, P94 (cilium adhesin) and P85 proteins. (B) Immunoblot of J strain proteins reacted with hyperimmune serum raised to *M. hyopneumoniae* (Beaufort strain) proteins. (C) Immunoblot analysis of J strain proteins reacted with a pool of convalescent sera recovered from swine naturally infected with *M. hyopneumoniae*.

P85 but the convalescent sera identified only P85. The cilium adhesin P94 is shown in both blots reacting with hyperimmune and convalescent sera and served as a control protein in these experiments. This data illustrates the immunoreactivity of the two P216 antigens and in addition shows that P85 may be more naturally immunogenic than P120.

5.3.9.4. Processing of P216 During Different Stages of the Growth Cycle

The processing of P216 into cleavage products P120 and P85 was examined through immunoblot analysis of synchronous cultures of *M. hyopneumoniae* harvested at early log (8 h), mid-log (16-28 h), late-log (32-40 h) and stationary (48-56 h) phases. Immunoblots of cell lysates were separately reacted with anti-$F2_{P216}$ and anti-P27 serum in order to identify the cleaved fragments. The results can be seen in Figure 5.20 and show the prominence of P120 and P85 throughout all stages of the growth cycle indicating that processing is not significantly affected by growth cycle. Several weakly reactive lower mass proteins were also evident.

5.3.9.5. Heparin Binding of P216

The non-ionic detergent Triton X-114 has been used to study the properties of mycoplasma membrane proteins and associated structures. Immunoblotting studies show P85 and P120 were both detectable in the aqueous (A) and detergent (D) phases, but these two proteins predominantly resided in the insoluble (I) pellet (data not shown). Ligand blots of TX-114-extracted, high mass proteins of *M. hyopneumoniae* probed

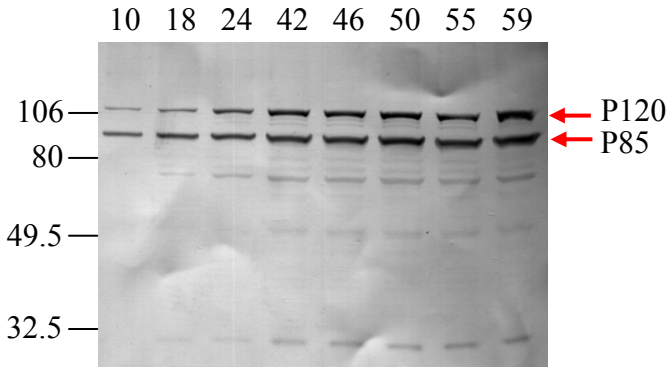


Figure 5.20. Immunoblot of cell lysates of synchronised cultures of *M. hyopneumoniae* strain J. Lysates from cultures harvested at 10, 18, 24, 42, 46, 50 55 and 59 hours post inoculation are shown. The blot was reacted sequentially with anti-P27 sera (detecting P85) followed by anti-$F2_{P216}$ sera (detecting P120). Molecular mass markers are as indicated in kDa. Due to limited growth of M. hyopneumoniae at 10 h post inoculation, approximately 50% of protein (compared to other lanes) was loaded.

with biotinylated heparin primarily identified proteins with masses of approximately 120, 85 and 60 kDa (Figure 5.21 (A)). MALDI-TOF mass spectrometry of trypsin digests of gel-extracted proteins (identified by arrows) identified the 120 kDa N-terminal fragment (P120) of Mhp493 (Mascot scores of 155 and 221 respectively) and the 85 kDa C-terminal fragment (P85) of Mhp493 (Mascot score 150). Mhp493 has a predicted mass of 216 kDa but despite repeated attempts we were unable to detect the P216 preprotein using this approach. MALDI-TOF mass spectrometry of tryptic peptides derived from the heparin-binding 60 kDa protein identified three proteins each with significant matches to the *M. hyopneumoniae* genome. Further studies are required to identify which of these bind heparin.

Recombinant P216 antigens $F1_{P216}$, $F2_{P216}$ and $F3_{P216}$, but not rP27 bound biotinylated heparin in ligand blotting studies (Figure 5.21 (B)). These fragments bound biotinylated heparin irrespective of the recombinant proteins being treated with (panel B) or without (data not shown) cracking buffer and heating to 100˚C for 5 min prior to loading. A higher mass protein of unknown composition bound faintly with biotinylated heparin (lane 5). To examine the kinetics of heparin binding to $F1_{P216}$, $F2_{P216}$ and $F3_{P216}$ proteins, microtitre plate-based heparin binding studies were performed. $F1_{P216}$, $F2_{P216}$ and $F3_{P216}$ bound heparin in a dose-dependent and saturable manner with Kd values of 46.57 ± 8.0, 84.16 ± 7.5 and 23.61 ± 2.9 nM respectively (Figure 5.22 (A)). Binding to heparin was largely abolished by the addition of unlabelled heparin, mucin and fucoidan but not by chondroitin sulphate B (Figure 5.22 (B)). These studies suggest that a minimum of two heparin binding domains exist in P120 and that P85 is also able to bind heparin.

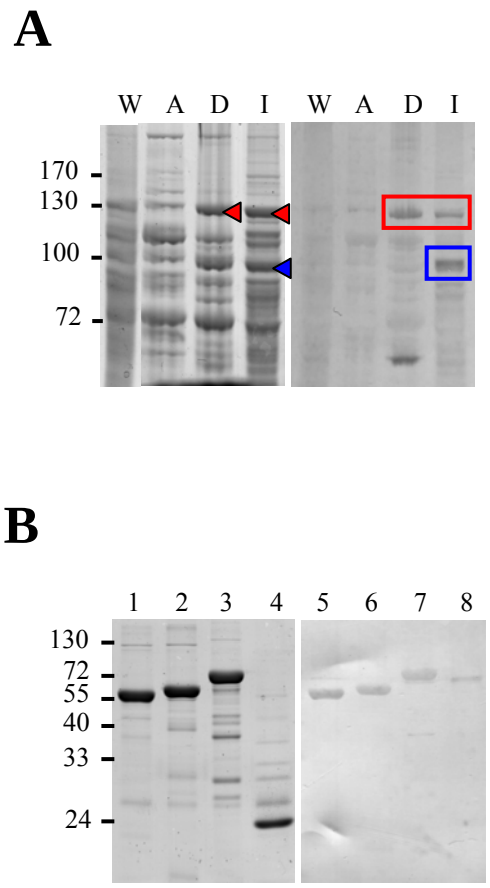


Figure 5.21. Binding of P216 to biotinylated heparin in ligand blots. (A) 7% SDS-polyacrylamide gel loaded with *M. hyopneumoniae* whole cell proteins (W), TX-114 aqueous phase proteins (A), TX-114 detergent phase proteins (D), and TX-114 insoluble proteins (I) stained with colloidal Coomassie Blue (left panel) and exposed to biotinylated heparin (right panel). Red arrows and boxed region shows P120 while blue arrow and boxed region shows P85 binding to biotinylated heparin. (B) 12% SDS-polyacrylamide gel showing $F1_{P216}$ (lanes 1 and 5), $F2_{P216}$ (lans 2 and 6), $F3_{P216}$ (lanes 3 and 7) and rP27 (lanes 4 and 8) stained with colloidal Coomassie Blue (left panel) and exposed to biotinylated heparin (right panel). Heparin bound to $F1_{P216}$, $F2_{P216}$, $F3_{P216}$ but not rP27. Molecular mass markers are as indicated in kDa.

