The Development of Methods to Investigate the Mechanisms underlying Serum Resistance of *Ureaplasma* Species

By

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DEDICATION

This thesis is dedicated to my beloved mother, wife and children (Fatma, Enaas, Aya, and Mayar)

Summary of Thesis

The human *Ureaplasma* species are among the smallest and simplest self-replicating bacteria known to date. These microbes cause infection in humans, particularly in the upper genital tract during pregnancy, leading to several adverse outcomes including preterm birth, chorioamnionitis, and respiratory diseases of neonates. Little is known about the pathogenesis of *Ureaplasma* and mechanisms by which they avoid recognition and killing by the complement system. In this thesis, some mechanisms underlying serum resistance of *Ureaplasma* spp. were investigated. This goal was achieved by creating serum-resistant models of serum-sensitive laboratory *Ureaplasma* strains and developing and using some proteomic and molecular biology methods to study the role of potential factors, which mediate serum resistance and play a role in pathogenesis of *Ureaplasma*. My original contribution to the knowledge in this work was the development of transposon mutagenesis method that can now be used to study virulence genes of *Ureaplasma.* This method will also allow genetic manipulation of *Ureaplasma* for future studies. Monitoring and investigating induced serum-resistant strains using immunoblot analysis and proteomics revealed significant changes in two candidate proteins coincident with serum resistance. The first was the elongation factor Tu protein that found to be immunogenic and had altered *pI* isoforms. The observed change in this protein was consistent in all serum-resistant strains, which suggests a possible role in mechanism of serum resistance, possibly as a mediator for binding complement regulators, such as factor H and C4BP, at the cell surface of *Ureaplasma*. The second candidate protein was a novel 41 kDa protein that was uniquely expressed in all induced serum-resistant strains. Expression of this protein in all resistant strains strongly indicates its involvement in mechanism(s) of serum resistance of *Ureaplasma*. The possible gene that encodes for this 41 kDa protein has putatively been identified as UUR10_0137 in the genome of *U. urealyticum* serovar 10 (strain ATCC 33699) using the transposon mutagenesis method developed in this study. Although the gene product of UUR10_0137 gene is not known (hypothetical protein), this protein is now identified and proposed to have a role in serum resistance of *Ureaplasma.* The product of the UUR10_0137 gene could function as a complement regulator or inhibitor that prevents the activation of complement system, protecting *Ureaplasma* from the complement attack. The contribution of the multiple-

banded antigen, MBA, was proven to be unimportant to serum resistance. Sole antigenic variations in this major surface antigen of *Ureaplasma* did not play any role in mediating serum resistance. Confirmation of a gene that mediates complement resistance would dramatically increase our understanding of *Ureaplasma* pathogenicity and provide a target for future human studies with preterm birth and *Ureaplasma* infection.

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May God Bless You All

Abbreviations

5’	5 prime
3’	3 prime
16S rRNA	16S ribosomal RNA
23S rRNA	23S ribosomal RNA
1-DE	One Dimensional Electrophoresis
2-DE	Two Dimensional Electrophoresis
A	Adenine
Ab	Antibody
Ag	Ag
AP	Alternative pathway
ATCC	American Type Culture Collection
BAL	Bronchioalveolar lavage
BPD	Bronchopulmonary dysplasia
BCA	Bicinchoninic acid
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
C1 INH	C1 inhibitor
C4BP	C4-binding protein
CCU	Colour changing unit
CBS	Central Biotechnology Services
CFU	Colony forming unit
CLD	Chronic Lung Disease
CNS	Central nervous system
CP	Classical pathway
CR1	Complement receptor 1
CRP	C-reactive protein
CSF	Cerebrobral spinal fluid
Da	Dalton
DAF	Decay accelerating factor
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate

DR	Direct repeats
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tertraacetic acid
ETA	Endotracheal aspirate
FH	Factor H
FHL-1	Factor H- like protein 1
FHRs	Factor H-related proteins
G	Guanine
GPI	Glycosylphosphatidylinositol
HAE	Hereditary angioedema
HBSS	Hanks buffered saline solution
HI-NHS	Heat-inactivated normal human serum
HPA	Health Protection Agency
HPE	Heath Protection England
HRPO	Horse radish peroxidase
ICE	Integrative conjugative element
IEF	Isoelectric focusing
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
IR	Inverted repeats
JCVI	J. Craig Venter Institute
Kb	Kilo bases
kDa	Kilo Dalton
Kg	Kilo grams
LDS	lithium dodecyl sulphate
LPS	Lipopolysaccharide
MAb	Monoclonal antibody
MAC	Membrane attack complex
MASP-2	MBL-associated serine peortease-2
MBA	Multiple banded antigen
MBL	Mannose binding lectin
MCP	Membrane cofactor protein
MIC	Minimal inhibitory concentration

M. genitalium	*Mycoplasma genitalium*
M. hominis	*Mycoplasma hominis*
M. pneumoniae	*Mycoplasma pneumoniae*
MS	Mass spectrophotometry
MALDI TOF/TOF	Matrix- assisted laser desorption ionization time-of-flight
mRNA	Messenger RNA
NGU	Non-gonococcal urethritis
NHS	Normal human serum
NICU	Neonatal Intensive Care Unit
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween-20
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Isoelectric point
PMN	Polymorphonuclear
Ply	Plymouth
PMF	Protein mass fingerprint
PRRs	Pattern recognition receptors
RCA	Regulators of complement activation
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SAK	Staphylokinase
S. aureus	*Staphylococcus aureus*
SEM	Standard error of the mean
SNP	Single nucleotide polymorphism
Spp.	Species
SV	Serovar
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
T	Thymine
TEAB	Tri-ethyl ammonium Bicarbonate
TFA	Trifluoroacetic acid
TNF-α	Tumour necrosis factor alpha
TLRs	Toll-like receptors
U	Uracil
U. parvum	*Ureaplasma parvum*

Upvmp376	*Ureaplasma* phase-variable membrane protein 376
U. urealyticum	*Ureaplasma urealyticum*
USM	Ureaplasma Selective Medium
VBS	Veronal buffered saline
W.B	Western blot

Table of Contents

Chapter 3

Chapter 4

List of Figures

List of Tables

Chapter 1

Introduction

1. Introduction

1.1 Taxonomy and classification of *Ureaplasma*

Ureaplasma spp. are a group of bacteria belonging to the class *Mollicutes* that contains four orders, five families, eight genera, and about 200 known species that are capable of infecting humans, vertebrate animals, arthropods, and plants (Razin *et al.*, 1998; Waites *et al.*, 2005). *Ureaplasma* spp. are recognised within the genus *Ureaplasma* that is taxonomically classified within the family *Mycoplasmataceae*, the order *Mycoplasmatales*, the class *Mollicutes*, and the phylum *Firmicutes*. Members of *Mollicutes* are believed to have evolved from Gram-positive bacterial ancestors, which had DNA with low G+C contents (Dybvig and Voelker, 1996). *Ureaplasma* can inhabit both human being and animal hosts, in which they can cause diseases (Razin *et al.*, 1998).

Ureaplasma spp. were first recognised as human pathogens in 1954, when isolated from men suffering from non-gonococcal urethritis (NGU) (Shepard, 1954). *Ureaplasma* spp., as well as all other members of the class *Mollicutes*, are phenotypically distinguished from other bacterial species by a total absence of a classical bacterial cell wall and small genome sizes (Razin *et al.*, 1998). Initially, human *Ureaplasma* spp. were described as T- strain mycoplasmas because of the tiny colonies they produce (ranging from 5 µm to 20 µm) compared with those of other members of the same family (Shepard, 1956). However, due to their ability to hydrolyse urea and use it as a primary source of energy, they were proposed as a single genus, consisting of 14 serovars, based on metabolic inhibition tests and colony indirect epifluorescence assays, and designated as *Ureaplasma* (Robertson and Stemke, 1982; Shepard *et al.*, 1974). From that time up until 2002, human *Ureaplasma* genus was believed to be comprised of only one single species, referred to as

Ureaplasma urealyticum, which was divided into two biovars: biovar parvo or biovar 1 comprised of serovars (SVs) 1, 3, 6, and 14, and biovar T960 or biovar 2 included serovars (SVs) 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13. However, Robertson and co-workers (2002) reclassified the biovars of *Ureaplasma urealyticum* into two distinct human associated species: *Ureaplasma parvum,* previously biovar 1 and *Ureaplasma urealyticum,* previously biovar 2. The reclassification was based on similarity data obtained from phenotypic and genotypic methods that include DNA-DNA hybridisation homology (Christiansen *et al.*, 1981), restriction endonuclease cleavage patterns (Razin *et al.*, 1983) polyacrylamide gel electrophoresis of cellular proteins (Swenson *et al.*, 1983), sequences of 16S rRNA, urease and multiple banded antigen genes (Knox *et al.*, 1998; kong *et al.*, 1999a) and genome size (Kakulphimp *et al.*, 1991). *U. parvum* has smaller genome size (0.75 - 0.77 Mb) than *U. urealyticum* (0.83 - 0.94 Mb) with the standard strains serovar 3 (strain 27) and serovar 8 (strain T960) as prototype strains for each species, respectively (Robertson *et al.*, 2002).

1.2 Description of *Ureaplasma*

Ureaplasma are among the smallest and simplest self-replicating free-living prokaryotes known to date. They have a pleomorphic shape due to the absolute absence of a cell wall that maintains structural integrity of the cellular form for other types of bacteria (Figure 1.1). Instead of the cell wall, these micro-organisms are reported to have a triple layered membrane with micropili and an electron dense outer surface surrounding the cell (Black *et al.*, 1972). As a result of the absence of a cell wall, *Ureaplasma* spp. are inherently resistant to many antimicrobial agents that interfere with cell wall synthesis, and are unstainable by Gram stain and very sensitive to dehydration (Waites *et al.*, 2005). In fact, the lack of cell wall and the minute genome sizes make *Ureaplasma* unique amongst

prokaryotes. Mollicutes, to which *Ureaplasma* belongs, are believed to be evolved via a genomic reduction that has resulted in a significant loss of genes. The small genome size has limited the biosynthetic capability of *Ureaplasma* and rendered them fastidious microbes that necessitate a complex growth media for their culture. *Ureaplasma* spp. require sterols for their growth *in vitro* provided by an essential serum supplement added to growth media (Razin *et al.*, 1998; Waites *et al.*, 2005). *Ureaplasma* are very tiny bacteria with cells of approximately 0.2 µm, this small size allows them to pass through filters usually used to sterilise solutions (Robertson *et al.*, 2002). Another unique characteristic of *Ureaplasma*, which is attributed to the genome reduction, is the lack the cell division FtsZ protein. This protein is required for forming a `Z` like ring between cells during cell division; therefore, the mechanism of their cell division is still elusive (Glass *et al.*, 2000).

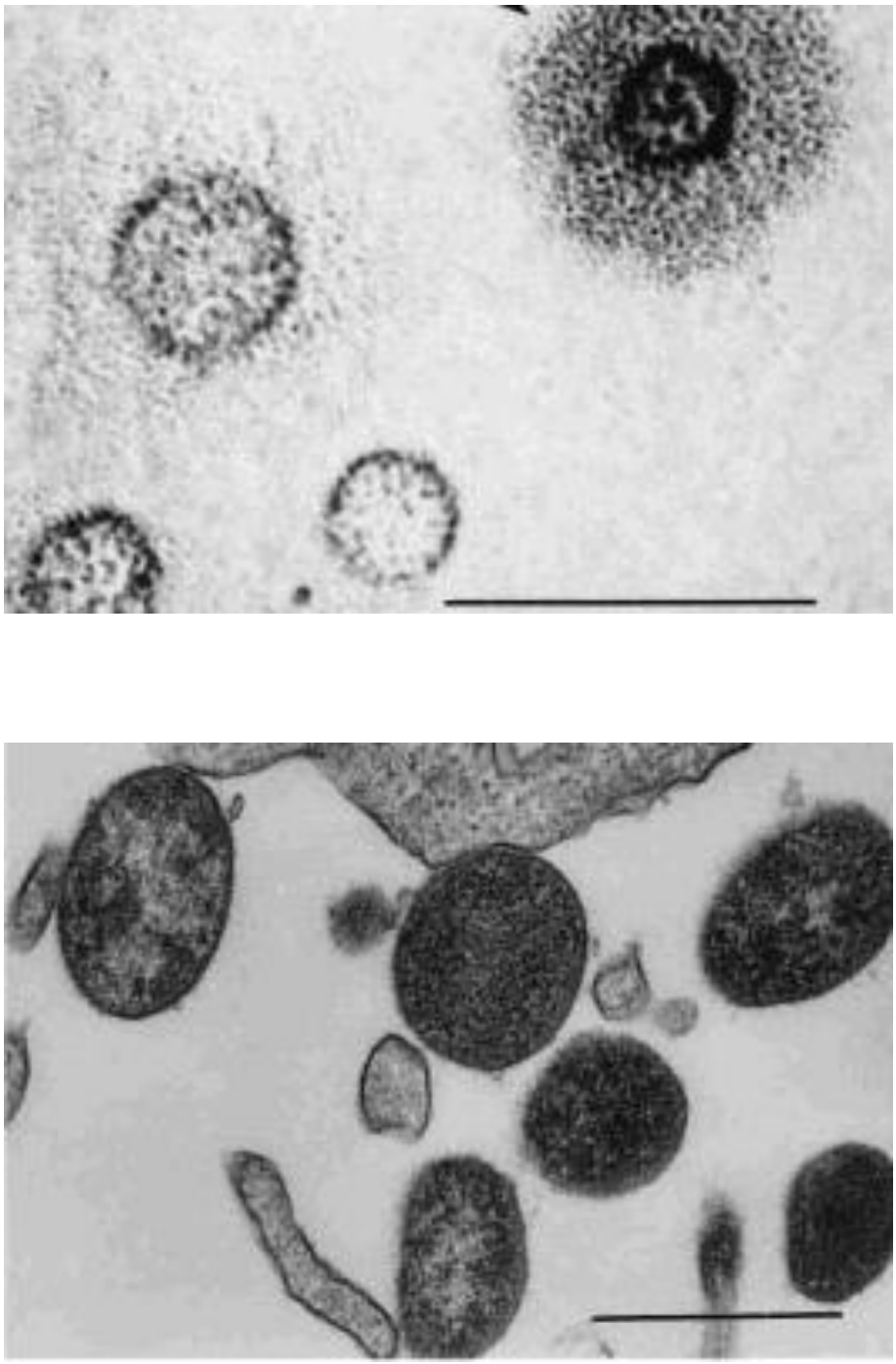

Figure 1.1 The Colony morphology of *Ureaplasma.* **(A)** Tiny colonies of *Ureaplasma* spp. (bar=100μm). **(B)** Electron micrograph shows pleomorphic small size cells of *Ureaplasma* cells (bar = 0.5μm) (Robertson *et al.*, 2002).

1.3 Genome structure and base composition

Ureaplasma spp. possess a small circular chromosome that range in size from 0.75 - 0.77 Mb for *U. parvum* and 0.83 - 0.94 Mb for *U. urealyticum*. The variability in genome sizes of mollicutes has been attributed to one of two factors; the first is the presence of repetitive elements, which are comprised of gene segments varying in size and number, and the second is variability in insertion sequence (IS) elements. In addition, it has been suggested that integration of viral sequences into the chromosome may result in differences in genome size within the same species (Razin *et al.*, 1998). The complete genome sequence of *U. parvum* serovar 3 (ATCC strain 700790) was published in 2000, which is available in the GenBank under accession no. NC_002162, (Glass *et al.*, 2000). Furthermore, the full genome sequence of *U. urealyticum* serovar 10 (ATCC 33699 strain) is also available on the GeneBank (accession no. NC_011374); the genome size of this strain is 874,478 bp. Analysis of gene content showed that that serovar 3 whole genome contained 751,719 bp with a G + C content of 25.5%. A total of 613 predicted protein-encoding genes and 39 genes encoding RNAs, comprising 93% of the genome, were identified. The low G + C genome content is a distinctive feature of the *Mollicutes*, and the distribution of the G + C in the genome is believed to be irregular (Razin, 1985). In a current comparative genome analysis study, 538 genes were identified as the minimal functional core genes for *U. parvum*. Additionally, 175 genes (35% of the genome) were indicated to be unique to *U. parvum*, while 407 genes of the 613 *U. parvum* genes also existed in the *U. urealyticum* genome (Momynaliev *et al.*, 2007).