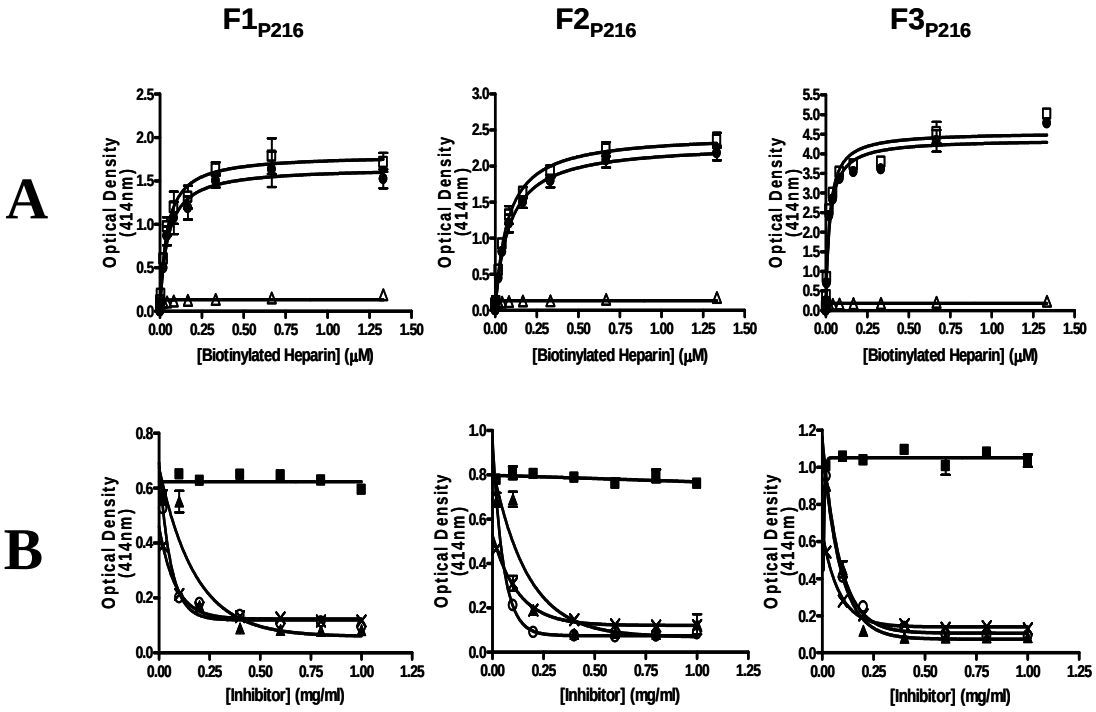


Figure 5.22. Binding of heparin to recombinant fragments $F1_{P216}$–$F3_{P216}$. (A) 96-well microtitre plates coated with 20 nM of each of the three recombinant Mhp493 proteins that had been pretreated with dithiothreitol and heated to 100°C prior to coating. Total binding was determined by the standard microtitre assay protocol while non-specific binding was determined in the presence of a 50-fold excess (2.4 mg ml-1) of unlabelled heparin. □ represents the total binding and Δ represents non-specific binding. The specific binding curve, represented by the symbol ●, was obtained by subtracting non-specific binding from the total binding values. Error bars represent ±SEM from triplicate wells. (B) Competitive binding assays. Various glycosaminoglycans were examined for their ability to inhibit the binding of recombinant Mhp493 proteins to biotinylated heparin. Unlabeled heparin (x), mucin (O) and fucoidan (▲) but not chondroitin sulphate B (■) inhibited the ability $F1_{P216}$, $F2_{P216}$ and $F3_{P216}$ to bind biotinylated heparin. Error bars represents mean values ± SEM from triplicate wells.

5.4. Discussion

Identification of surface associated proteins of *M. hyopneumoniae* not only increases our knowledge of the surface topology of this organism but also potentially provides information on the mechanism of disease. The novel surface molecule characterised in this chapter is particularly interesting for a number of reasons. It was selected for characterisation based on its fractionation into a group of *M. hyopneumoniae* antigens that displayed immunoreactivity and that had afforded some protection against disease in pigs in an experimental trial (Djordjevic *et al*., unpublished data; Djordjevic *et al*., 1997).

Cloning the gene encoding the protein selected for characterisation in this study involved an unconventional multi-step process beginning with a novel internal tryptic peptide sequence. A degenerate oligonucleotide designed from the peptide sequence was used in a method of DNA fractionation and cloning (described in detail in the previous chapter) but when the clone failed to contain the entire gene the process continued with inverse PCR. Failure to identify the 5' end of the gene when the inverse PCR product was sequenced co-incided with an affiliation made with Minion and colleagues (Iowa State University, USA) who were in the process of sequencing the genome of *M. hyopneumoniae* strain 232 (Minion *et al*., 2004). Access to preliminary genome sequence information allowed completion of cloning and sequencing as well as identification of the Beaufort homolog of a 216 kDa strain 232 protein, since designated P216.

Cloning for expression of P216 antigens followed with the generation of four overlapping polyhistidine fusion recombinants ($F1_{P216}$, $F2_{P216}$, $F3_{P216}$, and rP27) covering 75% of the full length protein. This was made possible despite the number of TGA codons within the gene through provision of two Mhp493 clones which had 8 of the 17 TGAs converted to TGGs by site-directed mutagenesis. The C-terminal 435 amino acids (25%) containing the remaining 9 TGAs was unable to be expressed in *E.coli*. The subsequent generation of polyclonal antisera to the four recombinants (anti-$F1_{P216}$, anti-$F2_{P216}$, anti-$F3_{P216}$, and rP27) enabled further characterisation of P216.