Another striking feature of *Ureaplasma*, which is a shared property among almost all mollicutes, is the unusual codon usage. The UGA codon in all other prokaryotes functions as a terminal stop codon (opal), whereas in *Ureaplasma* spp. UGA encodes for the amino acid tryptophan (Blanchard, 1990). It has been suggested that this unusual

codon usage was a consequence of the low G + C and the high A + T genome content of mollicutes; this is a bias towards A-T base pair during their evolution. Using UGA codon as a tryptophan has resulted in deletion of the peptide chain release factor 2 (RF2) that recognises the stop codons UGA and UAA. However, the peptide chain release factor 1 that recognises UAG and UAA was conserved (Razin *et al.*, 1998). Therefore, it can be anticipated that *Ureaplasma* spp., as a member of mollicutes, uses only UAA and UAG as stop codons. The use of UGA as tryptophan codon makes the ability of expressing mollicute genes in *Escherichia coli* challenging, as *E. coli* recognises UGA as the opal stop codon. Consequently, cloned mollicute proteins expressed in *E. coli* are usually truncated. There have been attempts to overcome this problem by using *E. coli* strains with suppressed stop codon usage (Neyrolles *et al.*, 1996; Renbaum *et al.*, 1990; Smiley and Minion, 1993), but the efficiency of the expression was proven to be low.

During their genome reduction process, *Ureaplasma* spp. has lost several essential genes, which are usually present in other prokaryotes. This, in fact, has limited their biosynthetic capabilities and required them to adopt a parasitic life style relying on in their host to survive. An example of gene loss limitations is the absolute dependence of *Ureaplasma* and all other mollicutes on the salvage pathway for synthesis of DNA precursors (purine and pyrimidine nucleotides). This pathway initiates via a thymidine kinase (TK) that helps the transfer of phosphates from nucleotides, e.g. ATP, to the 5' hydroxyl group of pyrimidine deoxyribonucleoside (Carnrot *et al.*, 2003).

1.4 *Ureaplasma* virulence factors

Although *Ureaplasma spp. are* usually found as commensal bacteria with a low virulence in the urogenital tract of both healthy men and women, it can occasionally cause invasive infections under specific circumstances (Waites *et al.*, 2005). Several virulence factors of

Ureaplasma that may play a role in the pathogenesis have been proposed (Glass *et al.*, 2000; Momynaliev *et al.*, 2007). These virulence factors include the following:

1.4.1 The multiple banded antigen

The multiple banded antigen (MBA) is a major surface antigen of *Ureaplasma* that was suggested to be one of the predominant antigens recognised during infection with *Ureaplasma* (Watson *et al.*, 1990). Watson and colleagues (1990) showed that human sera collected from patients infected with *Ureaplasma* were able to bind a 71 kDa band of *Ureaplasma* antigens. Further immunoblot analysis with monoclonal antibodies against the MBA (MAbs) has detected a unique laddering pattern (multiple banded) of this surface membrane protein, therefore this antigen was annotated the multiple banded antigen (Figure 1.2).

Further investigations of *Ureaplasma* antigens showed high variations in the MBA size *in vitro*; it was also found that this antigen has both serovar-specific and cross-reactive epitopes, which indicates that both serovar-specific and cross-reactive monoclonal MBA antibodies (MAbs) can react with the MBA. Additionally, it was also demonstrated that *Ureaplasma* expressing MBA with altered size (*in vivo*) evolved from inoculating strains, and it was thought that the number of MBA variants may be associated with pathogenicity of *Ureaplasma* (Dando *et al.*, 2012; Knox *et al.*, 2010; Robinson *et al.*, 2013). The importance of this antigen in host defence was also addressed when it was shown that monoclonal antibodies (mAbs) against the MBA were able to inhibit *Ureaplasma* growth *in vitro* (Watson *et al.*, 1990; Zheng *et al.*, 1994; Zheng *et al.*, 1992).

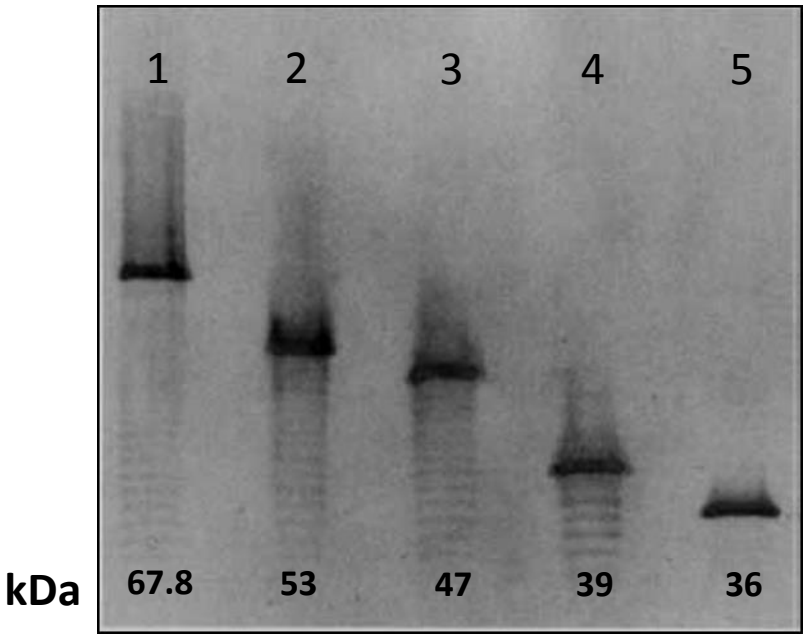

Figure 1.2 Size variation and unique laddering pattern of the multiple banded antigen. Immunoblot analysis of four clones of *U. parvum* SV3 (clones 2-4 were generated from a clinical isolate and clone 5 was clinical isolate from amniotic fluid) compared to SV3 reference strain (Lane 1). The blot shows the MBA size variation detected by mAb. Adopted from: (Zheng *et al.*, 1994).

The MBA gene (*mba*) consists of two regions: the N-terminal region (5' region) that contains a signal peptide and acylation site (a membrane lipoprotein lipid attachment site at a cysteine) and C-terminal region (3' region), which is composed of multiple tandem repeat units (consisting of 18 nucleotides encode for six amino acids QPAGKE for *U. parvum* serovar 3). The C-terminal region (the variable part) was proven to be surface exposed, hydrophobic, antigenic and contains epitopes that are serovar-specific, and also it was determined that alterations in the copy number of the tandem repeats of this region was responsible for size variations in the MBA (Zheng *et al.*, 1994; Zheng *et al.*, 1995) . The N-terminal region of the MBA gene was shown to be conserved among all 14 serovars of *Ureaplasma* (Zheng *et al.*, 1995). The 5' region of the MBA gene has been

used as a target for PCR methods of detection and speciation for *Ureaplasma* spp., because it contains species-specific nucleotide polymorphisms (Teng *et al.*, 1994).

As stated earlier, size variations in the MBA can occur *in vitro* as well as *in vivo*. In addition, the MBA, as a major surface antigen, was shown to undergo phase variation (silencing of gene expression through genomic rearrangement). This mechanism is a common strategy that has been well-documented in the closely related genus *Mycoplasma,* where several genes encoding for immunogenic membrane proteins have been found to undergo phase variation. Examples of such genes are vsp genes of *Mycoplasma bovis*, vsa genes of *Mycoplasma pulmonis* and vpma genes of *Mycoplasma agalactiae* (Bhugra and Dybvig, 1992; Bhugra *et al.*, 1995; Chopra-Dewasthaly *et al.*, 2008; Citti *et al.*, 2010; Glew *et al.*, 2000; Lysnyansky *et al.*, 2001). In general, phase variation is a well-known tactic used by most pathogenic bacteria to alter their surface-exposed antigens to avoid recognition by the host immune system and to adapt to harsh environments (Salaun *et al.*, 2003; van der Woude and Baumler, 2004). In *Ureaplasma*, phase variation has also been reported following a selection pressure with specific antibodies *in vitro*. In 2003, Monecke and colleagues showed that putting *Ureaplasma* spp. under immunological pressure *in vitro* via passaging in a medium containing rabbit polyclonal anti-MBA antibodies resulted in emerging MBA-negative variants, which showed significant variations in the MBA gene when compared with the parental strains following PCR analysis and sequencing (Monecke *et al.*, 2003). A phase variation (an ON/OFF switching) employed by *Ureaplasmas*, therefore, was suggested as a strategy for altering the expression of MBA to adapt to harsh environment and maintain survive; however the mechanism underlying that switch in expression of the MBA was not clear at that time. Another study by Zimmerman and co-workers (2009) has also shown that *Ureaplasma* is able to undergo phase switching in the MBA (locus UU375) expression

with an adjacent gene locus (UU376 gene) by inverting the MBA promoter to drive expression of the UU376. In this study, *Ureaplasma* was also subjected to immunological pressure through culturing and passaging in broth medium supplemented with antisera against either of the antigens. The authors of that study pointed out that the shift in the MBA expression to UU376 expression occurred as a result of a DNA inversion event (Figure 1.3). The genes UU375 and UU376 face in the opposite orientation (only true for strain ATCC 27815, not ATCC 700970, and the authors predict an additional inversion event to explain this difference) and inversion of the intervening DNA sequence, which holds a single uni-directional promoter, determines which of these two genes was expressed. Therefore, the resulting change in antigenicity was proposed to be as a mechanism of immune evasion used by *Ureaplasma* to a void eradication by host immune defences (Zimmerman *et al.*, 2009).

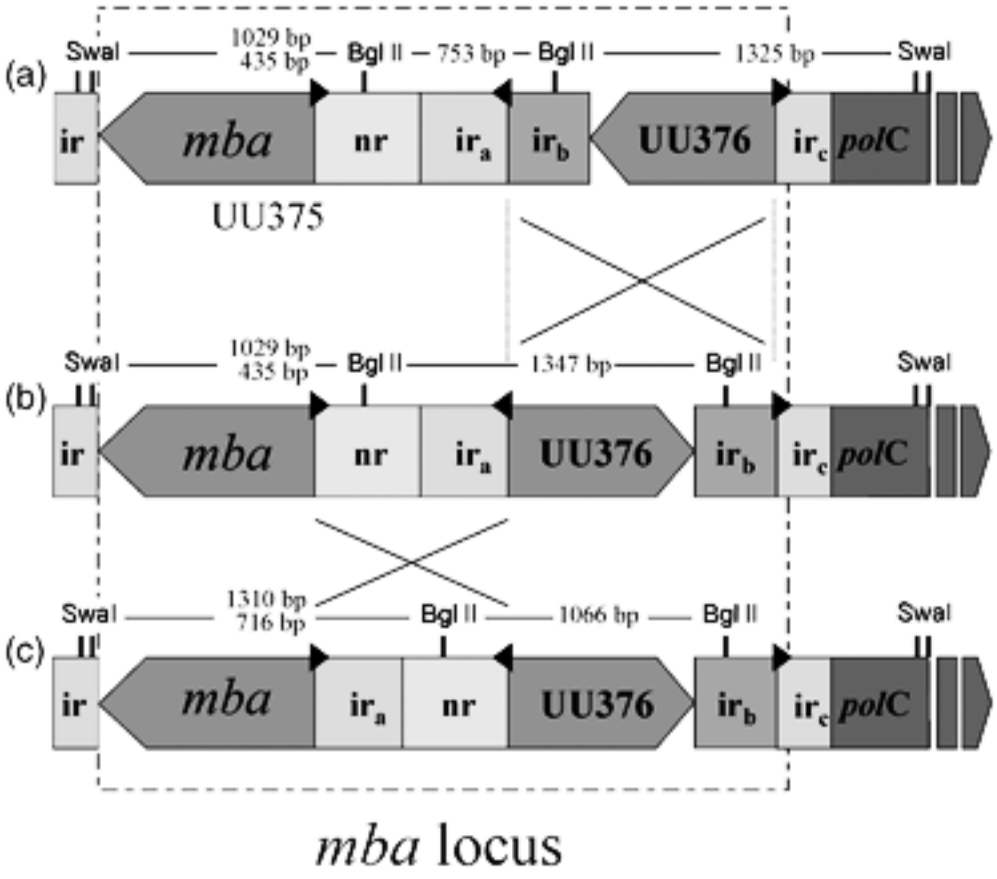

Figure 1.3 Schematic illustration of the possible DNA inversion events within the *mba* locus during phase variation based on published sequence information (a , b and c); ir (a, b and c), intergenic regions; nr, nonrepetitive region of mba. Locus configurations of (a) *U. parvum* serovar 3 strain ATCC700970, (b) *U. parvum* serovar 3 type strain ATCC27815T, and (c) *U. parvum* serovar 3 expressing UU376. The upper number above the mba gene indicates the length of the region containing 40 repeat units, and the lower, the length of a region containing 7 repeat units. Black triangles indicate repeat regions that are putative recombination sites. Reproduced from: (Zimmerman *et al.*, 2009), with permission from the publisher John Wiley and Sons.

Colonisation and establishment of an infection by a particular pathogen is somewhat attributed to its capability to attach to the mucosal surfaces of a susceptible host. *Ureaplasma spp. are* also found to adhere to different types of human cells such as spermatozoa (Busolo *et al.*, 1984; Knox *et al.*, 2003), erythrocytes (Saada *et al.*, 1991), urethral epithelial cells (Razin *et al.*, 1998) and human respiratory cells (Torres-Morquecho *et al.*, 2010). Although the receptors on host cells are believed to be sialyl residues and sulphated compounds, adhesin molecules that mediate *Ureaplasma* cytadherence have not been extensively defined. As a major surface antigen of *Ureaplasma*, MBA was proposed to be one of the possible adhesion molecules that facilitate the attachment of *Ureaplasma* cells to both erythrocytes and HeLa cells (Monecke *et al.*, 2003).

Although the MBA has been suggested as one of the virulence factors for *Ureaplasma* spp., its precise role in *Ureaplasma* pathogenesis is still elusive. There have been some attempts to understand the role played by MBA in intra-amniotic infection and chorioamnionitis caused by *Ureaplasma* in pregnant sheep (Dando *et al.*, 2012; Knox *et al.*, 2010). Knox and colleagues (2010), for example, demonstrated size variations of the tandem repeat region of the MBA gene; and suggested that these variations in MBA expression *in vivo* could be a mechanism by which *Ureaplasma* escape recognition by host immune arsenals to mediate disease (Knox *et al.*, 2010).

1.4.2 Urease activity

Ureases play an important role in the pathogenesis of several microorganisms via their enzyme activity. For example, urease activity is recognised as an important virulence factor for *Proteus mirabilis*, which causes urinary tract infections and *Helicobacter pylori* that causes gastritis and stomach cancer in human (Mobley *et al.*, 1995; Olivera-Severo *et*

al., 2006). *Ureaplasma* spp. produces an abundant amount of urease that is used to hydrolyse urea to ammonia and release ATP, as a sole source of energy, the hydrolysis resection is as following: $CO(NH_2)_2 + H_2O \rightarrow 2\ NH_3 + CO_2$. Urease activity is also proposed as a significant virulence factor for these species (Mobley *et al.*, 1995). Colonisation of the upper urinary tract leading to the formation of infective kidney stones by *Ureaplasma* is believed to be mediated by urease production (Hedelin *et al.*, 1984; Reyes *et al.*, 2006). Hydrolysis of urea via urease causes increased pH and ammonia toxicity. It was found that injecting viable *Ureaplasma* into mice led to death of these animals within 5 minutes; this outcome was prevented in another group of mice that were injected with bacteria pre-treated with a potent urease inhibitor (flurofamide). This indicated that urease activity and toxicity of ammonia was the inevitable cause of mortality in these animals. However the researchers argued that the bacterial dose used in their study was high and may not reflect the conditions in a naturally-occurring infection (Ligon and Kenny, 1991).