Preliminary Western blots using the four sera against *M. hyopneumoniae* whole cell lysates indicated a possible cleavage event when antiserum generated against N-terminal expression products $F1_{P216}$ and $F2_{P216}$ recognised a 120 kDa antigen (P120) whereas antiserum generated against C-terminal rP27 recognised an 85 kDa antigen (P85), their sizes approximately summing to the predicted 216 kDa of the full-length protein. Antiserum raised to $F3_{P216}$ which bridges the gap between $F2_{P216}$ and rP27 (as well as overlapping with rP27) recognised both P120 and P85 suggesting cleavage is occurring between amino acids 908 and 1049. This cleavage pattern was duplicated in 2D gels of *M. hyopneumoniae* and confirmed by peptide mass fingerprinting which identified two series of protein spots at approximate masses of 85 kDa (P85) and 120 kDa (P120). The peptides identified by MALDI-TOF MS for the two sets of spots did not overlap when mapped to Mhp493 and indicated cleavage to occur somewhere between amino acids 1040 and 1093, a region in the vicinity of that mapped by Western analysis. The exact location of the cleavage site was unable to be specifically mapped by N-terminal amino acid sequencing.

Subsequent Western blots using anti-$F2_{P216}$ and rP27 to detect the N- and C-terminal cleavage fragments of P216 respectively identified P120 and P85 in geographically diverse strains of *M. hyopneumoniae* from the UK, USA and Australia. A similar cleavage profile was reported for a proteomic study of strain 7448 from Brazil (Pinto *et al*., 2007). The same sera suggested rapid processing of P216 into P120 and P85 when immunoblot analysis of synchronous *M. hyopneumoniae* cultures showed the prominence of P120 and P85 throughout all stages of the growth cycle. Immediate processing of P216 among strains originating from different regions indicates that this event is likely to be important and possibly effecting function of the protein. An inability of the P216 sera to detect full-length P216 even in continued analysis enriched for the detection of high mass proteins suggests that P120 and P85 are the functional units of P216 (Wilton *et al*., 2009).

P216 is the fourth (P97, P102, P159) protein of *M. hyopneumoniae* shown to undergo post-translational cleavage. Cleavage fragments of the other three proteins have been shown to reside on the surface of *M. hyopneumoniae* indicating that these molecules are likely to play important roles in pathogenesis (Burnett *et al*., 2006, Djordjevic *et al*., 2004). Pinto *et al*. (2007) also identified the cleavage of these and other surface proteins of *M. hyopneumoniae* in proteomic analysis of strain 7448. Detailed proteome studies of two other *Mycoplasma* species (Jaffe *et al.*, 2004a, Jaffe *et al.*, 2004b) have not reported processing of surface adhesins to the extent so far described in *M. hyopneumoniae*.

The surface location of both P120 and P85 were confirmed when whole cell shaving experiments using anti-$F2_{P216}$ and rP27 sera detected the sensitivity of these two proteins to digestion by trypsin. Both proteins were undetectable at a trypsin

concentration of 30 ug/mL. A similar method was used to demonstrate the surface location of *M. hyopneumoniae* adhesins P97 (Wilton *et al*., 1998 and Djordjevic *et al*., 2004) and P159 (Burnett *et al*., 2006), both completely digested by trypsin at concentrations between 10 and 50 ug/mL.

The rapid processing and surface location of P216 suggests that cleavage occurs external to or during translocation across the membrane. If cleavage occurred cytosolically, separate transport mechanisms would be required to export the two cleavage products as P85 does not possess a secretion signal or hydrophobic region (as predicted by the TmPred algorithm) sufficient to span the cell membrane (Wilton *et al*., 2009). P120 may however retain the putative transmembrane domain identified between amino acids 7 – 30 providing it with a mechanism for cell surface attachment. Edman sequence analysis of N-terminal cleavage fragments of adhesins P97 and P159 of *M. hyopneumoniae* show these proteins to possess intact hydrophobic leader sequences (Djordjevic *et al.*, 2004; Burnett *et al.*, 2006). How P85 remains associated with the cell surface is largely unknown but it is possible that the KEKE motif and two regions of glutamine repeats present in P85 play a role in maintaining its association with the cell surface.

KEKE motifs are believed to encourage association between proteins (Realini *et al*., 1994) but their occurrence in prokaryotes is unusual and their role in *M. hyopneumoniae* is yet to be established. The strict sequence rearrangement needed to define them however means they rarely occur by chance (Realini *et al*., 1994). Interestingly this motif was completely conserved in P216 from different strains of *M. hyopneumoniae*. Repeat region R1 of the P97 adhesin of *M. hyopneumoniae* has been well established as

the region of the molecule responsible for binding porcine cilia (Hsu and Minion, 1998; Minion *et al*., 2000). These were AAKPV(E) repeats which differ from the glutamine (Q) repeats of P85. Glutamine repeats are known to promote protein aggregation (Perutz *et al*., 2002) and are often found in host adapted pathogens (Mrazek *et al*., 2007). Faux *et al*. (2005) showed that single sequence repeat (SSR) regions, like the CAA nucleotide repeat encoding the two strings of glutamine residues in P85, can act as a flexible linker that bridges functional domains. It has also been noted that repeat regions displaying amino acid bias in proteins may represent sites for protein-protein interactions and function as the focal point of protein interaction networks (Dunker *et al*., 2005). Membrane-associated, high mass proteins that have a demonstrated role in adherence and other complex protein-protein interactions often partition to the Triton X-114 insoluble fraction in *Mycoplasma* spp. (Stevens and Krause, 1991; Layh-Schmitt and Herrmann, 1992; Stevens and Krause, 1992; Popham *et al*., 1997). P85 partitioned exclusively to the insoluble phase and P120 primarily to the insoluble phase and also the detergent phase suggesting their potential role in protein binding complexes.

The host extracellular matrix (ECM) components to which adhesins including P97 and P159 bind have become a focus of more recent research. The molecules of the ECM include fibronectin, collagen, laminin and glycosaminoglycans (GAGs) such as heparan sulphate and chondroitin sulphate (Kreis and Vale, 1993). Bacterial pathogens have been shown to produce adhesins that specifically target heparin sulphates (Rostand and Esko, 1997; Menozzi *et al*., 2002). Heparan sulphate has been identified as the predominant GAG found along the surface of cilia and microvilli in the porcine respiratory tract as well as in the basement membrane of the bronchial epithelium (Erlinger, 1995). Interactions between basement membrane GAGs and the epithelial cytoskeleton are believed to

contribute to the stability of the epithelium (Erlinger, 1995). Heparin has also been shown to block the binding of *M. hyopneumoniae* to porcine cilia (Zhang *et al*., 1994a,b). For these reasons, the identification and characterisation of heparin-binding proteins on the surface of *M. hyopneumoniae* is considered essential to understanding how this pathogen colonises ciliated epithelial cells to initiate cell pathology.

Biotinylated heparin is frequently used to identify heparan sulphate binding proteins (Mulloy and Linhardt, 2001; Rabenstein, 2002). Biotinylated heparin was used in this study in ligand blots as well as binding assays for assessment of the potential of P216 to bind heparan sulphates. P120 and P85 were identified in ligand blots as two of the three major heparin binding proteins detected in TX114 extracted proteins of *M. hyopneumoniae*. The recombinant fragments of P216 were also shown to bind heparin in standard ligand blots involving the transfer of proteins from denaturing polyacrylamide gels. Microtitre plate-based heparin binding assays using the recombinant P216 proteins $F1_{P216}$-$F3_{P216}$ bound heparin in a dose-dependent, saturable manner with physiologically significant Kd values. Binding to heparin was largely abolished by the addition of unlabelled heparin, mucin and fucoidan but not by chondroitin sulphate B. Collectively these data suggests that sequences within P120 and P85 are binding glycosaminoglycans through charge interactions (Jenkins *et al*., 2006). Binding of all three P216 fragments suggests that P120 contains at least two heparin-binding domains and P85 at least one, but further studies are required to define the exact location of these sites.

Studies conducted after the completion of the research component of this thesis also showed that P120 and P85 have cell and cilium binding capabilities (Wilton *et al*., 2009). Cell binding was demonstrated in a latex bead adherence assay in which beads separately

coated with $F1_{P216,}$ $F2_{P216}$ and $F3_{P216}$ were shown to adhere to a monolayer of PK15 cells with comparable affinity to $F4_{P159}$ adherence. $F4_{P159}$ had been included as a positive control for its previously demonstrated ability to bind PK15 cells in a similar assay (Burnett *et al*., 2006). $F2_{P216,}$ $F3_{P216}$ and rP27 all bound cilia in microtitre plate cilial binding assays. $F3_{P216}$ spans the C-terminus of P120 and the N-terminus of P85 and encompasses the major cleavage site identified in P216 and so further studies are required to define the cilium binding domain (Wilton *et al*., 2009).

Although the heparin and cilium-binding domains in P216 have not been clearly defined by this thesis or subsequent research, it is clear that sequences within P120 and P85 bind cilia. P216 is only the second cilium adhesin to be described, with P97 being the first, from which it is differentiated by its lack of an R1 cilium-binding domain. Further studies are required to clearly define the cilium- and heparin-binding domains in P216. The data generated in this thesis and the research that continued from it indicates that the P120 and P85 cleavage fragments of P216 are important components of the surface architecture of *M. hyopneumoniae* with a significant role in its interaction with porcine respiratory tract cells of its host. This makes P216 a potential vaccine candidate for which further studies are required.

Chapter Six

General Discussion and Future Work

Mycoplasma hyopneumoniae is the primary aetiological agent of porcine enzootic pneumonia. *M. hyopneumoniae* induce disease by colonising ciliated epithelial cells that line the respiratory tract causing ciliostasis, cilial shedding and epithelial cell death (Jacques *et al*., 1992). These events contribute significantly to the pathogenicity of *M. hyopneumoniae* but the specifics of these processes remain largely unknown. Surface proteins of *M. hyopneumoniae* and their ability to bind target cells are believed to play an important role in the colonisation process. Defining the surface architecture of *M. hyopneumoniae* may not only increase the current understanding of the organism's pathogenic mechanisms but may also play an important role in the development of vaccines that target surface proteins and binding processes to prevent colonisation and subsequent development of disease.

Identifying surface proteins of *M. hyopneumoniae* when the research for this thesis began was directed largely by the reaction of *M. hyopneumoniae* antigens in immunoblots with convalescent sera. The antigens characterised in this thesis were three of multiple selected for further analysis after being produced as part of an *M. hyopneumoniae* expression library or as components of size fractionated *M. hyopneumoniae* proteins in research completed previously in the laboratory. The characterisation techniques used in this thesis enabled the successful identification of the three antigens and investigation of their potential as surface antigens of *M. hyopneumoniae* with a role in binding host cells.

Preliminary analysis of the first antigen identified it as the carboxy-terminus of a previously identified adhesin of *M. hyopneumoniae*. The adhesin, P97 from strain 232, had been designated as such after a series of experiments showed it to be a surface

located protein of *M. hyopneumoniae* with an ability to adhere to cilia (Zhang *et al*., 1995). Upon sequencing the clone encoding the adhesin antigen, two regions of reiterated repeats (identified as RR1 and RR2 in this research) were identified and shown to vary in number among strains of *M. hyopneumoniae* from varying geographic localities (Wilton *et al*., 1998). RR1 displayed greatest variation in its five amino acid repeats with 9 copies in the highly passaged non-pathogenic J strain and 15 copies in the lower passage virulent 232 strain. R1 of the adhesin was demonstrated to mediate the adherence of *M. hyopneumoniae* to swine cilia by Minion and colleagues (2000) with results indicating that eight R1 repeating units were required for cilium binding. All *M. hyopneumoniae* strains sequenced had at least 8 repeats so no correlation between the number of R1 repeats and virulence could be made, as initially hypothesised.

Early investigation by Zhang *et al*. (1995) suggested that the adhesin was only responsible for approximately 65% of *M. hyopneumoniae* adherence suggesting the involvement of other surface molecules. More recent evidence of proteolytic processing of the adhesin (Djordjevic *et al*., 2004) into multiple cleavage products presents a paradox with the evidence of its surface association. A complex binding process is therefore hypothesised where protein-protein interactions are believed responsible for maintaining association of R1 to *M. hyopneumoniae*. The proteins involved may be either mycoplasma or host derived (Djordjevic *et al*., 2004). Host proteins, in particular components of the ECM, are frequently the targets of pathogenic bacteria and have been shown to facilitate binding, colonisation and invasion of host cells (Patti *et al*., 1994; Ljungh and Wadström, 1995).

Fibronectin was the first ECM protein shown to be involved in this host-pathogen interaction (Kuusela, 1978) and is ubiquitous in the ECM. Fibronectin and its interaction with bacteria via fibronectin binding proteins is believed to contribute to virulence in a number of microorganisms (Joh *et al*., 1999). Fibronectin binding by the P97 adhesin, specifically a C-terminal antigen encompassing RR1 and RR2, was demonstrated by Jenkins *et al*. (unpublished) to bind fibronectin. Fibronectin binding proteins have also been reported in a number of other mycoplasma species (Giron *et al*., 1996; Dallo *et al*., 2002; May *et al*., 2006). Jenkins *et al*. (2006) also demonstrated the binding of both the N- and C-terminal fragments of P97 to heparin. Together these findings suggest that firstly the repeat regions can bind multiple ligands and secondly that P97 has a role in colonisation as well as adherence. The potential of P97 as a vaccine antigen is therefore reinforced and requires further investigation.