1.4.3 Phospholipases

Phospholipases (PLs) have been indicated as virulence factors in many bacterial pathogens (Songer, 1997). In *Ureaplasma*, they have also been proposed as an important virulence factor (De Silva and Quinn, 1991). Phospholipases have both phosphodiesterase and acyl hydrolase activity and are able to hydrolyze one or more ester bonds in phospholipids (Istivan and Coloe, 2006). It has been suggested that phospholipases stimulate premature labor, which is often associated with *Ureaplasma* infection, via their action to hydrolyze phospholipids in the placental membrane. This process, consequentially, leads to release of arachidonic acid and formation of elevated levels of prostaglandins, which can trigger preterm birth (Bennett *et al.*, 1987). Three types of PLs have been identified and localized in *Ureaplasma* spp., PLA1, PLA2 and

PLC. It has been found that the activity of these enzymes may vary among *Ureaplasma* serovars; for instance, it was demonstrated that the activity of PLA2 was 2-fold higher in serovar 8 membranes, whereas PLA1 was 2-fold higher in serovars 3 membranes (De Silva and Quinn, 1991). Furthermore, these authors found the activity of PLA2 in serovar 8 to be 3-fold greater than that of SV3 and SV4. In spite of studying the activity of these enzymes, their putative orthologous sequences have not been identified in the fully sequenced genome of serovar 3 (Glass *et al.*, 2000). This may be due to a sequence difference compared with that of other bacteria. Both PLA1 and PLA2 have been shown to have significant variations in their specific characteristics, indicating that they are unique enzymes for *Ureaplasma* relative to phospholipases from other prokaryotes (De Silva and Quinn, 1999). Ureaplasmal PLA2 activity has also been suggested to inhibit pulmonary surfactant production and cause lung damage (Cultrera *et al.*, 2006). This assumption is based on a study by Schrama and colleagues (2001) who demonstrated that PLA2 activity in meconium inhibits pulmonary surfactant *in vitro* (Romero *et al.*, 2013; Schrama *et al.*, 2001).

1.4.4 Immunoglobulin A (IgA) protease

IgA plays an important role in immune defence at mucosal membranes, as it is the most abundant immunoglobulin at these sites (Woof and Russell, 2011). However, many Gram-negative pathogenic bacteria, such as *Neisseria gonorrhoeae*, are capable of producing extracellular IgA proteases as a virulence factor that inhibit IgA function and allow them to colonise and invade host surfaces membranes (Klauser *et al.*, 1993; Mistry and Stockley, 2006; Pohlner *et al.*, 1987). Similarly, *Ureaplasma* spp. has also been found to produce IgA1 proteases, which are serine proteases (Kilian *et al.*, 1984; Robertson *et al.*, 1984; Spooner *et al.*, 1992). Human *Ureaplasma* proteases were found to degrade human IgA1, but not IgA2, and were host specific as they could not cleave

murine, porcine, or canine IgA (Kapatais-Zoumbos *et al.*, 1985). The inability of ureaplasmal IgA proteases to cleave IgA2 was suggested to be due to the omission of the specific region at which IgA is cleaved. Human IgA1 was found to be cleaved between proline and threonine residues located at positions 235 and 236, respectively (Spooner *et al.*, 1992).

1.4.5 Haemolysins

Haemolysins are extracellular toxins produced by a number of Gram-positive and Gram-negative bacteria (e.g. *Staphylococci aureus* and *E. coli*) and play an important role as virulence factors (Bhakdi *et al.*, 1988; Wiseman, 1975). In *Ureaplasma*, two haemolysins encoded by two genes *hlyA* and *hlyC* in the genome of *U. parvum* serovar 3 have been repored and shown to have an enzymatic activity (Glass *et al.*, 2000). Of these two haemolysin genes, *hlyA* has been suggested as the main haemolysin that may serve as a virulence factor for *Ureaplasma* spp. Such implication was made because this hemolysin has an orthologue with hemolytic and cytotoxic activity in some pathogenic bacteria, while *hlyC* gene is orthologous to *M. pneumonia* hemolysin which has its activity mediated by H_2O_2 (Glass *et al.*, 2000).

1.4.6 Pathogenicity islands (PAI)

Pathogenicity islands (PAIs) are distinctive genetic elements found on the genomes of many bacterial pathogens and are absent from non-pathogenic strains of the same or closely related species. PAIs are believed to be acquired by horizontal gene transfer via transduction, conjugation and transformation. These genetic elements contain genes that encode numerous virulence factors (Schmidt and Hensel, 2004). A pathogenicity island of *Ureaplasma* was also proposed and characterised by Momynaliev and co-workers (2007), when they identified 4 regions of hypervariable plasticity on the chromosome of

U. parvum clinical isolates. Of these regions, the region containing genes annotated as UU145-UU170 in the genome of serovar 3 (ATCC 700970 strain) was proposed to be a pathogenicity island because of its genetic features: this region was not found in all investigated clinical isolates, it has a conserved length in the genomes that was found in, and its insertion site into the genome was directly linked to tRNA genes, which are well known as anchorage points for horizontally transferred genetic elements (Momynaliev *et al.*, 2007). Supporting evidence for this includes the existence of genes encoding proteins essential for horizontal gene transfer such as the ripX integrase-recombinase enzyme and phage recombinase enzyme. Studying genes within the proposed PAI showed adhesin gene homologues that could be of importance to virulence. PAI normally have a skewed G + C content relative to the rest of the host genome, but in this case the G + C contents were very similar. Further comparative genomic analysis showed that tetranucleotides GCGC and CGCG were not present within the element, but abundant among other regions of the chromosome of *Ureaplasma.* The authors concluded that an association between this hypothetical PAI and the clinical outcome of *U. parvum* could happen (Momynaliev *et al.*, 2007) , but no direct experimental evidence was provided.

In a current study, similar insertion regions to that mentioned above were also identified through a comparative genome analysis of 19 *U. parvum* and *U. urealyticum* strains (Paralanov *et al.*, 2012). Two gene insertion events were identified: the first one was as a result of a transposon insertion; and the DNA segment inserted contained 8 genes, 6 encode hypothetical proteins, one hypothetical protein containing a subtilase domain, and one Type I specificity subunit of a restriction enzyme-like protein. The second insertion event is believed to be due to a phage insertion into the chromosome, as the insert has three phage genes, The first gene encodes an integrase-recombinase protein that contains a phage integrase domain (UPA3_0153 [GenBank: YP_001752228]). A phage

recombination protein Bet (UPA3_0162 [GenBank: YP_001752237] is located further downstream of the integrase and the final gene in the insert is a phage terminase large subunit of the pbsx family (UPA3_0176 [GenBank: YP_001752251]. The fact that these insertions were found in some isolates of the same serovar and not in other isolates of the same serovar may indicate horizontal gene transfer of a group of foreign genes or a 'pathogenicity island'.

1.5 Role of *Ureaplasma* in human diseases

In spite of being common commensals in genitourinary tract of healthy individuals, *Ureaplasma* spp. are considered opportunistic human pathogens. In the early 1950s, these species were first linked to human diseases when isolated from urethral discharges from men with primary and recurrent non-gonococcal urethritis (NGC) (Shepard, 1954; Taylor-Robinson *et al.*, 1977). A few years later, an association between *Ureaplasma* and both adverse pregnancy outcomes and low birth weight in neonates was established (Cassell, 1986; Cassell *et al.*, 1983). Currently, there is accumulating evidence that indicates the implication of human *Ureaplasma* in a broad range of medical complications in adult men and women as well as neonates. In adults, *Ureaplasma* have been associated with several diseases (genitourinary tract and non-genital infections) such as infertility, prostatitis, epididymitis, pyelonephritis, cystitis, suppurative arthritis, subcutaneous abscesses, osteomyelitis, urinary calculi, postpartum endometritis, chorioamnionitis, spontaneous abortion, premature birth, and stillbirth. Furthermore, it has been shown that *Ureaplasma spp. are* associated with diseases in premature babies such chronic lung disease of prematurity (CLD), pneumonia and meningitis (Viscardi, 2010; Viscardi and Hasday, 2009; Waites *et al.*, 2005; Waites *et al.*, 2009).

It has been estimated that 40% - 80% of healthy adult women are asymptomatically colonised by *Ureaplasma* spp. in their cervix or vagina. Transmission of *Ureaplasma* infection occurs via venereal as well as vertical modes. Colonised mothers are more likely to transfer the infection to their babies through vertical transmission either *in utero* or during delivery, with an infection rate reaching 90% (Waites *et al.*, 2005). *Ureaplasma spp. are* a less common coloniser of the lower urogenital tract of men and the colonisation rate was estimated to be about 20% -29% (Kong *et al.*, 2000a; Xiao *et al.*, 2010) *U. parvum* is more commonly detected than *U. urealyticum* in samples from urogenital tract of adults or respiratory tract of neonates (Waites *et al.*, 2005); however, no significant difference in pathogenicity between the two species has been observed (Heggie *et al.*, 1994; Katz *et al.*, 2005; Sung *et al.*, 2011). In contrast, it was noticed that premature babies colonised by *U. urealyticum* have a higher rate of broncho pulmonary dysplasia (BPD) compared with those colonised by *U. parvum* (Abele-Horn *et al.*, 1997). Despite of all these considerable efforts to define which one of the two human *Ureaplasma* species is more virulent in several human infections, no consensus about this issue has been reached so far and investigations continue. It has been speculated that there may be variation in pathogenicity among the 14 serovars, indicating that differential virulence is more likely to be related to the serovars and even different isolates from the same serovar, rather than to species. Moreover, a current study has highlighted the issue of horizontal gene transfer among *Ureaplasma* clinical isolates, generating genetic hybrid forms from different serovars that cannot be assigned to any of the 14 known serovars using genetic methods (Xiao *et al.*, 2011b). This phenomenon may also a play a role in variations in pathogenicity among *Ureaplasma* as some virulence genes could be acquired this way.

Interaction between *Ureaplasma* and the host immune system and the resultant inflammatory response has also been indicated to be an important contributor in ureaplasmal diseases, especially BPD in preterm babies. *Ureaplasma* spp. has been demonstrated to be recognised by the innate immune system via Toll-like receptors (TLRs) (Shimizu *et al.*, 2008; Triantafilou *et al.*, 2013). In the study by Shimizu and colleagues (2008), it was reported that *U. parvum* serovar 3 lipoproteins were able to initiate activation of nuclear factor-kappa B (NF-κB) *in vitro* through TLR1, TLR2, and TLR6 signalling. Triantafilou *et. al.*, (2013) have also revealed the involvement of TLR2, TLR6 and TLR9 in the innate immune response against *Ureaplasma* serovars, and have shown activation with release of inflammatory cytokines tumour necrosis factor-α (TNF-α), interleukin1β (IL-1β), IL-6 and IL-8 in human amniotic epithelial cells by *Ureaplasma*. A relationship between the activation of inflammatory cytokines (e.g. TNF-α, IL1β, IL-8) triggered by *Ureaplasma* infection and the development of BPD in preterm babies has been reported by a number of studies of preterm babies (Viscardi, 2010; Viscardi and Hasday, 2009; Waites *et al.*, 2005).

1.6 The complement system overview

In 1894, the complement system was first discovered by Jules Bordet as a heat-sensitive component in human serum that has anti-bacterial activity. The name complement was given because it was believed that this system has the ability to complement antibodies in opsonizing and killing bacteria. Although it was seen only as an effector support for the antibodies at that time, complement is now known to be able to activate in the absence of antibodies during early stages of infection via other pathways (Janeway *et al.*, 2001). The complement system is an integral arm of the innate immune defenses against invading pathogens. Currently, the complement system is composed of more than 35 proteins and

glycoproteins circulating in plasma and expressed on human cell surfaces (Carroll and Sim, 2011). In humans, most of the complement components are produced in the liver (about 90%), the most notable exception is a critical component of the terminal pathway (C7) which is produced primarily in granulocytes. Although a normal range of circulating complement proteins is produced by hepatocytes, the majority of complement components are also acute phase reactants and synthesis can be induced locally in other cell types such as fibroblasts, endothelia, astrocytes during infection by primary acute cytokines such as IL-6, IL-1β and TNF-α (Carroll, 2004; Marsh *et al.*, 2001; Wurzner *et al.*, 1994). In general, complement proteins function as enzymes, enzyme inhibitors, or enzyme cofactors, many of them circulate in plasma and body fluids in inactive forms (zymogenes), expressing their activity only after activation of the complement system. Besides, some of these proteins are bound to cell membranes and act as regulators of complement activation protecting host self-cells and tissues (Janeway *et al.*, 2001).

The complement system is activated via three different activation pathways in response to different initiators, the classical pathway, the alternative pathway, and the lectin pathway. Each single pathway depends on specific molecules (components) for its initiation. However, all these pathways converge at the hydrolysis of a central C3 component by their respective pathway C3 convertases to generate the same set of effector molecules (Figure 1.4). C3 convertases, in the absence of regulation, proceed to cleave enzymatically intact C3 components. C3 is cleaved to C3a that is released and C3b, which binds covalently to the activating surface. If the activation continues, proteins of terminal pathway generate the membrane attack complex (MAC) that forms pores and in sufficient numbers, directly lyses the target cells. Surface bound C3b is an opsonin that interacts with phagocyte receptors and mediate killing of invading microbes via phagocytosis. Furthermore, the complement system also participates in generation of

inflammatory response fragments, called chemotaxins and anaphylotoxins (Walport, 2001a).

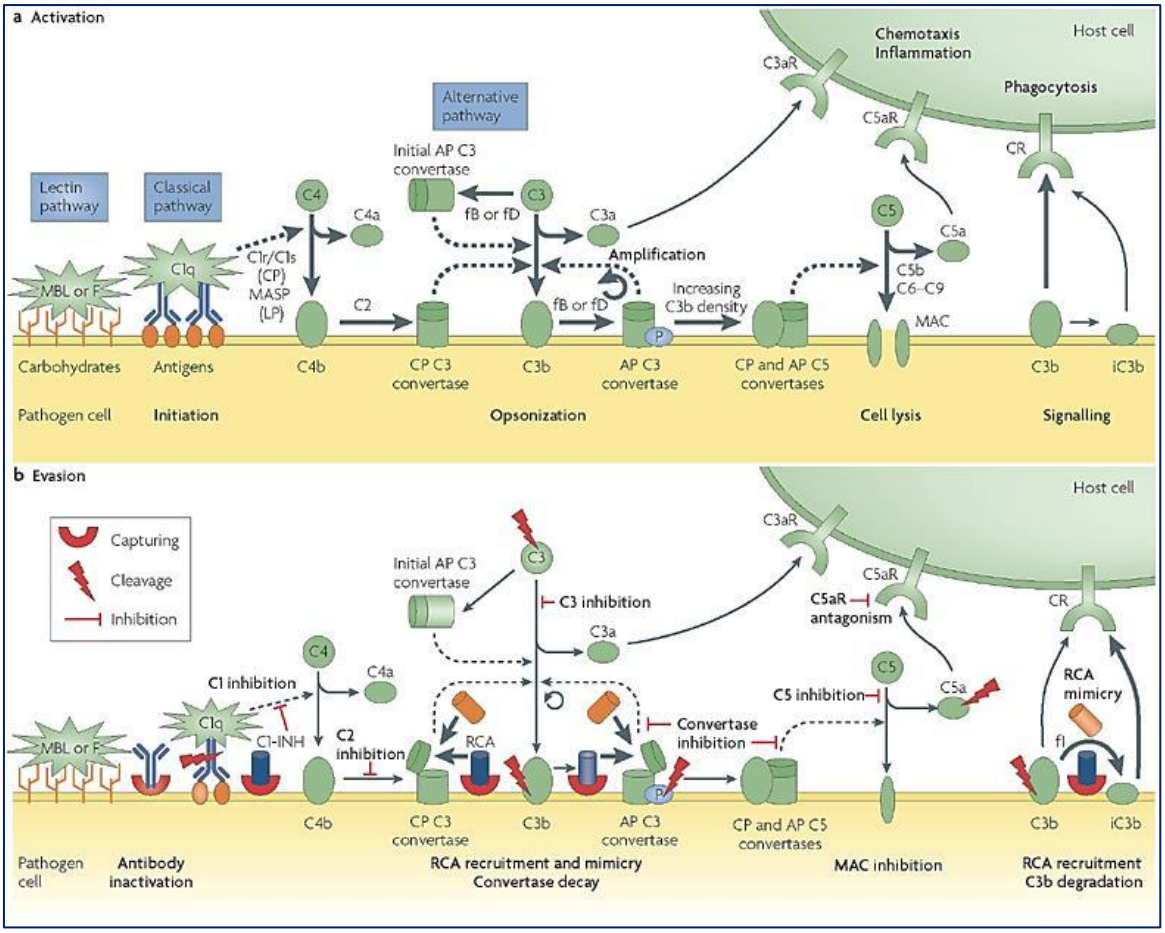

Figure 1.4 Activation and evasion of complement. **a)** Overview the human complement system activation cascade. The initial steps of the complement system activation occur via three different pathways (the classical, lectin and alternative pathways), which are triggered differently by their specific molecules (components) binding to an activating surfaces such as invading microbes. However, they all converge at the hydrolysis of a central C3 component by their respective pathway C3 convertases to generate the same set of complement effector molecules, which play role in opsonization, recruitment of host effector cells and direct killing of invading pathogens. **b)** Microorganisms have developed many ways to evade complement actions. Suppression of CP activation can be achieved by trapping endogenous C1 inhibitor (C1-INH) to the surface or by inactivating antibodies through the capture of their Fc regions. Whereas the recruitment of soluble regulators by capturing host proteins is a common strategy to impair downstream complement actions, certain viruses also produce structural mimics of these regulators. In addition, some microbial proteins have similar activities to CD59 in preventing MAC formation. Direct inhibition of C3, the C3 and C5 convertases, C5 or the C5a receptor (C5aR) is a prominent strategy of *Staphylococcus aureus*. Finally, a set of different microbial proteases can degrade many of the crucial components of the complement system. These proteases act directly or by capturing and activating a human protease. Reproduced from: (Lambris *et al.*, 2008), with permission from the publisher Nature publishing group.