Characterisation of the second *M. hyopneumoniae* antigen in this thesis identified it as pyruvate dehydrogenase subunit E1α (PdhA). As part of the pyruvate dehydrogenase complex that catalyses the first step in the pyruvate roundhouse it was assumed that PdhA was a cytosolic enzyme and therefore an unlikely vaccine candidate. PdhA had however been found complexed with the P1 adhesin on the surface of *M. pneumoniae* (Layh-Scmittet *et al*., 2000). The R2 subunit of the cytosolic enzyme ribonucleotide reductase had been found on the surface of *M. hyopneumoniae* (Fagan *et al*., 1996) as had glycolytic enzymes in other bacteria (Pancholi and Fischetti, 1992; Pancholi and Fischetti, 1998; Bergman *et al*., 2001; Chhatwal *et al*., 2002; Wilkins *et al*., 2003). These research findings warranted its continued investigation.

Trypsin and triton studies both indicated the intracellular location of PdhA in *M. hyopneumoniae* but a small amount retained in the insoluble pellet of triton extractions suggested that it may also function as part of some kind of complex membrane associated structure. This possibility requires further exploration. Although an unlikely vaccine candidate, the capacity for the 12 kDa C-terminal PdhA antigen characterised in this research to provide protection against disease would only be assessable by conducting a vaccine trial and challenge in swine. Data generated in the characterisation of PdhA also complemented research being conducted by J. Matic at the University of Wollongong and contributed to an increased understanding of the pyruvate dehydrogenase complex in *M. hyopneumoniae* (Matic *et al*., 2003).

A novel protein of *M. hyopneumoniae* designated P216 was the third antigen in this thesis to be characterised. Characterisation was aided by pre-release access to the *M. hyopneumoniae* genome sequence during preliminary cloning and sequencing experiments. Molecular analysis of P216 revealed its sequence similarity to P97, identifying it as an adhesin paralog. Generation of recombinant expression fragments covering 75% of the molecule and antiserum raised to them enabled extensive characterisation. P216 was identified predominantly as cleavage fragments P120 and P85. Full length P216 was unable to be identified in experiments enriched for its detection and appeared to be processed almost immediately into P120 and P85 in growth cycle experiments. P120 and P85 were also identified among strains of *M. hyopneumoniae* originating from different regions suggesting the cleavage products as the functional units.

P120 and P85 were shown to reside on the surface of *M. hyopneumoniae* as have cleavage products of P97 (Djordjevic *et al*., 2004) and P159 (Burnett *et al*., 2006). This raises the question as to how these cleavage products remain associated with the organism. A transmembrane domain was identified at the N-terminus of P120, and has also been identified in P97 and P159, which may account for attachment of these fragments to the mycoplasma membrane. How the C-terminal fragments of all three molecules maintain their association with the *M. hyopneumoniae* surface remains largely unknown. Active mechanisms and protein-protein interactions have been hypothesised for the surface location of P97 cleavage fragments (Djordjevic *et al*., 2004). KEKE motifs are believed to encourage association between proteins (Realini *et al*., 1994) and glutamine repeats are known to promote protein aggregation (Perutz *et al*., 2002). Both are found in P85, which supports the hypothesis of protein interactions playing a crucial role in this phenomenon.

Protein-protein interactions between cleavage fragments and other surface proteins have been reported in the attachment organelle of *M. pneumoniae* (Popham *et al*., 1997; Krause *et al*., 1998). Although *M. hyopneumoniae* does not possess an attachment organelle, similar interactions may exist between surface proteins in a concerted effort for host binding and colonisation; another area requiring further research. Several potential proteases have been identified in the genome sequence of *M. hyopneumoniae* (Minion, unpublished) but have not been confirmed responsible for the observed cleavage patterns. Cleavage has been observed in the molecules mentioned here and Djordjevic *et al*. (unpublished data) has observed this proteolytic process among other high molecular weight surface proteins of *M. hyopneumoniae*. Processing to the extent observed for *M. hyopneumoniae* has not been reported for other mycoplasmas

suggesting it as an important phenotype of this organism and reinforcing the need to identify cleavage mechanisms. Identification of cleavage fragments and other proteins interacting at the surface of *M. hyopneumoniae* that might be functioning together in adherence is pertinent to understanding its pathogenesis. An increased understanding of what proteins are involved and how they are bound to each other means blocking the interaction between proteins in a complex required for binding could be achieved and has implications for therapeutics and vaccination.

Another interesting feature supporting the idea that these surface molecules of *M. hyopneumoniae* are interacting is their location within operons. Transcription and translation are tightly coupled in bacteria so genes within operons are generally co-expressed and the likelihood of a co-ordinated function is increased. Co-transcription of adhesins and accessory proteins has been demonstrated for *M. pneumoniae* (Sperker *et al*., 1991). Mhp183 encoding P97 is located in an operon with Mhp182 encoding P102, also a surface associated molecule of *M. hyopneumoniae* (Hsu and Minion, 1998; Djordjevic *et al*., 2004). Six P97 and P102 paralogs were identified when the genome was sequenced (Minion *et al*., 2004). Paralogous families of genes like these have the potential to provide *M. hyopneumoniae* with an armoury of adhesins capable of binding various ECM components and a range of tissues.

The gene encoding P216 (a P97 paralog) is located in an operon with Mhp 494, the gene encoding P159. P159 shows similarity but is not significantly homologous to P102 making it an exception to the above mentioned gene arrangement. In very recent studies mapping the proteome of *M. hyopneumoniae*, P216 and P159 consistently show significantly high expression levels (Djordjevic *et al*., unpublished data) reinforcing

their importance in pathogenesis and the disease process. Identifying the ECM components which they are binding is the focus of current research. This study showed the ability of P216 fragments to bind heparin in a dose-dependant manner. Similar studies have demonstrated the same for fragments of P159 (Burnett *et al*., 2006) and P97 (Jenkins *et al*., 2006). Future research will include mapping the binding domains of these and other surface proteins of *M. hyopneumoniae* in an effort to understand how surface proteins are interacting with each other and the extracellular milieu.