1.6.1 The classical pathway

The classical pathway was the first complement activation pathway to be discovered. The first component of this pathway is the C1-complex, which consists of one molecule of the hexameric glycoprotein C1q and two molecules of each of the serine protease proenzymes C1r and C1s (C1qr2s2) and depends on Ca^{++} for its assembly (Busby and Ingham, 1990). C1q plays an integral role as it bridges the gap between the innate and the adaptive immune response by binding antibodies (Fc-regions of IgG or IgM) attached to antigens such as bacteria, viruses or cell debris (Walport, 2001a). The C1q component is also able to bind directly to the surface of some pathogens such as lipopolysaccharides (LPS), nucleic acids, immune complexes, some viruses and C-reactive protein (CPS) in the absence of antibodies and activate the classical pathway of the complement system (Kishore and Reid, 2000; Loos and Clas, 1987; McGrath *et al.*, 2006). C1q belongs to a protein family called the collectins, and requires calcium for achieving maximum binding (Roumenina *et al.*, 2005). A single molecule of C1q component consists of 3 combined polypeptide chains, which in turn combine in a complex of 6 collagen-like triple helical arms each with a globular head domain. Each globular head domain is a binding site for C1q that binds to Fc region of aggregated or antigen-bound IgG (IgG1 or IgG3) or IgM antibodies found on a target surface such as invading microorganisms (Kishore and Reid, 2000). Binding of C1q to antibodies needs at least two molecules of IgG attached close enough to each other on a target surface to engage 2 globular heads of C1q; this means that a relatively high surface density of IgG is required for the activation of the classical pathway. In contrast, only one pentameric molecule of IgM bound to a target antigen can activate the classical pathway, as each molecule has five separate domains that could work as C1q binding sites; Free IgM (not bound to antigen) cannot bind to C1q and initiate complement activation in the circulation because it is in the form of a planar

molecule, but once bound to an immune complex, a conformational change in IgM occurs (from a planar to bent form) allowing tight binding to C1q(Schumaker *et al.*, 1987).

Binding of C1q causes a conformational change in the C1-complex that leads to stimulation of an autocatalytic enzyme activity in C1r, the active form of C1r, which in turn, cleaves and activates C1s. The active form of C1s is an active serine protease that functions on two zymogens (substrates): the components C4 and C2. The component C4 is activated upon enzymatic cleavage by C1s into a small fragment C4a and large fragment C4b. C4b fragment binds covalently to the surface target in the area of C1, while C4a fragment, which is a soluble weak anaphylatoxin (Gorski *et al.*, 1979), is released. C1s also cleaves C2 into C2a and C2b fragments; C2b is released and C2a binds to C4b, which is bound to the activating surface, in the presence of Mg^{++} to generate the C4b2a complex (Xu *et al.*, 2001). The resulting C4b2a complex is called the classical C3 convertase that cleaves C3 into C3a that is released and C3b, which is an opsonin that covalently binds to activating surfaces such as microbial surfaces. C3a is anaphylatoxins and chemoattractants that attracts and activates immune effector cells (basophils, mast cells, neutrophils and macrophages), and has also been reported to act directly as an antimicrobial and antifungal agent (Nordahl *et al.*, 2004).

1.6.2 The lectin pathway

The lectin pathway was the latest defined complement activation pathway, as it was discovered in the 1980's (Ikeda *et al.*, 1987). The lectin pathway is initiated upon binding of soluble pattern recognition molecules (PRM) (types of lectins): mannan binding lectin and ficolins (M-ficolins, L-ficolins and H-ficolins) that are structurally similar to C1q, to certain carbohydrate structures characteristic of non-self surfaces, e.g. microbial sugars (Holmskov *et al.*, 2003; Runza *et al.*, 2008). MBL and ficolins activate

the lectin pathway in association with serine proteases called MBL-associated serine proteases (MASPs): MASP-1, MASP-2, MASP-3 and a MBL-associated protein 19 (MAp19), which are functionally similar to the proteases of the classical pathway C1 complex (C1r and C1s)(Schwaeble *et al.*, 2002; Thiel, 2007). Following binding of MBL/MASP-2 complex to microbial surface through recognition of prokaryotic carbohydrate motifs such as mannose, MASP subsequently cleaves the complement components C4 (to C4a and C4b) and C2 (to C2a and C2b) resulting in the formation of the C3 convertase, the C4bC2a complex, as above. The C4bC2a activates C3 by cleavage into C3a and C3b. C3a is an anaphylatoxin released away and C3b an opsonin binds to activating surfaces (e.g. microbes) (Turner, 1996; Sorensen et al., 2005).

1.6.3 The alternative pathway

Unlike the classical and lectin pathways, the alternative pathway is independent of activation by antigen-antibody complexes or pattern recognition. The activation of the alternative pathway requires four serum proteins: C3, factor B, factor D and properdin and uses its own C3 and C5 convertases. The initiation of alternative pathway activation occurs via slow spontaneous hydrolysis of an unstable internal thioester bond in C3 to generate C3(H_2O) that is a C3b-like molecule; this mode of initiating the activation of alternative pathway is also called 'tick-over'. In the presence of Mg^{2+}, C3(H_2O) molecule binds to factor B, which is a single chain plasma glycoprotein structurally homologous to C2 (Bentley, 1986). Binding C3(H_2O) to factor B makes it unstable and exposes a site on factor B that serves as a substrate for factor D, which subsequently cleaves factor B into Ba and Bb fragments. Ba molecule is a smaller fragment that is released, while Bb fragment remains bound to C3(H_2O) and forms C3(H_2O)Bb complex, which is an unstable fluid-phase C3 convertase that, at this stage, still unbound in plasma. The free

C3 convertase promptly cleaves a few C3 molecules into C3b and C3a fragments, which are rapidly degraded in the absence of binding to activating surfaces of foreign cells or are quickly inactivated by regulators on the surface of host cells. However, C3b molecules, in presence of an infection, covalently bind to microbial surfaces via their thioester linkages and remain active for a longer time (Le Friec and Kemper, 2009).

Surface-bound C3b, in the presence of Mg^{2+}, binds factor B, which is in turn cleaved by factor D to form the C3bBb complex or the alternative C3 convertase, which is similar to C4b2a complex of the classical pathway. C3 convertase activity of C3bBb complex is unstable at this point and needs to bind to properdin (a plasma protein also called factor P) to form stabilized C3bBbP complex or the active alternative pathway C3 convertase. Subsequently the surface-bound C3 convertases of alternative pathway cleave more C3 and generate enormous aggregates of C3b molecules on microbial surfaces, which in turn, bind to factor B enabling its cleavage and activation by factor D and causing an intense spontaneous amplification. Properdin has also been reported to recognize and bind to surfaces of certain pathogens such as *E. coli*, *Neisseria* and yeasts and facilitates complement activation; therefore, it has been suggested to function as a pattern recognition receptor (PRR) to aid the initiation of the alternative pathway activation (Hourcade, 2006; Kemper and Hourcade, 2008; Le Friec and Kemper, 2009; Spitzer *et al.*, 2007). The active surface-bound C3b fragment, which is required to initiate the alternative pathway cascade, can also be generated from the other cascades (the classical and lectin pathways), providing an amplification feed-back loop mechanism linking the all complement activation pathways. It is important to note that surface bound C3b on host cells is rapidly inactivated by the regulatory enzyme factor I (FI) into iC3b in the presence of host co-factors membrane cofactor protein (MCP,CD46) or factor H (FH). The iC3b is unable to bind to factor B and cannot form the C3 convertase. Therefore, the

alternative pathway on its own is only really effective for surfaces that lack the ability to regulate C3b (Zipfel and Skerka, 2009). The C3 convertase activity of C3bBb generates the C3bBb3b complex, or the alternative C5 convertase (equivalent to the classical and lectin pathways C5 convertases, C4b2a3b complex), which then cleaves C5 into C5a and C5b (Kinoshita *et al.*, 1988). C5a is another potent anaphylatoxin diffuses way to the nearby environment, while C5b associates non-covalently to the activating surface and then recruits binding of C6 to initiate the common terminal pathway that leads to formation of the terminal complement complex (TCC), also known as the membrane attack complex (MAC), which form pores in the targets surface membrane, such as microbes and causes cell lysis (Pangburn and Rawal, 2002).

1.6.4 The terminal pathway

The final result of activation of all three complement pathways is the formation of C5 convertase (composed of C4b2a3b complex for both the classical and lectin pathways and C3BbC3b complex for the alternative pathway) and initiation of the terminal pathway. C5 is the first component in the terminal pathway, which is cleaved by C5 convertases into C5a and C5b as mentioned above. Active C5b molecule has affinity for binding sequentially and non-covalently to C6, C7, C8 and C9. Binding of activated C5b to C6 has to happen rapidly, because the C5b molecule is unstable and prone to conformational change that could inactivate it and prevent C6 binding. But once bound to C6, C5b molecule becomes stable (DiScipio *et al.*, 1999). Sequentially, the formed complex C5b6 binds to C7 to form a hydrophobic complex (C5b67) that undergoes conformational change, which results in exposure of hydrophobic binding regions, and attaches itself to the surface membrane of the target pathogen. These hydrophobic binding regions in the complex are believed to facilitate the insertion of the C5b67 complex into the

phospholipid bilayer following attachment to microbial membranes (Muller-Eberhard, 1986). If the location of that reaction was on an immune complex or other non-cellular activating surfaces, the hydrophobic binding sites cannot attach to the immune complexes and then the C5b67 complex will be released. Free C5b67 complexes can bind to and deposit on the membranes of nearby host cells resulting in cell lysis, however, such lysis is controlled by the complement regulator CD59 in normal situations (Podack *et al.*, 1979). Binding C8 to C7 in the C5b67 complex causes a conformational change in C8 that exposes its hydrophobic domain, allowing the insertion into the interior of phospholipid membranes. In absence of C9, the C5b678 complex is capable of forming a small pore that can induce lysis of red blood cells and some nucleated cells (Gee *et al.*, 1980; Sodetz, 1989). The last step of in the terminal pathway and formation of the MAC is the binding and polymerization of 12- 18 molecules of C9 to the C5b678 complex (Muller-Eberhard, 1986). After binding to the complex, C9 also undergoes conformational changes that allow its insertion into the membrane. Composed of C5b678 complex surrounded by a poly-C9 complex (C5b6-9 complex), the final complete MAC complex is now able to insert into and form large circular pore in the target cell membranes resulting in an osmotic cell lysis (Bhakdi *et al.*, 1978).

1.6.5 The regulation of complement activation

The main function of complement system as an arm of the innate immunity is to aid the elimination of invading pathogens and the clearance of modified self-cells such as apoptotic cells. However, uncontrolled amplified complement activation can lead to several adverse effects on host self-cells. Therefore, the complement activation, under normal conditions, is firmly controlled and regulated to prevent any potential destruction

of self-cells and tissues. The complement activation cascades are regulated by a number of membrane-bound and circulating proteins called regulators of complement activation (RCA) (Zipfel and Skerka, 2009). These regulators act at all stages of complement activation and can be divided into two distinct groups: Fluid phase regulators and surface-bound regulators.

1.6.5.1 Fluid phase complement regulators

Fluid phase regulators are soluble proteins present and circulating in plasma and body fluids (e.g. the synovial and vitreous). These regulators include the following:

1.6.5.1.1 C1 inhibitor (C1INH)

C1 inhibitor (C1INH) is one of the soluble regulators that control the activation of both the classical and the lectin pathways (Davis *et al.*, 2008). C1INH is a member of proteins called serine protease inhibitors, and it regulates the classical pathway by either binding the active site of serine proteases C1r and C1s and dissociating them from the C1q-complex or via blocking the antibody-independent C1q activation, thus preventing the subsequent activation of C4 or C2 (Ziccardi, 1982; Ziccardi and Cooper, 1979). C1INH is the only protease inhibitor that controls both the classical and lectin pathways (Davis *et al.*, 2008). C1INH is also regulates the lectin pathway by inhibiting the proteolytic activities of both MASP-1 and MASP-2 by binding to them and forming a stable equimolar complexes (Kerr *et al.*, 2008; Matsushita *et al.*, 2000). Moreover, it can regulate the alternative pathway via inhibiting C3b and preventing the binding of factor B (Jiang *et al.*, 2001)

Inherited or acquired C1INH deficiency in humans can cause a lethal disease known as hereditary angioedema (HAE), which is characterized by edema in the skin and the

mucus membranes of both the gastrointestinal tract and the upper respiratory tract (Carugati *et al.*, 2001).

1.6.5.1.2 C4 binding protein (C4BP)

C4 binding protein (C4BP) is a major fluid phase regulator that controls the activation of both the classical and the lectin pathways. It is a glycoprotein composed of 7 identical long α-chains (75 kDa) and one short β-chain (40kDa) linked together by disulphide bonds. Structurally, the chains of C4BP consist of short consensus repeats (SCRs); each α-chain has eight SCRs, while the β-chain has only three SCRs (Blom *et al.*, 2004a, b). C4BP regulates the classical and lectin pathways by binding the C4b molecule and preventing the assembly of the C3 convertase C4b2a (Fujita and Nussenzweig, 1979). C4BP has the capacity to act as a cofactor to FI in cleaving the membrane-bound C4b into two inactive fragments (C4c and C4d), C4c diffuses away and C4d fragment remains bound to the target surface, hence blocking the formation as well as the reassembly of the C4b2a complex (Fujita and Tamura, 1983; Seya *et al.*, 1995). It can also prevent the formation of the classical and lectin C3 convertases by binding nascent C4b fragments (Gigli *et al.*, 1979). Additionally, C4BP can accelerate the decay of the C4b2a complex by separating C2a from C4b2a complex and preventing the re-assembly again (Gigli *et al.*, 1979). It is also believed that C4BP could act as cofactor to FI in mediating the degradation of soluble C3b, thus additionally accelerating the decay of the alternative C3bBb complex (Blom *et al.*, 2003).

Beyond complement regulation, C4BP has also additional activities such as binding to the vitamin K-dependent regulator protein S, which is one of the regulators of the coagulation system. While such binding does not interfere with regulatory action of C4BP, as it is the β-chain that is responsible for this activity, it affects the cofactor role of protein S for

anticoagulant activated protein C (APC) activity (Dahlback and Hildebrand, 1983; van de Poel *et al.*, 1999).

1.6.5.1.3 Factor H (FH)

While C4BP is a major regulator of the classical pathway, factor H (FH) is considered to be a major fluid phase regulator for the alternative pathway. FH is an abundant plasma glycoprotein of approximately 155 kDa in size (previously described as β1H-globulin) that is composed of 20 SCRs, which function as binding domains for FI, C3b and host cell surfaces (Nilsson and Mueller-Eberhard, 1965; Whaley and Ruddy, 1976). FH controls the activation of the complement cascade at the C3 level and is able to inactivate both soluble C3b and membrane-bound C3b. Binding and regulating the membrane-attached C3b by FH is influenced by several membrane structural components of the host cell surfaces such as sialic acids, polyanionic substances (e.g. glycosaminoglycans) or sulphated polysaccharides (e.g. heparin) (Fearon, 1978). The FH regulatory action occurs via three ways: i) it acts as a main cofactor for the serine protease FI in the degradation of C3b into inactive form, iC3b; thus preventing the formation of the alternative C3 convertase (C3bBb), ii) it prevents the assembly of the C3bBb complex by binding C3b and blocking the attachment of factor B, and iii) it functions as a decay accelerating factor for C3bBb complex by dissociating Bb from the C3bBb complex (Pangburn *et al.*, 1977; Weiler *et al.*, 1976; Whaley and Ruddy, 1976). In the absence of FH, a spontaneous, amplified activation of complement occurs, resulting in consumption of the C3 and factor B complement components (Schreiber *et al.*, 1978).