Although the research in this thesis began prior to the release of the *M. hyopneumoniae* genome sequence and was completed some time ago, it provided a solid foundation for continued research on P97 and P216, which remain the only two cilium adhesins of *M. hyopneumoniae* identified to date. Research in this area continues with the search for surface proteins and an improved understanding of their interaction with each other and the host. Blocking *M. hyopneumoniae* adherence and subsequent colonisation by vaccination is believed to be necessary for the effective control of PEP (Hsu and Minion, 1998). Studies aimed at an increased understanding of potential vaccine antigens are therefore of primary importance in this field of research.

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Appendix A: Buffers and Reagents

Commonly used buffer solutions

Phosphate buffered saline (PBS) pH 7.2
136 mM NaCl
2.5 mM KH_2PO_4
7 mM K_2HPO_4

TE buffer pH 7.4
10 mM Tris.Cl
1 mM EDTA

Tris buffered saline (TBS or TS)
25 mM Tris
150 mM NaCl
Adjust pH with HCl to pH 7.2

Triton buffer
0.0606 g/50 mL Tris
0.438 g/50 mL NaCl
0.1 mL/50 mL 0.5M EDTA

Culture Reagents

LB media
10 g/l Tryptone
5 g/l Yeast
5 g/l NaCl

LB agar
10 g/l Tryptone
5 g/l Yeast
5 g/l NaCl
15 g/l Agar

SOC medium
2 g/100 mL Tryptone
0.5 g/100 mL Yeast Extract
0.06 g/100 mL NaCl
0.02 g/100 mL KCl
0.1 g/100 mL $MgCl_2$
0.12 g/100 mL $MgSO_4$
0.36 g/100 mL Glucose

1-D SDS-PAGE gels/4 gels

	12% resolving gel	15% resolving gel	4% stacking gel
MilliQ water	13.2 mL	9.2 mL	10.8 mL
30% acrylamide	16 mL	20 mL	1.95 mL
1.5 M Tris pH 8.8	10 mL	10 mL	
1.0 M Tris pH 6.8			1.95 mL
10% w/v SDS	0.4 mL	0.4 mL	0.15 mL
APS	45 mg	45 mg	10 mg
TEMED	16 µl	16 µl	11 µl

Electrophoresis reagents

SDS-PAGE buffer
3.03 g/l Tris base
14.33 g/l Glycine
1.0 g/l SDS

Laemmli Reducing solution
25% v/v 1 M Tris.HCl (pH 6.8)
8% w/v SDS
20% v/v β-mercaptoethanol
40% v/v Glycerol
0.01% w/v Bromophenol Blue

Coomassie stain 1-D
0.25% w/vCoomassie R250
45% Methanol
10 % Acetic Acid

Coomassie de-stain 1-D
10% v/v Acetic acid
4.5% v/v Methanol

1X TAE for agarose gels
200 mM Tris.HCl
83 mM NaAcetate
5 mM EDTA

0.5X TBE for agarose gels
45 mM Boric acid
220 mM Tris base
5 mM EDTA

Bromophenol blue loading dye
0.05% w/v bromophenol blue
75% v/v glycerol
25% v/v TE buffer

2-D Gel Electrophoresis Reagents

5X Tris buffer pH 8.8
227 g/l Tris

Equilibration buffer

Urea		21.6 g/60 mL
SDS		1.2 g/60 mL
5X Tris buffer	pH 8.8	12 mL/60 mL
Glycerol		12 mL/60 mL
DTT		1.2 g/60 mL
40% Acrylamide		3.75 mL/60 mL

Lysis buffer

Urea	0.54 g/100 mL
DTT	1 g/100 mL
CHAPS	4 g/100 mL
Ampholytes pH 3-10	0.8 mL/100 mL
Tris	0.42 g/100 mL
PMSF	10 µl/mL
EDTA 0.5 M	9 µl/mL

Standard solubilisation solution (SSS)

Urea	9.6 g/20 mL
DTT	0.308 g/20 mL
CHAPS	0.8 g/20 mL
Ampholytes pH 3-10	0.4 mL/20 mL
Tris	0.097 g/20 mL

Multiple surfactant solution (MSS)

Urea	6.0 g/20 mL
Thiourea	3.04 g/20 mL
DTT	0.2 g/20 mL
CHAPS	0.4 g/20 mL
Sulfobetaine	0.4 g/20 mL
0.8% Ampholytes 3-10	0.4 mL/20 mL
Tris	0.1 g/20 mL

Colloidal Coomassie
17% (w/v) Ammonium sulfate
3.6% (v/v) Phosphoric acid
0.1% (w/v) Coomassie G250
34% (v/v) Methanol

Colloidal Coomassie de-stain
1 % (v/v) Acetic acid

Western Transfer Reagents

Western transfer buffer
3.03 g/l Tris
14.33 g/l Glycine
200 mL/l Methanol

DAB solution
0.05% w/v DAB
100 mM Tris pH 7.6
0.03% v/v µl Hydrogen peroxide

ELISA Reagents

Carbonate coating buffer pH 9.6
20 mM $NaHCO_3$
30 mM Na_2CO_3

ABTS solution
0.1 M citric acid
0.2 M Na_2HPO_4
pH to 4.2
0.55% w/v ABTS
0.3% v/v hydrogen peroxide (30%)

Southern and Colony Hybridisation Reagents

Denaturation solution (Southern and colony hybridisations)
0.5 M NaOH
1.5 M NaCl

Neutralisation solution (Southern hybridisation)
0.5 M Tris.HCl (pH 7.5)
3 M NaCl

Neutralisation solution (Colony hybridisation)
1.0 M Tris.HCl (pH 7.5)
1.5 M NaCl

10X SSC buffer pH 7.0
1.5 M NaCl
150 mM Na CitrateSodium citrate

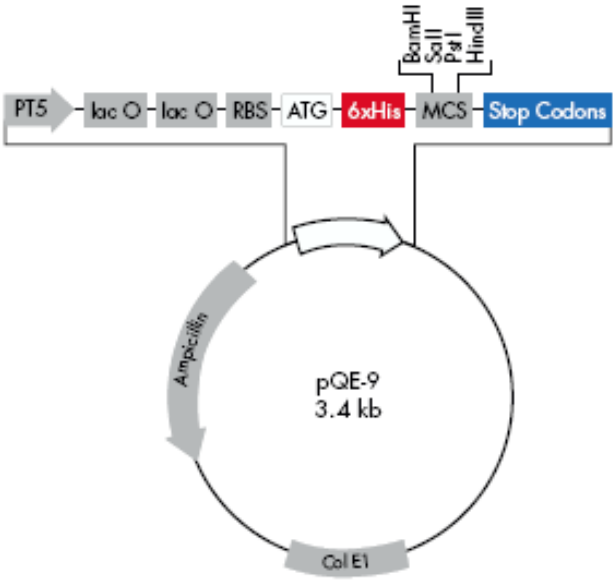
Maleic Acid buffer pH 7.5
0.1 M Maleic acid
015 M NaCl

Ni-NTA purification buffer for His-tag clones

8 M Urea
0.1 M NaH_2PO_4
0.01 M Tris
Same buffer used but lysis buffer B at pH 8.0, wash buffer C at pH 6.3, elution buffers D and E at pH 5.9 and pH 4.5, respectively.

Appendix B: Plasmid Maps

pQE9 Vector Map



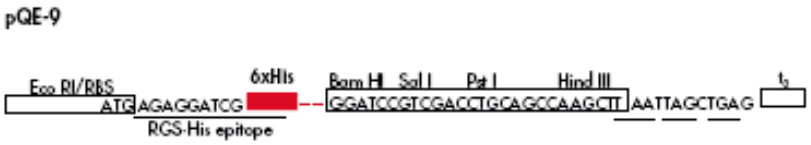


Figure A1. Circular map of the pQE9 vector for N-terminal 6xHis tag constructs. Major features and enzyme sites in the MCS are indicated. The nucleotide sequence is available at *www.Qiagen.com* (Figure taken from website).

pPCR-Script Amp SK(+) Vector Map

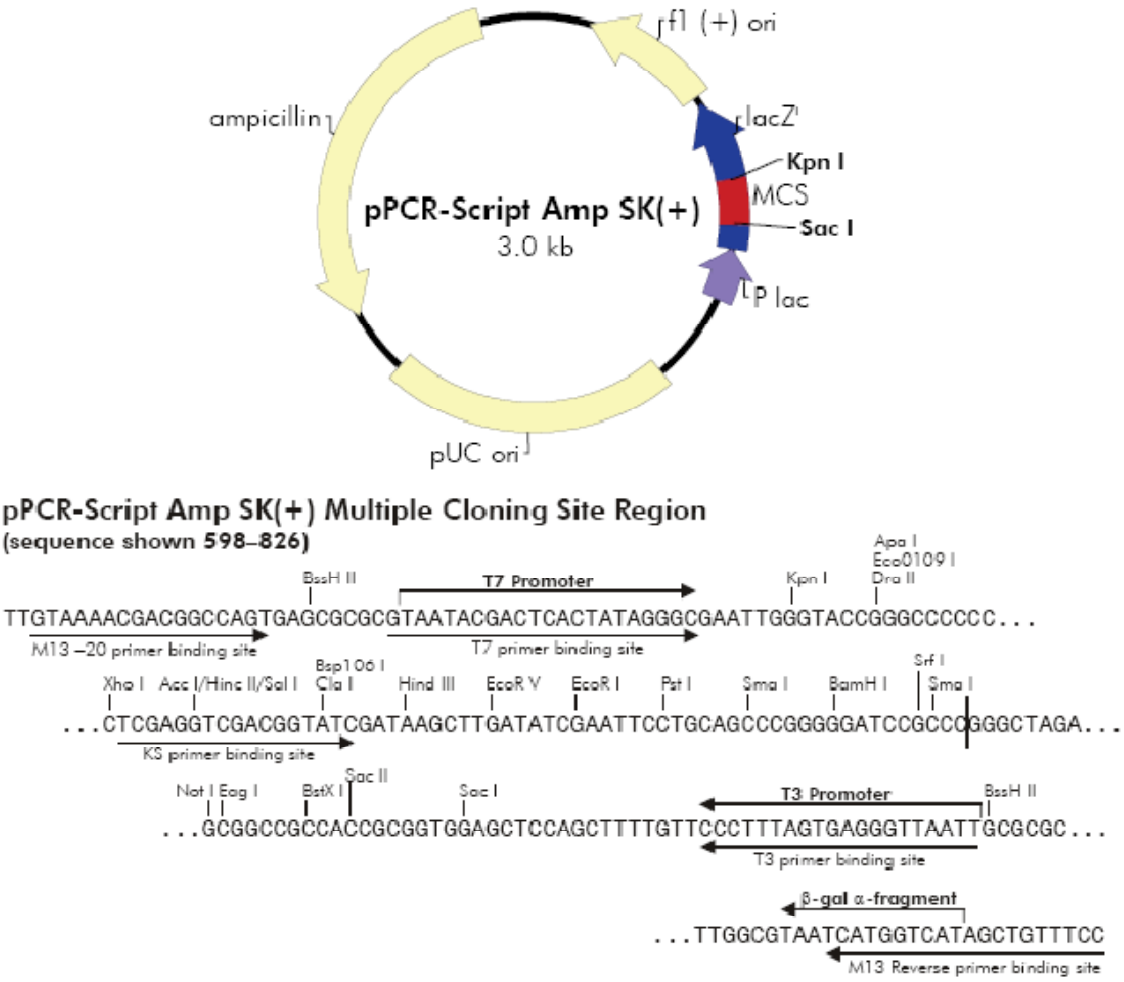


Figure A2. Circular map and polylinker sequence of the pPCR-Script Amp SK(+) cloning vector. Major features and enzyme sites in the MCS are indicated. The nucleotide sequence and additional information is available at *www.stratagene.com* (Figure taken from website).