Beyond the control of the complement activation, FH is also a multifunctional protein that has further activities such as mediating cell attachment and acting as an adhesin (DiScipio *et al.*, 1998; Nabil *et al.*, 1997). It has also been shown that FH is able to interact with and bind to heparin, adrenomedullin (AM) and a 52-amino acid peptide

belonging to the calcitonin gene peptide superfamily, and it is anticipated that this interaction modulates the biological activities of these proteins (Pangburn *et al.*, 1991; Pio *et al.*, 2001).

FH deficiency or dysfunction are associated with several diseases in humans such atypical haemolytic uremic syndrome (aHUS), membranoproliferative glomerulonephritis type II (MPGN-II) and recurrent microbial infections and chronic inflammation and immune evasion of tumour cells (Zipfel *et al.*, 2006). This, in fact, indicates the importance of FH as an effective regulator of complement activation.

FH has closely related glycoproteins that share common features and are encoded by genes located in the FH family gene cluster on chromosome 1q32 ; these family members are factor H-like protein 1 (FHL-1) and five factor H-related proteins (FHR1, FHR-2, FHR-3, FHR-4 and FHR-5) (Skerka *et al.*, 2013; Zipfel *et al.*, 2002).

FHL-1 is an alternative spliced product of a 42 kDa of the gene that encodes for FH. This protein has 7 SCRs which are homologous to the N-terminal SCRs of FH and a four amino acid tail at the C-terminus that are FHL-1 specific; FHL-1 has a similar complement regulatory function to that of FH, as it can act as a cofactor for FI mediating the cleavage of C3b (Misasi *et al.*, 1989). An additional role in complement control as a decay accelerating factor for C3 and C5 convertases has also been reported (Kuhn and Zipfel, 1996). FHL-1 has also been proposed to facilitate cell adhesion to the fibroactin matrix (Hellwage *et al.*, 1997).

FHRs are closely related proteins homologous to FH encoded by separate genes that are located on the same chromosome downstream the FH gene within the RCA (Regulation of Complement Activation) gene cluster (Diaz-Guillen *et al.*, 1999). Like FH, all FHR proteins, with exception of FHR-4 are composed of SCRs and are able to identify self

from non-self-cell surfaces; These proteins can be divided into two main groups based on their covered domains: Group I composed of FHR-1, FHR-2 and FHR-5 and group II includes FHR-3 and FHR-4 (Skerka *et al.*, 2013). Similar to FH, all five FHR proteins have been shown to play a role in complement regulation as well as other functions beyond complement, however, the defined mechanism of complement control of some of them is still not clear (Skerka *et al.*, 2013). FHR-1 prevents the cleavage of C5 by binding and inhibiting the C3b component of the C5 convertase, but the exact mechanism still not fully defined. In addition, it has been shown to regulate the terminal pathway via preventing the assembly of the MAC complex (Heinen *et al.*, 2009). FHR-2 has been shown to regulate the complement activation via inhibiting the alternative C3 convertase activity as well as preventing the formation of the terminal pathway complex (Eberhardt *et al.*, 2013). Although the defined mechanism in complement regulation is still not understood, FHR-3 and FHR-4 have been shown to bind to C3b, C3d and heparin (Hellwage *et al.*, 1999). FHR-5, in high concentrations, has been found to act as a cofactor for the serine protease FI as well as a decay accelerating factor, additionally, it can bind to C3b and heparin (McRae *et al.*, 2005). With the exception of FHR-2, the majority of FHRs have been found to compete with factor H for binding to C3b (Goicoechea de Jorge *et al.*, 2013).

1.6.5.1.4 Clusterin

Clusterin is a fluid phase complement regulator that has been described by several different names based on its multifunctional activities such as SP-40-40, Apo-J, complement lysis inhibitor (CLI) and sulphated glycoprotein 2 (SGP2) (Jenne and Tschopp, 1992). This soluble regulator consists of two chains (α-chain and β-chain) connected together by a disulphide bond and has an affinity to bind to aggregated cells such as erythrocytes and Sertoi cells. It has also been demonstrated within soluble C5b-9

complexes in human glomeruli (Rosenberg and Silkensen, 1995). Clusterin regulates the terminal complement pathway by binding to the TCC complexes (C5b67 complex, C7, C8 and C9) and forming soluble complexes that are unable of insert into cell membranes and cause cell lysis (Tschopp *et al.*, 1993). In addition, clusterin is also thought to play a role in some other biological activities such as the clearance of cellular debris and regulation of lipid transport (Jenne and Tschopp, 1992).

1.6.5.1.5 Vitronectin

Vitronectin, also known as S-protein, is a multifunctional glycoprotein that is involved in several other activities besides complement regulation (Preissner, 1991). Vitronectin is one of the regulators of the terminal complement pathway that inhibits the assembly of MAC complex. It does that by binding to C5b67, C5b6-8 and C5b6-9 complexes and prevents their attachment to host cell membranes and the formation of lytic pores (Podack and Muller-Eberhard, 1979). S-protein is also able to inhibit the polymerization of C9 and assembly of MAC complex on cell surfaces (Podack *et al.*, 1984). As stated earlier, vitronectin has also other functions such as mediating both cellular adhesion and invasion as well as playing a role in the regulation of coagulation (Hayman *et al.*, 1985; Singh *et al.*, 2010).

1.6.5.1.6 Carboxypeptidase N (CPN)

Carboxypeptidase N is an enzyme (a plasma zinc metalloprotease) produced by hepatocytes that acts to remove carboxy-terminal arginines and lysines from peptides (e.g. kinins, creatine kinase MM-skeletal muscle and complement anaphylatoxins (C3a and C5a)) that circulate in bloodstream (Matthews *et al.*, 2004). CPN was shown to inactivate the anaphylatoxins C3a and C5a as well as anaphylatoxins derived from bradykinin. Carboxypeptidase N inactivates C3a and C5a by cleaving terminal arginine and forming C3a(desArg) and C5a(desArg), which are markedly reduced in their ability

to bind their receptors (C3aR and C5aR, respectively) and induce signalling (Bokisch and Muller-Eberhard, 1970). By functioning this way, Carboxypeptidase N protects the human body from unnecessary accumulation of possibly harmful peptides that have either local autocrine or paracrine activity (Skidgel and Erdos, 2007).

1.6.5.2 Membrane bound complement regulators

The membrane bound complement regulators are present on the surface of host cells and their main role is to mark host cells as self-cells and prevent the complement attack upon activation (Kim and Song, 2006). Several surface attached complement regulators have been characterized and defined such as complement receptor 1(CR1, CD35), decay accelerating factor (DAF, CD55) and membrane cofactor protein (MCP, CD46).

1.6.5.2.1 Complement receptor 1 (CR1, CD35)

Complement receptor 1 is a glycoprotein expressed on the surface of a number of circulating host cells such as erythrocytes, T lymphocytes, B lymphocytes, neutrophils, monocytes and eosinophils but not on platelets (Fearon, 1980). Expression of CR1on the surface of other human cells, including follicular dendritic cells, peripheral nerve fibers and glomerular podocytes, has also been demonstrated (Appay *et al.*, 1990; Reynes *et al.*, 1985; Vedeler and Matre, 1990). CR1 regulates both the classical and alternative pathway as it acts as a cofactor for FI degradation of C3b and C4b as well as cleavage of iC3b into C3c and C3dg fragments (Medof *et al.*, 1982). It also accelerates the decay of the classical and alternative C3 and C5 convertases that are assembled either intrinsically on the same cell or extrinsically on nearby cells (Krych-Goldberg *et al.*, 1999). Furthermore, CR1 expressed on erythrocytes plays an exceptional role in mediating the delivery of

immune complexes bound to C3b from the circulation to the reticuloendothelial system for further processing (Medof *et al.*, 1982).

1.6.5.2.2 Decay accelerating factor (DAF, CD55)

Decay accelerating factor (DAF) is a glycosylphosphatidylinositol (GPI) linked glycoprotein (70 kDa) that is extensively expressed on a variety of human cells including circulating blood cells, endothelial cells and epithelial cells as well as being present in a soluble form in different body fluids (e.g. in plasma, synovial, and cerebrospinal fluids) (Medof *et al.*, 1987).This complement regulator functions on and controls the activation of both the alternative and classical complement pathways via accelerating the decay of their respective C3 convertases (C3bBb and C4b2a, respectively) (Nicholson-Weller *et al.*, 1983). DAF is able to bind to and remove C2a from C4b2a complex and Bb from C3bBb complex and prevents the assembly of these C3 convertases (Fujita *et al.*, 1987).

1.6.5.2.3 Membrane cofactor protein (MCP, CD46)

Membrane cofactor protein (MCP or CD46) is an integral glycoprotein (a type I transmembrane protein) that is expressed on almost all surfaces of human cells and peripheral blood cells except erythrocytes (McNearney *et al.*, 1989; Seya and Atkinson, 1989). MCP is able to regulate the complement activation and protect autologous host cells from complement attack by binding to C3b and acting as a cofactor for FI in mediating the cleavage of C3b, and also, with a weak cofactor activity, facilitating the cleavage of C4b (Seya and Atkinson, 1989). Furthermore, MCP has been shown to work as a receptor for some microorganisms such as measles virus (MV), *N. gonorrhoeae* and *Neisseria meningitides* mediating their adherence to epithelial cells and entry to nucleated cells (Lindahl *et al.*, 2000).

1.6.5.2.4 Protectin (CD59)

CD59 is another complement regulator that is known by a number of different names, due to its initial description by different research groups, such as protein p18, membrane attack complex inhibitory factor (MACIP), homologous restriction factor 20 (HRF20), membrane inhibitor of reactive lysis (MIRL) (Holguin *et al.*, 1989; Meri *et al.*, 1990; Okada *et al.*, 1989; Sugita *et al.*, 1988). Protectin is a glycoprotein that is ubiquitously expressed on the surfaces of human cells and tissues. CD59 is found on the surfaces of endothelial cells, epithelial cells, circulating blood cells, spermatozoa as well as neural tissues (Meri *et al.*, 1991). Similar to DAF, CD59 has a GPI anchor and both its free circulating and membrane- bound forms can be found in the human body (Hakulinen and Meri, 1995; Rooney and Morgan, 1992; Vakeva *et al.*, 1994) CD59 is a regulator of the terminal pathway as it specifically inhibits the final steps of the MAC formation via binding to the C5b-8 complex and blocking the assembly of polymeric C9 complex thereby protects self-cell from the complement-mediated cell lysis (Meri *et al.*, 1990). Combined deficiency in both CD59 and DAF is associated with a rare acquired haemolytic disorder called paroxysmal nocturnal haemoglobinuria (PNH) that is characterised by excessive lysis of red blood cells and leukocytes in a pattern earlier than normal (Risitano, 2013).

1.7 Evasion of complement by pathogenic bacteria

Despite the important role played by the complement system in the clearance of the invading microorganism, microbial pathogens, during their evolution, have developed many sophisticated tactics to avoid recognition and destruction by the complement system. These mechanisms either directly target and inactivate complement proteins or indirectly manipulate and modulate the complement activation by exploiting the

complement regulators, therefore, mimicking host self-cells and tissues (Figure 1.4). Microbial interference with complement activation occurs at any level of complement activation: at the initial stages of activation, at the C3 convertase generation or at the final stage of MAC formation. Microbial mechanisms and strategies of evading the complement system are comprehensively reviewed in a number of good reviews (Blom *et al.*, 2009; Kraiczy and Wurzner, 2006; Lambris *et al.*, 2008; Zipfel *et al.*, 2013; Zipfel *et al.*, 2007).

1.7.1 Blocking the initial stages of complement activation

Evading the initial levels of activation can happen by different ways. Some Gram-positive bacteria, for example, express surface proteins that are able to bind the Fc region of immunoglobulins (IgG) and interfere with C1q binding thereby inhibiting the activation of the classical pathway. Staphylococcal protein A (SPA) and protein G of both *S. aureus* and *Streptococci* are good examples (Atkins *et al.*, 2008; Nitsche-Schmitz *et al.*, 2007). Moreover, some microbes are able to cleave immunoglobulins and C3/C3b and block the complement activation either directly with their own endogenous proteases or indirectly by acquiring and using human proteases. Examples of these are two cysteine proteinases of *Streptococcus pyogenes*: IgG degrading enzyme of *S. pyogenes* (IdeS) and streptococcal endotoxin B (SpeB) are able to bind to and dissociate the Fc region of membrane-bound IgG hence disrupt complement activation (von Pawel-Rammingen and Bjorck, 2003). *S. aureus* is also able to achieve the same outcome in an indirect way by binding and activating human plasminogen to plasmin, using its secreted enzyme staphylokinase (SAK). Activated plasmin then binds to bacterial membranes and cleaves both IgG and C3b (Rooijakkers *et al.*, 2005). Blocking the early stages of complement activation can also be achieved via recruiting the complement regulator C1INH, therefore, preventing the activation of classical pathway. Examples of pathogenic bacteria

that bind to C1 INH, which functions on and inhibits the activation of both the classical and lectin pathways, are *E. coli* O175:H7 and *Bordetella pertussis* (Lathem *et al.*, 2004; Marr *et al.*, 2007).

1.7.2 Interfering with the formation of the C3 convertases

Invading microorganisms have also evolved strategies to elude complement attack at the C3 convertases level. For example, *S. aureus* produces a secreted protein called staphylococcal complement inhibitor (SCI) that is able to bind to C3 convertases of both the classical and lectin pathways, hindering their activity to cleave the complement component C3 as well as preventing the formation of new C3 convertases (Rooijakkers *et al.*, 2005). Moreover, Group A *Streptococci,* in order to protect itself from C3b deposition and phagocytosis, uses its cysteine protease SpeB to cleave properdin, which is required for the assembly of C3bBb complex. Therefore, SpeB inhibits the formation of the C3 convertase of the alternative pathway and blocking further cleavage of C3 (Tsao *et al.*, 2006). On the other hand, a number of pathogenic microbes are also able to mimic complement regulators that act as cofactors for factor I-mediated cleavage of C3b fragments. An example of such modulation is used by vaccinia virus, which has a protein known as vaccinia virus complement control protein (VCP) that has homology to C4BP and acts by functioning both as a decay accelerating factor for the C3 convertases, as well as being able to bind C4b and prevent the formation of the C4b2a complex (Kotwal *et al.*, 1990). Other microbes can indirectly interfere with the C3 convertase formation by binding to regulators of complement activation (RCA) that control complement activation at this level. Three fluid phase complement regulators (FH, FHL-1 and C4BP) that regulate the complement activation have been shown to be exploited by different pathogens to protect themselves from the complement activity. For example, *S. pyogenes* have been shown to used their M proteins to bind to FH, FHL-1 and C4BP and inactivate

C3 convertases of the alternative and lectin pathways and thereby avoiding the subsequent phagocytosis (Horstmann *et al.*, 1988; Johnsson *et al.*, 1996; Kotarsky *et al.*, 1998). Another example of bacterial binding to complement regulators is *N. gonorrhoeae*, which expresses outer membrane proteins called porins that are able to bind both FH (via por1A) and C4BP (via por1B) and inhibit activation of both the alternative and classical pathways (Ngampasutadol *et al.*, 2005; Ngampasutadol *et al.*, 2008; Ram *et al.*, 1998). Further examples are reviewed in detail by (Blom *et al.*, 2009).

1.7.3 Evading the late stages of activation

Even at the final stages of complement activation that lead to the formation of the cytolytic MAC, microbial pathogens have developed mechanisms to interfere with and prevent the activation of the terminal pathway of complement. Gram-positive bacteria are inherently resistant to direct lysis by complement attack due to the specific structure of their cell wall (which contains a thick peptidoglycan layer) that provides a physical barrier to block the insertion of the C5b-9 complex into the bacterial inner membrane (Joiner *et al.*, 1983). Furthermore, the polysaccharide capsule produced by some bacteria such as *S. pneumoniae* act as a mechanical block that prevents both the direct killing by the complement and impedes the opsonophagocytic killing by professional phagocytes (Hyams *et al.*, 2010). Many Gram negative bacteria can be directly lysed and killed by the complement activity (Fierer *et al.*, 1972); however, it was found that the presence of long O-polysaccharide side chains of LPS in the cell wall of some serum resistant strains of the Gram negative bacteria, *E. coli*, provide a protective shield against the complement-mediated serum killing (Porat *et al.*, 1987). The role of long O-polysaccharide side chains of LPS in avoiding direct complement killing was also demonstrated among *Salmonella Minnesota* strains, as it was found to provide a physical

barrier preventing the insertion of the C5b-9 complex into the membrane of bacterial cells, thereby inhibiting the MAC-mediated cell lysis (Joiner *et al.*, 1986).

Moreover, some bacterial pathogens can directly modulate the late stages of complement activation (the terminal pathway) using their secreted proteins that have ability to inhibit the activation of the terminal pathway. *S. aureus*, for example, produces a family of 11 proteins called staphylococcal superantigen-like (SSLs) that have a variety of biological functions; of these proteins, SSL7 has been found to bind to C5 preventing its cleavage into C5b and C5a fragments and thereby blocks the formation of the MAC and the complement-mediated cytolysis (Langley *et al.*, 2005). Another example, the streptococcal inhibitor of complement (SIC) that is expressed by Group A streptococci can bind to the soluble C5b-7 complex and blocks its insertion into the cellular membranes (Fernie-King *et al.*, 2001). *Borrelia burgdorferi* is another pathogen that is able to avoid the complement attack by producing a protein known as a CD59-like protein, which has a homology with the human membrane-bound complement regulator CD59; this protein is able to interfere with the final stage of the MAC formation by preventing the polymerisation of C9, hence inhibits the cell lysis (Pausa *et al.*, 2003).

On the other hand, other bacteria can indirectly inhibit the activation of the terminal pathway by acquiring and exploiting its regulators. A number of pathogenic bacteria, have been reported to recruit and bind the fluid phase complement regulator vitronectin that inhibits the MAC formation, examples include: *H. influenzae, Moraxella catarrhalis, Pseudomonas aeruginosa, S. aureus, Streptococcus pneumoniae,* and *S. pyogenes* (Singh *et al.*, 2010).

1.7.4 Modulating the anaphylatoxins, C3a and C5a

C3a and C5a are the small fragments that are cleaved and released from both the components C3 and C5, respectively, upon activation. These molecules are anaphylatoxins that act as chemoattractants to mediate effector cell migration and activation thereby triggering an inflammatory response. C5a particularly is able to recruit neutrophils and macrophages to phagocyte opsonized foreign cells such as pathogens or apoptotic cells. C5a interacts with its receptor C5aR that is widely expressed on many host cells, including inflammatory cells such as neutrophils, resulting in their stimulation to release their metabolic granules which intensify the immune response. Besides their activity as chemoattractants, C3a and C5a also have reported to have direct antimicrobial activity (Gavrilyuk *et al.*, 2005; Hugli, 1986). Microbial pathogens have also developed ways to inactivate these anaphylatoxins and avoid their action. For example, *S. aureus* produces protein called chemotaxis inhibitory protein of *S. aureus* (CHIPS) that was shown to interfere with C5a signalling to neutrophil and monocyte via binding to C5aR on the surfaces of these inflammatory cells thereby blocking C5a binding (de Haas *et al.*, 2004; Postma *et al.*, 2004).

1.8 The complement system and *Ureaplasma*

The complement activity against *U. parvum* has previously been investigated (Beeton *et al.*, 2012). In that comprehensive study, the authors have found that complement mediated killing of this cell wall-less bacteria occurs by the activation of the classical complement pathway, with no involvement of the alternative and lectin pathways. Furthermore, the results revealed significant variations in susceptibility to complement-mediated killing among the investigated serovars and clinical isolates of *U. parvum,* as

some were highly sensitive to complement-mediated serum killing, even in the absence of detectable anti-*Ureaplasma* antibodies suggesting direct C1q binding to *Ureaplasma* membranes, while others were resistant. As mentioned above, bacterial resistance to complement mediated killing is not uncommon phenomenon among bacterial pathogens; as this phenotypic trait renders resistant strains more virulent and enables them to cause systematic infections compared with sensitive strains (McCabe *et al.*, 1978; Porat *et al.*, 1987). While several mechanisms responsible for complement resistance among pathogenic bacteria have been defined, strategies used by *Ureaplasma* to elude the complement system and avoid clearance have yet to be elucidated.

1.9 Hypothesis

Resistance to complement mediated killing of *Ureaplasma* is key to its pathogenic potential, and induced serum resistance of laboratory strains of *Ureaplasma* will result in identifiable phenotypic and genotypic alterations that can be correlated to pathogenicity in future studies. Transposon mutagenesis can be used as a tool to identify serum resistant genes through removing resistance by gene disruption or delivering resistance genes to susceptible strains under control of strong exogenous high-expression promoters.

1.10 Aims

The objectives of this research work were:

- To examine the association between serum resistance and MBA type and MBA size in *U. urealyticum*.
- To generate serum-resistant *Ureaplasma* strains, through repeated sublytic challenge with NHuS, and identify proteins that are induced or cease to be expressed coincident with induction of serum resistance.
- To use proteomics to identify proteins with altered size or *pI* isoforms co-incident with induction of *Ureaplasma* serum resistance.
- To develop successful transposon mutagenesis for *Ureaplasma*, and characterise disrupted genes in strains with altered complement susceptibility or any other alteration in phenotype.

- To utilise red fluorescent protein gene expression as a measurable delivered foreign gene to determine the optimal promoter, codon usage and copy number to facilitate future expression and testing of any identified serum resistance genes.

- Achieving these goals will definitely enhance our understanding of immune-escape mechanisms of *Ureaplasma*. In addition, identification of virulence factors that mediate *Ureaplasma* infection will be of a great importance as it will provide new insights towards developing novel vaccines and therapeutics.

Chapter 2

Materials and methods

2. Materials and Methods

2.1 *Ureaplasma* spp. serovars and clinical isolates

All of the original characterised prototype serovars for all known 14 serovars (Robertson and Stemke, 1982) and several clinical isolates from both groups of *Ureaplasma* spp. (*U. parvum* and *U. urealyticum*) were investigated in this thesis. Selection of some representative serovars or identified clinical isolates for some experiments was performed to determine variations between groups and among serovars from the same group.

2.1.1 Source of *Ureaplasma* spp.

Ureaplasma spp. serovars (SV) and clinical isolates were kindly provided by Dr. Victoria Chalker from the Health Protection Agency (HPA) (Colindale, London; now known as Health Protection England (HPE)). Hence, the prefix HPA represents samples obtained from that source. While the HPA provided all representative samples for each serovar, all reference strains were originally received from either the Institute of Medical Microbiology, University of Aarhus, Denmark or Prof. Janet Robertson (University of Calgary, Canada). All isolates were frozen and stored as stocks at -80 ˚C in my host laboratory. *U. parvum* was represented by serovars 1, 3, 6 and 14, and *U. urealyticum* by serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13. In addition, several clinical *U. urealyticum* isolates were also provided by the HPA. During the tenure of this studentship, weekly samples were also received (following appropriate consent by collecting physician) from Dr. Nicola Maxwell at Deriford Hospital in Plymouth U.K. Samples were anonymised and coded sequentially from 101, where each subsequent sample was given a sequential letter (i.e. sample Ply144C, corresponds to the 44th recruited preterm neonate from

Plymouth and this sample represents the 3rd consecutive collected sample from that baby). The results of these samples were not fed back to the collecting Neonatal Intensive Care Unit until the breaking of the codes for retrospective analysis. In contrast, samples submitted to the laboratory from the University Hospital of Wales (UHW) NICU (7 in total) were positive samples submitted for diagnostic evaluation and were anonymised prior to being given to me. A series of *U. urealyticum* clinical isolates from Prof. Cecile Bebear (University of Bordeaux, France) were also provided and include samples (UUf1 & UUg1) that were from sexual partners (Beeton *et al.*, 2009a). A list of isolates used in this study is presented in Table (2.1).

Table 2.1 *Ureaplasma* serovars and clinical isolates used in this study

Isolate	Species	Serovar
DKF-1	*Ureaplasma parvum*	SV1
HPA5	*Ureaplasma parvum*	SV3
HPA2	*Ureaplasma parvum*	SV6
HPA32	*Ureaplasma parvum*	SV14
M0063/92 (T23)	*Ureaplasma urealyticum*	SV2
M0089/92 (DFK-4)	*Ureaplasma urealyticum*	SV4
SV5 (Western)	*Ureaplasma urealyticum*	SV5
SV7 (KDF-7)	*Ureaplasma urealyticum*	SV7
SV8 / T960 (CX8)	*Ureaplasma urealyticum*	SV8
SV9 (Vancouver strain)	*Ureaplasma urealyticum*	SV9
SV10 (Western)	*Ureaplasma urealyticum*	SV10

Continued

Table 2.1 (continued)

SV11 (K2)	***Ureaplasma urealyticum***	**SV11**
SV12 (U24)	*Ureaplasma urealyticum*	SV12
SV13 (U38)	*Ureaplasma urealyticum*	SV13
HPA3	*Ureaplasma urealyticum*	SV9/SV7/SV11 [I]
HPA4	*Ureaplasma urealyticum*	SV4/SV12/SV13 [I]
HPA6	*Ureaplasma urealyticum*	SV4/SV12/SV13 [I]
HPA7	*Ureaplasma parvum*	SV3
HPA8	*Ureaplasma parvum*	SV3
HPA9	*Ureaplasma parvum*	SV3
HPA12	*Ureaplasma urealyticum*	SV4/SV12/SV13 [I]
HPA17	*Ureaplasma urealyticum*	SV2/SV12 [I]
HPA20	*Ureaplasma urealyticum*	SV4/SV12/SV13 [I]
HPA24	*Ureaplasma urealyticum*	SV10 [I]
HPA31	*Ureaplasma urealyticum*	SV10 [I]
HPA43	*Ureaplasma urealyticum*	SV9 [I]
HPA44	*Ureaplasma urealyticum*	SV10 [I]
HPA56	*Ureaplasma parvum*	SV3
HPA57	*Ureaplasma parvum*	SV3
HPA60	*Ureaplasma parvum*	SV3
HPA78	*Ureaplasma parvum*	SV1
W11	*Ureaplasma urealyticum*	SV12 [I]
U6	*Ureaplasma parvum*	SV3
UHW5	*Ureaplasma parvum*	SV3
UHW6	*Ureaplasma parvum*	SV3

[I] *U. urealyticum* clinical isolates identified and assigned in this study, HPA: isolates obtained from the Health Protection Agency (now known as Health Protection England, HPE); UHW: isolates obtained from the University Hospital of Wales.

2.1.2 Culturing of *Ureaplasma* spp.

Ureaplasma spp., either from clinical samples or frozen stocks, was cultured in Ureaplasma Selective Medium (USM), unless stated otherwise. This medium was purchased from Mycoplasma Experience Ltd, Surrey, UK. While the exact formula is proprietary, the medium is thought to consist of a simple broth base medium supplemented with yeast extract 2.5 g/L, Urea 1 g/L , and 10% heat-inactivated porcine serum, with a pH indicator (phenol red), medium pH= 6.65, and 2.5 µg/ml Amphotericin B and 0.25 mg/ml Ampicillin. Cultures were done in flat-bottom 96-well plates and sealed with adhesive sealing tape (Elkay, Basingstoke) and incubated in a humidified tissue culture incubator set at 37 °C with atmospheric CO_2 concentration.

Culturing of *Ureaplasma* spp. on USM-Agar medium (Mycoplasma Experience Ltd, Surrey, UK) was done by inoculating and spreading 1 µl of *Ureaplasma* growth (~10^4-10^5 CCU) in USM on the agar surface. The plates were incubated at 37 °C with atmospheric CO_2 concentration for 24-48 h. Colonies were viewed under X4 and X10 objective microscope lenses (Eclipse TS100 Inverted Routine Microscope, Nikon Instruments Europe B.V., UK). While colonies were viewed under the microscope, picking single colonies was also performed using sterile disposable loops. Single colonies were then transferred and inoculated into USM medium, where they were titrated out (1:10) and incubated as previously mentioned.

2.1.2.1 Culturing *Ureaplasma* spp. from clinical samples and frozen stocks

All clinical samples collected from Plymouth or Cardiff patients arrived at the laboratory as endotracheal aspirate samples. Upon receiving a sample, it was homogenised with a 1 ml sterile pipette tip, and then 20 µl cultured by titration (1:10) in USM in a single

column (8 wells) of flat-bottom 96-well plate: 20 µl of well-mix sample was taken and inoculated in 180 µl USM in the first well and then serially diluted to 10^7 (7^{th} well) in USM, and last well (8^{th} well) was left uninoculated as a negative control. Each sample was cultured in triplicate. Plates were sealed with air tight clear adhesive seals. Cultures were then incubated at 37°C for 24 h. Plates negative at 24h were re-examined at 48h to confirm the negative result. Positive cultures were indicated as a change in the colour of USM from yellow to red as the sole carbon source for *Ureaplasma* is urea which is converted to ammonium ions increasing the pH of the medium to >10. *Ureaplasma* isolates from positive cultures were collected and frozen as 20 µl stock aliquots at -80 °C until needed. When *Ureaplasma* clinical isolates were needed for an experiment, a 20 µl frozen aliquot was thawed on ice and then cultured by titration (1:10) in USM, as previously stated. This method usually provides a bacterial growth of about 10^4- 10^5 CCU/ml after 24 h incubation. If a poor growth was obtained, i.e. < 10^4 CCU, re-subculture was done by diluting the final well that was starting to show colour change by 1:10 serial dilution into USM to provide adequate growth for an experiment. Samples were subsequently prepared by a method according to whether they were to be used in complement studies, proteomic studies or genomic and transformation studies.

2.1.2.2 Quantification of *Ureaplasma* spp. growth

The standard unit used to quantify *Ureaplasma* numbers in broth media is colour changing units (CCU). To estimate the number of CCU of *Ureaplasma* in a sample or culture, the sample has to be serially diluted 1:10, as mentioned above: 180 µl of USM was added to each well of a single column (8 wells) of a 96 well plate. Then 20 µl of sample was inoculated into the first well to give an initial 1:10 dilution, and then from the initial inoculum the sample was serially diluted down to 10^7, with the last well remained

as a negative control. After incubation for 48 h., plates were checked for colour change, and the last red well was designated as 1 CCU, which, in theory, contained between 1-9 viable organisms and is, therefore, directly related to a Colony Forming Unit (CFU) (the standard unit used to quantify the number of other kind of bacteria) if it had been cultured on an agar plate. Therefore, the number of CCU of *Ureaplasma* in original sample can be calculated.

2.2 Complement studies

2.2.1 Serum source and preparation

The human sera used in the complement studies were collected from healthy volunteers. The Normal human serum (NHuS) was collected from volunteer healthy females (designated 'Vx' for volunteer then 'F' followed by the number collected; therefore VxF4 is the fourth female volunteer) and males (VxM#). NHuS was collected from previously characterised volunteers (Beeton *et al.*, 2012) where presence or absence of anti-*Ureaplasma* IgG antibodies had been determined using western blot probing of prototype strains. Seropositive sera yielded specific immunoreactive bands whereas seronegative sera did not show immunoreactive bands. Blood from both sources was collected into 20 ml glass universal bottles and allowed to coagulate at room temperature for 1 h. Then the clot was released from the wall of the bottle and re-incubated again on ice for another an hour to allow the clot to contract further. The bottles were then centrifuged at 1000 x g for 10 min. The clear yellow supernatant (serum) was carefully transferred to clean universal bottles and aseptically dispensed in 400 µl aliquots in sterile microcentrifuge tubes and stored at – 80 °C until needed.

To prepare heat-inactivated serum (HI-NHuS), serum aliquots were incubated at 56°C for 30 min. This heat treatment inactivates C2 of the complement system, which is required for both the classical and lectin pathways, and inactivates factor B that is required for the alternative pathway. Sera was diluted to 50% (heat-inactivated or normal) for complement studies by mixing with an equal volume (1:1 v/v) of sterile veronal buffered saline, VBS, commercially available as Complement Fixation Buffer (Oxoid plc, Basingstoke, UK).