pCR 2.1 Vector Map

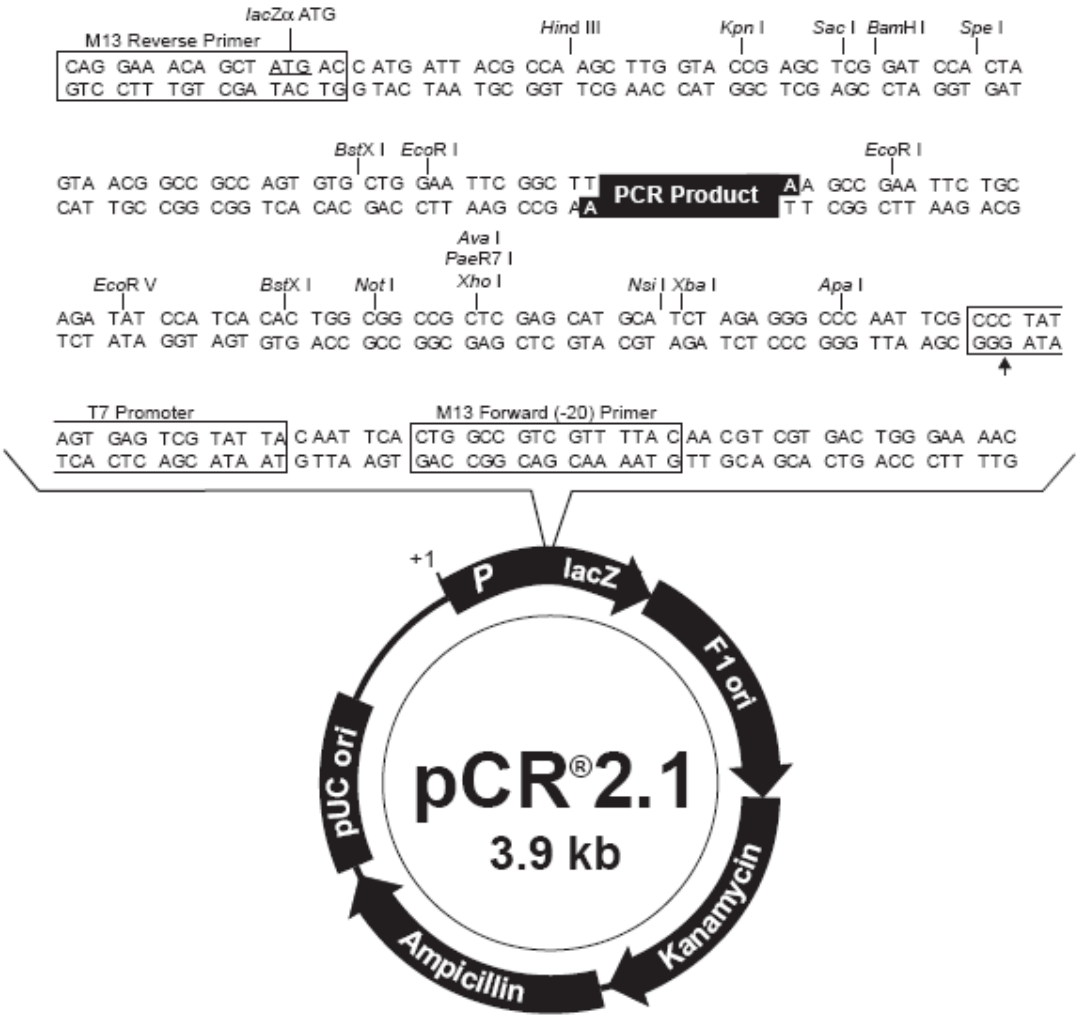


Figure A3. Circular map and polylinker sequence of the pCR2.1 cloning vector designed for direct cloning of *Taq* polymerase amplified PCR products. Major features and enzyme sites in the MCS are indicated. The nucleotide sequence and additional information is available at *www.invitrogen.com* (Figure taken from website).

pET161 Vector Map

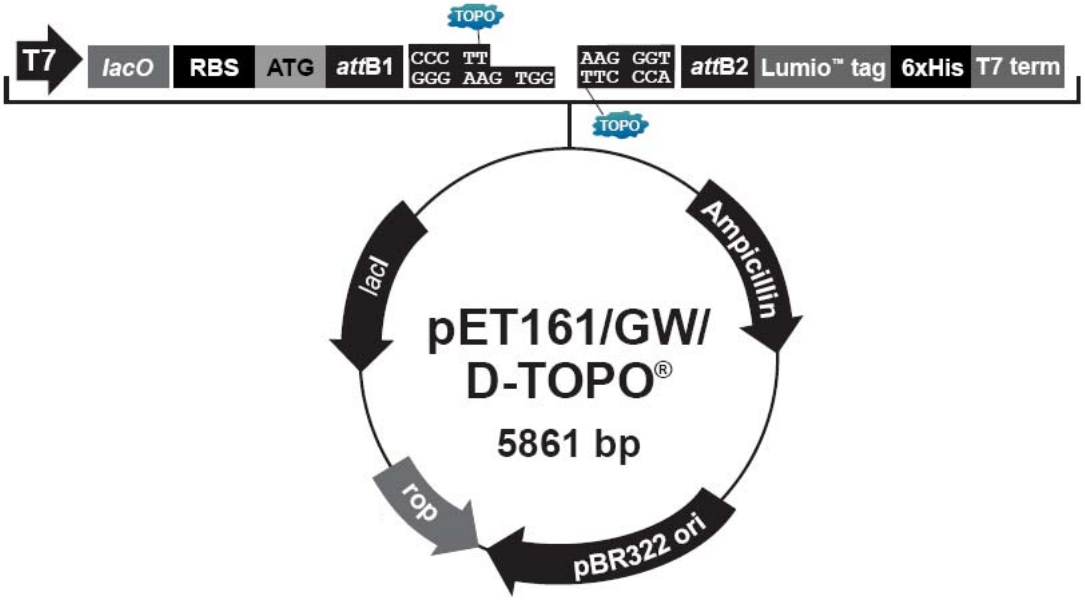


Figure A4. Circular map of the pET161 cloning vector designed for simple directional cloning and expression of PCR products amplified with *Pfu* polymerase. Major features and enzyme sites in the MCS are indicated. The nucleotide sequence and additional information is available at *www.invitrogen.com* (Figure taken from website).

Appendix C: Cloning Primer Sequences

RRF	5'-TCAGTATTCATTTGAAGCTA-3'
RRR	5'-CTCGATTAGTTCAACCTCTG-3'
Adh5’F	5’-AGT TAA ATA AAT TTT TCA CT-3’
Adh5'R	5’-CTT TAA TCT GAT TGT AAG GA-3’
AdhHisF	5’-GGGGATCCAAATTAGACGATAATCTTCAG-3’
AdhHisR	5’-GGAAGCTTAGGATCACCGGATTTTTGAAT-3’
P216 HisF	5’-GGGGATCCAACGGGCAAAGCCAAAATGTA-3’
P216 HisR	5’-GGCTGCAGTTATCCTTTAGCAATTTTAGGGGA-3’
ORF545F1	5’-CACCCGGGGTGTAAATCCAACTC-3’
ORF545AR1	5’-GATTTCAACATCACGGAAG-3’
ORF545F2	5’-CACCTTAGGTGATCAAGAACCTA-3’
ORF545R2	5’-TAGCTCAGCTATTTCTTCA-3’
ORF545F3	5’-CACCGAAGAAATAGCTGAGCTAG-3’
ORF545R3	5’-GGGAACACCAATTGATCGTTC-3’
PdhAF	5’-TCGCTACCATTTCAATATTGC-3’
PdhAR	5’- TAG ATA TGT TAA ACC TGG WCA AAT TAT GGC -3’
PdhBF	5’-TCGAGCAACTGAAGGTCTTCA-3’
PdhBR	5’-TCATAACCGGTAAGTCGAGCA-3’
PdhAHisF	5’-GGGGATCCCGCTATGTAAAGCCTGGTCAA-3’
PdhAHisR	5’-GGCTGCAGTTATCCAGGAATTCCGGCTGCCAT-3’