2.2.2 Complement killing assay

Complement killing assay was done according to the protocol described by Beeton *et al.* (2012). Log-phase growth *Ureaplasma* cultures were prepared as stated above (section 2.1.2.1). 200 µl of bacterial growth was transferred, in duplicate, into the wells of a V-bottom 96-well plate, the plate was sealed with clear adhesive film and centrifuged at 3600 g for 10 min, and the supernatant then carefully removed by pipetting. One of each matching pair of bacterial pellets was re-suspended in 200 µl of 50% NHuS diluted in VBS, and the other was re-suspended in a 50% heat-inactivated matched serum control (HI-NHuS), which was run in parallel as a direct comparison for later calculation and interpretation of killing. Plates then were incubated at 37° C for 1 hour, prior to centrifugation as above and removal of diluted serum. Pellets were then re-suspended in 200 µl USM and transferred to a flat-bottom 96-well plate. Surviving bacteria was quantified by titration in USM: both the test (NHuS exposed) and control (HI-NHuS exposed) were titrated out in 1:10 for 3 dilutions (1000-fold) and then 1:2 for 8 dilutions; this provided a dilution gradient to 2.56×10^5. Plates were sealed and surviving bacteria were measured after 48 hour incubation at 37°C to enable maximum growth. Killing was determined as the relative decrease in bacterial titre relative to incubation with heat-

inactivated serum (56°C, 30 min) as a control (Figure 2.1). All killing assays were performed in triplicate and repeated on at least three separate days with a range of characterised seropositive and seronegative human sera.

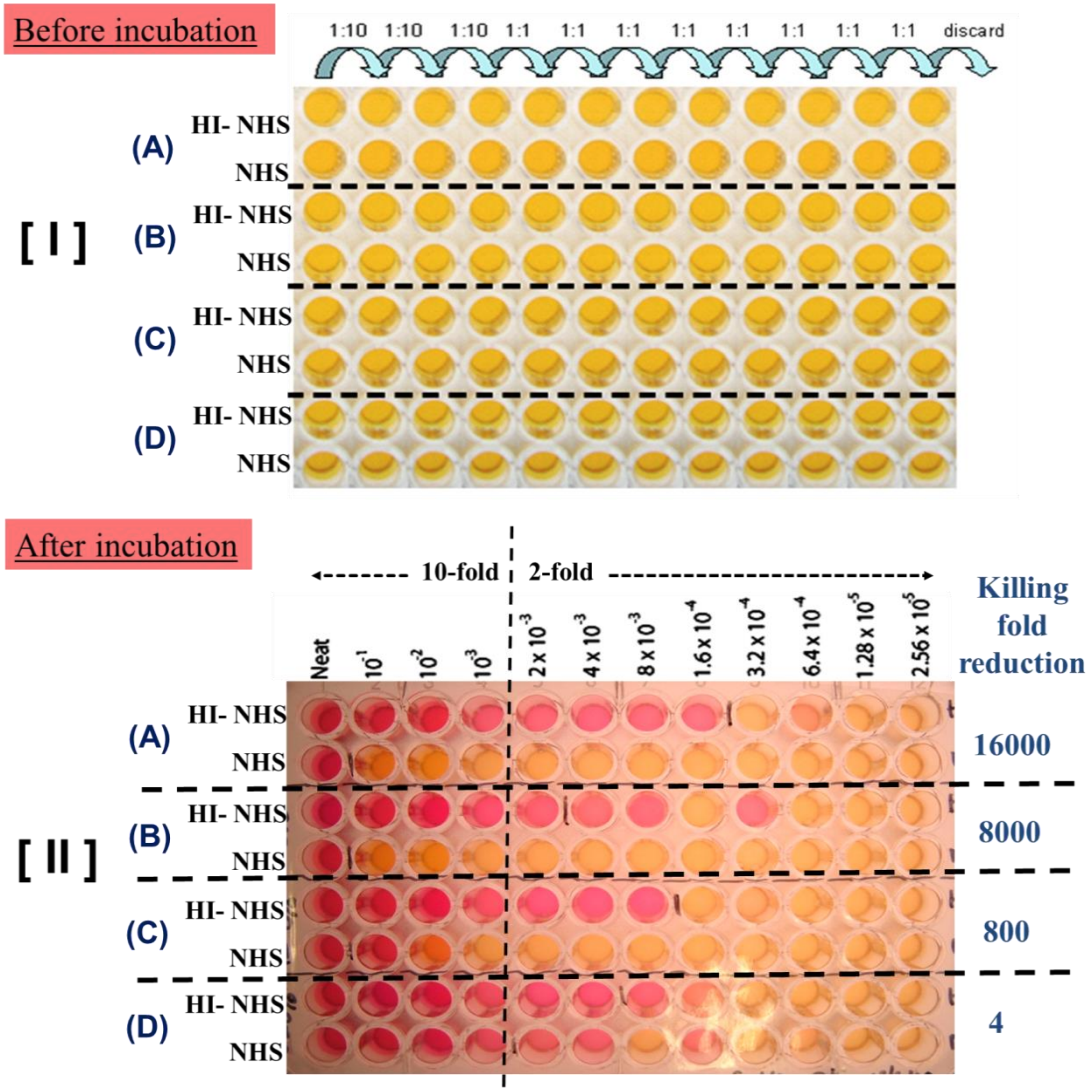

Figure 2.1 Schematic diagrams of the 96-well plate set up of complement killing assay. Four representative *Ureaplasma* isolates (A - D) were incubated under two conditions: 50% heat inactivated normal human serum (HI-NHuS) and 50% normal human serum (NHuS). Following complement attack *Ureaplasma* cells were titrated out as set out in the plate, initially 1:10 dilutions followed by 1:1 dilutions, to determine the fold reduction of colour changing units between HI-NHuS and NHuS (Plates I&II).

2.2.3 Complement-induced resistance assay

The complement killing assay was performed as above, but with a sublytic dilution of NHuS serum (although often the first 1-2 wells show survival following challenge with seropositive serum) and the surviving bacteria is used as the source for the subsequent serial challenge. In the primary challenge, *Ureaplasma* was incubated with 200µl of 50%, 25%, 12.5% or 6.25% NHuS (seropositive or seronegative) diluted in sterile VBS at 37° C for 1 h, and along the side, the tested cells were also incubated with the same dilutions of the matched HI-NHuS in a similar way as previously described in the complement killing assay. Surviving *Ureaplasma* from the highest concentration of serum (even if only the first well) was used at 24 h post-challenge (to ensure log-phase growth of bacteria for the subsequent challenge). The plates were then further incubated for 48 h to allow the assay to completely develop prior to recording killing results for each assay, however. The sublytic challenge process was repeated until a serum resistant strain emerged, generally requiring 5-6 serial challenges. After each challenge, 20 µl of the surviving bacteria were collected and frozen at -80° C for further investigations. Confirmation of induced serum resistance was done by performing complement killing assay, in triplicates, on all serum-induced resistant strain compared to the sensitive parent strain. The serum-induced strains were stored as 20µl aliquots at -80° C till needed for an experiment.

2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

2.3.1 Sample preparation

Ureaplasma total cell protein samples were prepared by centrifuging a scaled up 5 ml culture of bacterial growth (usually allowed to incubate for 48 h at 37^{o}C before harvest) at 16000 x g for 10 min in a refrigerated bench top microfuge. The supernatant was discarded and the remaining bacterial pellet was washed three times in 1X sterile phosphate buffered saline, PBS (160 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM K_2HPO_4, pH 7.4). The washed pellets were then re-suspended in 25 µl 1X PBS and 25 µl lithium dodecyl sulphate (LDS) sample loading buffer (Invitrogen, Paisley, UK). Samples were then heated at 95 ºC for 10 min to solubilise whole cell proteins and stored at -20° C till needed. For Western blot analysis, 7-10 µl of each sample was resolved on by SDS-PAGE.

2.3.2 Gel preparation

A gel casting system, Mini-PROTEAN 3 cell, was assembled as described by the manufacturer (Bio-Rad, Hertfordshire, UK). Separating (Resolving) gel was prepared according to the percentage of gel desired for depending on the mass of the proteins to be resolved. As indicated in table 2.1.; in this study, both 7.5 % and 12.5% polyacrylamide gels were used. After the resolving gel set, stacking gel (4%) was prepared as shown in Table 2.2 and poured on top, and a gel comb was inserted.

Table 2.2 Ingredients required for preparing resolving and stacking gels

Stacking gel	4%
40% bis/acrylamide	1.012 ml
dH_2O	6.4 ml
Upper buffer*	2.4 ml
10% APS w/v	100 µl
TEMED	40µl
Total volume	9.9 ml

Resolving gel	7.5%	12.5%
40% acrylamide	1.93 ml	3 ml
dH2O	5.470 ml	4.3 ml
Lower buffer¥	2.5 ml	2.5 ml
10% APS w/v	100 µl	100 µl
TEMED	10 µl	10 µl
Total volume	10 ml	10 ml

* Upper buffer: 0.5M tris-HCl (pH 6.8), and 0.4% SDS,; ¥ Lower buffer: 1.5M tris-HCl (pH 8.8), and 0.4% SDS.

2.3.3 Sample loading and electrophoresis

For Western blot analysis, 5 µl of MagicMark™ XP Western Protein Standard (Invitrogen, Paisley) and EZ-Run PreStained Rec Protein Ladder (Invitrogen, Paisley) was loaded on each gel. Proteins were resolved using 1x running buffer (25 mM Tris, 192 mM glycine and 0.1% SDS at pH 8.3) at constant 150 V and approximately 120 mA for about 1 hour or until the dye front reached the bottom of the gel. Following gel electrophoresis, gels with separated proteins were processed according to next experiment, i.e. Western blot analysis or protein gel staining with dyes.

2.4 Western blotting

Following separation by electrophoresis, proteins were transferred from gels to 0.22 µm nitrocellulose membranes using the Mini Trans-Blot® Electrophoretic Transfer Cell system, according to the manufacturer guidelines (Bio-Rad, Hertfordshire, UK). Gels, nitrocellulose membranes, sponge pads, and filter papers were initially submerged in 1X transfer buffer (14.4 g/L glycine, 3.03 g/L Tris base, 20 % methanol) and then arranged into transfer cassettes (gel sandwich) as recommended by the manufacturer. Gel sandwich was then assembled in the transfer tank, which was filled with transfer buffer. A cooling

unit (an ice pack) to keep the transfer buffer cool and a magnetic stir bar to circulate buffer were also added into the tank. Transfers were run at 100 V constant for 1 hour. After the electrophoretic transfer, the nitrocellulose membranes containing transferred proteins (the blots) were placed within a 50 ml falcon tube (Elkay, Basingstoke) and blocked for 1 hour with 25 ml blocking buffer (5% w/v non-fat milk in 1% PBS with 0.05% Tween-20, PBST) on a roller. Membranes were firstly probed with primary antibodies or NHuS diluted in blocking buffer, as shown in Table 2.3, overnight. Membranes were then washed in 1% PBST for 10 min (three times) followed by wash in 1% PBS for 10 min (two times). To detect the probing primary antibodies, blots were probed with an appropriate peroxidise-conjugated secondary antibody diluted in blocking buffer for 1 h (Table 2.3). Membranes were again washed three times in PBST and subsequently two times in PBS before detected with Pierce® ECL Western Blotting Substrate (Thermo scientific, Loughborough). The immunoreactions were visualised on FUJI Super RX X-ray films (Fujifilm, Tokyo, Japan), and immunoreactive bands on X-ray films were then scanned and images were stored as TIFF files.

Table 2.3 Human sera and primary and secondary antibodies used in this study

Serum or Primary Antibody	Dilution	Source	Secondary Antibody	Dilution	Source
NHuS (VxF1, F7, F9, M11, M12& M18)	1:400	Normal Human Volunteers (Beeton *et al.*, 2012)	Peroxidase-conjugated affiniPure F (ab) Fragment Donkey **Anti-Human IgG (H+L)**	1:10000	Jackson ImmunoResearch
Anti-mCherry polyclonal antibody	1:1000	BioVision	Peroxidase-conjugated AffiniPure Donkey **Anti-Rabbit IgG (H+L)**	1:10000	Jackson ImmunoResearch
Anti-elongation factor Tu (EF-Tu) monoclonal antibody	1:100 (2DE=1:400)	Hycult biotech	Peroxidase-conjugated AffiniPure Goat **Anti-Mouse IgG (H+L)**	1:10000	Jackson ImmunoResearch
Anti-His tag	1:5000	BioLegend	Peroxidase-conjugated AffiniPure Goat **Anti-Mouse IgG (H+L)**	1:10000	Jackson ImmunoResearch
Anti-MBA monoclonal antibodies	1:1000	(Watson *et al.*, 1990)	Peroxidase-conjugated AffiniPure Goat **Anti-Mouse IgG (H+L)**	1:10000	Jackson ImmunoResearch
Anti-UU280 polyclonal antibody	1:200	This study (Genosphere-Biotech, Paris, France)	Peroxidase-conjugated AffiniPure Donkey **Anti-Rabbit IgG (H+L)**	1:10000	Jackson ImmunoResearch

Table 2.4 Monoclonal anti-MBA antibodies used in this study

Antibody	*Ureaplasma* serovar	Reference or Source
MAb 10C6.6	SV3	(Watson *et al.*, 1990)
MAb 8A1.2	SV10	"
MAb 5B1.1	SVs 3&14	"
2G9	SVs 1 & 6	"
6522	All *Ureaplasma* spp. SVs	Virostat plc (Portland, ME)
6523	SV3 & 14	"
6525	All *Ureaplasma parvum*	"

2.5 Proteomic studies

The proteomic workflow used was as follows: *Ureaplasma* cell pellets were subjected to SDS protein extraction followed by protein assay, proteins separation by electrophoresis (2-D electrophoresis and/or 1-D electrophoresis), Colloidal Coomassie blue staining, spot picking, trypsin digestion and tandem mass spectrometry (MS/MS) analysis, a service provided by Cardiff University Central Biotechnology Services (CBS). In some occasions, two identical electrophoresis gels were prepared, one was stained with Colloidal Coomassie blue and the other was investigated by western blotting analysis to identify particular proteins to be picked from the other gel, which was stained with Colloidal Coomassie blue stain.

2.5.1 Preparation of 10B Urea Broth (Shepard's media)

Urea broth medium was used to scale-up the growth of *Ureaplasma* to 100 ml cultures. One litre stocks were prepared by adding 14g of Mycoplasma broth base without crystal violet, pH 7.8: beef heart, infusion 6 g, peptone 10 g, sodium chloride 5 g (Becton, Dickinson, France), 1 ml of 1% phenol red and 0.2g herring sperm DNA (Sigma, UK) to 688ml distilled water. Following adjustment of the pH to 5.5 with 2 N HCL, the media was autoclaved for 15 min at 121 °C; The following heat-labile supplements were then prepared and filter sterilised separately and added to the autoclaved base after cooling: 200 ml of horse serum, 4 ml of 10% urea (Sigma, UK), 2.5 of 4% L-cysteine (prepared fresh on day of use), 100 ml of 25% yeast extract (Fisher-Scientific, UK), 5 ml of BD BBL^{TM} IsoVitaleX Enrichment (Becton, Dickinson, France), 1 ml ampicillin (100 mg/ml) (Sigma, UK), and 10 ml amphotericin B (250 µg/ml) (Sigma, UK). The pH of media was adjusted again to 6.0 with 2 N HCL and then stored at 4 °C until needed.

2.5.2 Growing *Ureaplasma* spp. in 10B urea broth (scale up)

Ureaplasma isolates were initially cultured by titration in USM (two columns of 8 wells) as described above; the last four wells that showed growth (colour change) after 24 h incubation were pooled together and directly transferred to 250 ml of media. The inoculated media was incubated at 37 °C for 48 h. After maximum colour change in the bottles, the bacterial growth was then pelleted at 10,000 xg for 30 min. The supernatant removed and the pellet was washed with 1X sterile PBS and centrifuged at 10,000 xg for 10 min (washing was repeated 3 times). After washing, the bacterial pellet was ready for protein extraction and sample preparation for proteomic studies. This amount of growth was optimised to give a sufficient amount of protein from *Ureaplasma* cells for proteomic studies.

2.5.3 Protein extraction

Protein extraction was done using the cell lysis buffer (1% (w/v) SDS in Tri-ethyl ammonium Bicarbonate (TEAB)). The bacterial cell pellets were re-suspended in 100µl 1% (w/v) SDS and incubating for 30 min at room temperature. The mixture was then boiled for 10 min and centrifuged at 13,000 rpm (15890 x g) for 5 min at 4 ˚C to clarify. The clear supernatant containing fully solubilised protein was removed, and protein concentration was quantified using the bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich, UK).

2.5.4 Quantification of protein using BCA assay

Quantification of protein concentration was done using the BCA assay or the Smith assay (Smith *et al.*, 1985). The assay was performed in a 96-well plate and a standard curve

using a dilution series of bovine serum albumin, BSA (1 mg/ml) (Sigma) was used. Dilutions of BSA were prepared in plate column 12 using 200 µl of BSA solution in well H12 and 100 µl of distilled water in wells B12 to G12. 100 µl of solution from H12 was added to G12 and mixed, and then 100 µl was taken from G12 to F12 and so on until B-12 (i.e. two-fold dilution series). A12 was left blank. From each diluted standard, 25 µl was taken and put in corresponding wells 1, 2, 3 of same row (triplicate); this comprised the standard curve. 25 µl of each test sample for protein quantification was added in duplicate in columns 4 and 5. BSA working reagent was prepared by mixing 5 ml reagent A and 100 µl reagent B. 200 µl of dilute BSA working reagent was added to each well (Figure 2.2). The plate was then covered with clear adhesive sealing film and incubated for 30 min. at 37º C. After colour was developed, the readings were then taken on a Dynex MRX TC Revelation 96-well microplate reader (Dynex Technologies, Chantilly, VA) at 570 nm. If any sample readings were out of range, it was diluted and repeated again. The protein concentration of samples was calculated using MRX software intrinsic to the plate reader.

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.0											0.0
B	0.015625											0.015625
C	0.03125											0.03125
D	0.0625											0.0625
E	0.125											0.125
F	0.25											0.25
G	0.5											0.5
H	1											1
	BSA mg/ml (25 µl) (triplicate)			Samples (25 µl) (duplicate)								BSA (mg/ml) serial dilution

Figure 2.2 Schematic diagram of a 96-well plate illustrating the BCA protein assay

2.5.6 One Dimensional Electrophoresis (1-DE)

One dimensional electrophoresis was run using the XCell SureLock™ mini-cell electrophoresis system (Novex® Life technology). The electrophoresis unit was assembled as described by the manufacturer. Sample preparation was also done according the gel manufacturer recommendations (Invitrogen) (Table 2.5).

Table 2.5 Sample preparation for 1-DE (Mini Gels)

Reagent	Reduced Sample	Non-reduced sample
Sample	x µl	x µl
NuPAGE LDS sample buffer (4X)	2.5 µl	2.5 µl
NuPAGE Reducing Agent	1 µl	_
Deionized water	To 6.5 µl	To 7.5 µl
Total volume	10 µl	10 µl

Samples were heated at 70º C for 10 min before loading.

Gel cassettes were prepared by removing the NuPAGE gel from the pouch and rinsing in deionized water. The tape from the bottom of the cassette was then peeled off and the comb was gently removed, in one smooth motion, from the cassette. The sample wells were then rinsed with 1X NuPAGE SDS running buffer, 3-(N-morpholino) propanesulfonic acid (MOBS), and then inverted to remove the buffer. The two cassette gels were assembled in the XCell SureLock mini cell tank such that the notched well side of the cassette faced inwards toward the buffer core. In case of using one cassette gel, a buffer dam was used to occupy the other place of the second cassette gel. 10 µl of each sample (15 µg protein) and 15 µl of unstained marker (Mark 12™ unstained standard, Invitrogen) were then loaded into the gel wells. Proteins were separated at 200V (120

mA, 25.0 W) for about 55 min or until the dye front reached the ridge at the bottom of the gel. Gels were then processed for Colloidal Coomassie blue staining or western blotting.

2.5.7 Two-dimensional electrophoresis (2-DE)

Two-dimensional electrophoresis of proteins was developed in 1975 as a powerful technique for separating proteins based on two parameters: isoelectric point and molecular weight (O'Farrell, 1975). This method has been widely used in proteomics.

2.5.7.1 Protein precipitation using the 2D Clean-Up kit

Before separating proteins by 2-DE, protein samples were subjected to protein precipitation and clean-up process using a 2D Clean-up kit as described by the manufacturer (GE Healthcare, Amersham, UK). This process removed contaminating substances such as excess salts, charged detergent, lipids, phenolics, and nucleic acids from proteins, which may interfere with isoelectric focusing (IEF) and mass spectrophotometry (MS) analysis. In this procedure, 65 µg (1-100 µl) of whole cell protein sample was transferred to a 1.5 ml microcentrifuge tube, and then 300 µl of precipitant (a solution that renders proteins insoluble) was added and briefly vortex mixed. The tube was centrifuged at 12000 xg for 5 min and supernatant was discarded. Centrifugation was repeated again to bring any remaining supernatant to the bottom of tube, and the supernatant was then carefully aspirated from the sample using micropipette. Without disturbing the pellet, 40 µl of co-precipitant (a solution contains reagents that co-precipitate with proteins and enhances their removal from solution) was added on top of the pellet and the tube was incubated on ice for 5 min. Another centrifugation at 12000 xg for 5 min was performed and any remaining supernatant

removed and discarded. 25 µl of deionised water was added to the pellet and mixed by vortex for 5-10 sec to disperse the pellet, but not to dissolve it. 1 ml of chilled wash buffer (a solution that is used to remove non-protein contaminants from the protein precipitate) (kept at -20º C) and 5 µl of wash additive (this solution contains a reagent that promotes rapid and complete re-suspension of the sample proteins) was then added and the tube was incubated in -20º C freezer for 30 min with brief vortex mix for 20-30 sec every 10 min. The tube was spun at 12000 xg for 5 min and the supernatant was removed and discarded. Pellet was allowed to dry for no more than 5 min. The cleaned-up protein pellet was then re-suspended in 116 µl of the rehydration/ lysis buffer (7 M Urea, 2M Thiourea, 2% CHAPS) as described below in the isoelectric focusing (IEF).

2.5.7.3 Isoelectric Focusing (IEF)

The first step in 2DE is the IEF, in which proteins are first separated by their *pI* and then further separated according to molecular weight through SDS-PAGE. The principle of the method was according to that described by (Mathew *et al.*, 1975). IEF (the first dimension) was performed using isoelectric focussing strip (IPG), a 7 cm Immobiline Drystrip pH 3-10 NL (nonlinear) (GE Healthcare, Amersham, UK). 116 µl of protein sample prepared in the rehydration buffer was transferred into a 1.5 ml microcentrifuge tube and 1 µl of 1% Bromophenol Blue, 2 µl of Isoelectric focussing buffer (IPG buffer) (GE Healthcare, Amersham, UK) and 6.25 µl of 50 mM Dithiothreitol (DTT) were added. The total volume sample mixture was adjusted to be 125 µl using the rehydration buffer and all the sample was carefully pipetted into the strip holder (coffin) and distributed evenly over the 7 cm well. Using tweezers, to hold the anode (+ ve) end of the IPG strip, the cover foil was pulled starting from the anode side of the IPG strip. The strip was then layered (the anode end of the strip towards the pointed end of the strip

holder, gel side down, avoiding bubble formation) on the sample-rehydration solution carefully to avoid getting any of the solution on top of the strip. A note of the strip number was taken for each specific sample. The sample and IPG strip was covered with 0.5 ml of DryStrip cover fluid and then placed on an isoelectric focusing unit (IPGphor Isoelectric Focusing System, GE Healthcare, Amersham, UK) for first dimension electrophoresis. The system was switched on and set on protocol for a mini gel (7 cm) and the number of strips was altered accordingly. The protocol settings were as follows: Rehydration: 12 hours at 20º C and IEF parameters: 50 µA/strip, 20º C (Step 1: step and hold 500 V 1 hour; Step 2: Gradient 1000 V 2 hours; Step 3: step and hold 1000 V 1 hour; Step 4: Gradient 8000 V 2 hours; Step 5: Step and hold 8000 V 8 hours). After inputting the conditions, the IEF was allowed to run to completion (total running time 26 hours). When IEF was completed, the strips were taken from the coffin, and the strip was gently placed sideways on tissue to blot off excess liquid. Care was taken to avoid damaging the gel side of the strip. The IPG strips were then subjected to an equilibration process. Each strip was first put in a 15 ml Falcon tube containing 5 ml of reducing solution (0.5 ml of 10X NuPAGE Sample Reducing agent (Invitrogen) and 4.5 ml of 1X NuPAGE LDS sample buffer (Invitrogen)), with the gel side of the strip up, and incubated for 15 min. on a rocker. The reducing solution was then decanted and the strip was carefully taken from the tube, using tweezers, and any excess liquid was removed by blotting. The strip was then transferred into another 15 ml Falcon tube containing 5 ml alkylating solution (116 mg of iodoacetamide in 5 ml of 1X NuPAGE LDS sample buffer) and incubated for 15 min on a rocker. In parallel, SDS-PAGE running buffer (1X MOPS: 40 ml 20X MOPS and 760 ml deionised water) was prepared.

2.5.7.4 Second dimension electrophoresis

Second dimension electrophoresis was run using the XCell SureLock™ mini cell electrophoresis system according to manufacturer instructions as described in section 2.5.6. The NuPAGE 4-12 Bis-Tris Zoom Gel (1 mm x IPG well) was removed from the pouch and the gel cassette was rinsed with deionised water. Tape was removed from the bottom of the cassette and the comb removed from the cassette and sample wells were rinsed with 1X NuPAGE SDS running buffer. The cassette gels were assembled in the XCell SureLock mini cell tank and 1X MOBS running buffer added until the level of buffer reached above the strip and marker wells. The IPG strip was then taken carefully from the alkylating solution and blotted gently. The ends of the strip that have no gel were removed; the IPG strip was then transferred to the IPG well of NuPAGE 4-12% Bis-Tris Zoom gel with the +ve end placed on the left next to the marker well containing 5 µl of unstained marker (Mark 12™ unstained standard, Invitrogen) (for western blot 3 µl of 1:1 v/v mixture of Magic and pre-stained markers used). SDS-PAGE was then run at 200V (120 mA, 25.0 W) for about 50 min. or until the dye front reached the ridge at the bottom of the gel. Gels were removed from the gel cassettes as described in 1-DE and processed according to the next experiment, i.e. Colloidal Coomassie blue staining or western blotting.

2.5.8 Colloidal Coomassie blue staining

Proteins in polyacrylamide gels were stained using Colloidal Coomassie blue stain kit according to manufacturer guidance (Invitrogen, UK). The staining method of this kit is based on the modified and enhanced protocol by (Neuhoff *et al.*, 1985). Staining was done manually and solutions were prepared based on the number of gels to be stained (e.g. for one gel: the fixing solution was prepared by mixing 40 ml deionised water, 50 ml

methanol, and 10 ml acetic acid; and the staining solution was prepared by mixing 55 ml deionised, 20 ml methanol, 20 ml stainer A, and 5 ml of stainer B that was added in the second step of staining). After the end of electrophoresis, gels (7.5% SDS gels, NuPAGE Novex 4-12% Bis-Tris mini gels, or NuPAGE 4-12% Bis-Tris Zoom mini gels) were transfer to a weighing boat and soaked in the fixing solution for 10 min. at room temperature. The fixing solution was then removed and the staining solution without stainer B was added on top of gels. Gels were soaked in this solution for 10 min at room temperature before adding stainer B to the existing staining solution. Gels were soaked in the staining solution with stainer B for about 12 h at room temperature. The staining solution was then decanted and replaced with 200 ml deionised water per gel. The gels were left to gently rock in water for at least 7 h until gels had a clear background. Gels were then scanned using an Image Scanner with MagicScan software (Amersham Biosciences) to record electronic image. The stained gels were stored submerged in 1% acetic acid (or 20% ammonium sulphate solution for long storage) at 4º C till needed for spot picking.

2.5.9 Gel-based protein identification

Gel-based protein identification was done by Cardiff University Central Biotechnology Services (CBS). After separating proteins by electrophoresis and staining with Colloidal Coomassie blue stain, gel plugs (1.5 mm diameter) were manually excised and placed in a 96-well plate. Peptides were then recovered following trypsin digestion using a slightly modified version of the (Shevchenko *et al.*, 1996) method. Sequencing grade modified trypsin (Promega, UK) was used at 6.25 ng/µl in 25 mM NH_4HCO_3 and incubated at 37º C for 3 hours. Finally the dried peptides were re-suspended in 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA; 5µl) for mass spectrometry (MS) analysis. An

aliquot corresponding to 10% of the material (0.5μl) was spotted onto a 384-well MS plate. The samples were then allowed to dry and then overlaid with α-cyano-4-hydroxycinnamic acid (CHCA, Sigma, Dorset, UK); 0.5 μl prepared by mixing 5 mg matrix with 1 ml of 50% (v/v) acetonitrile in 0.1% (v/v) TFA).

Mass spectrometry was performed using a matrix- assisted laser desorption ionization time-of-flight (MALDI TOF/TOF) mass spectrometer (Applied Biosystems 4800 MALDI TOF/TOF Analyzer; Foster City, CA, USA) with a 200 Hz solid state laser operating at a wavelength of 355nm (Bienvenut *et al.*, 2002; Brennan *et al.*, 2009; Gluckmann *et al.*, 2007; Medzihradszky *et al.*, 2000). MALDI mass spectra and subsequent MS/MS spectra of the 8 most abundant MALDI peaks were obtained following routine calibration. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolution of each other were excluded from the selection and the peaks were analyzed with the strongest peak first. For positive-ion reflector mode spectra 800 laser shots were averaged (mass range 700-4000 Da; focus mass 2000). In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.6 x 10-6 Torr) and default calibration.

Combined protein mass fingerprint (PMF) and tandem mass spectrometry (MS/MS) queries were performed using the MASCOT Database search engine v2.1 (Matrix Science Ltd, London, UK) (Perkins *et al.*, 1999) embedded into Global Proteome Server (GPS) Explorer software v3.6 (Applied Biosystems) on the Swiss-Prot database (download date 01/08/2008), and the secured database for *Ureaplasma* spp. from The J. Craig Venter Institute (JCVI), USA. Searches were restricted to the *Ureaplasma* spp.: *U. parvum* serovar 3 (strain ATCC 2715/ 27/ NCTC 11736), *U. parvum* serovar 1 (strain ATCC 27813) and *U. parvum* serovar 6 (strain ATCC 27818) taxonomy with trypsin

specificity (one missed cleavage allowed), the tolerances set for peptide identification searches at 50 ppm for MS and 0.3 Da for MS/MS. Cysteine modification by iodoacetamide was employed as a fixed modification with methionine oxidation as a variable modification. Search results were evaluated by manual inspection and conclusive identification confirmed if there was high quality tandem MS (good y-ion) data for ≥2 peptides (E value $p < 0.05$ for each peptide; overall $p < 0.0025$) or one peptide (only if E value was $p < 0.0001$).

2.5.10 Glycoprotein staining

Glycoprotein staining was used to determine the presence of any glycosylation of proteins obtained for *Ureaplasma* whole lysate. Glycoprotein staining was performed by using a glycoprotein staining kit (Biosciences, USA) to detect glycoprotein carbohydrates in a gel electrophoresis matrix. The Glycoprotein staining kit is based on a modification of the Periodic Acid-Schiff (PAS) method (Zacharius *et al.*, 1969). In this study, the SDS-PAGE gel was placed in a clean weighing tray containing 100 ml of 50% ethanol and rinsed on a shaker for 30 min. The solution was discarded and 100 ml of 3% acetic acid was added and the gel was rinsed on a rocking platform for 10 min. The solution was discarded and this wash step was repeated again. After fixing the gels, 25 ml of Glyco-Oxidising Reagent was added and gently agitated for 15 min. The gel was then washed with 100 ml of 3% acetic acid for 5 min, and the solution was discarded and the washing step was repeated twice. 25 ml of Glyco-Stain was added and gently agitated on a shaker for 15 min. The gel was then washed three times with 100 ml 3% acetic acid for 10 min and rinsed with deionised water. The gel was scanned and an electronic image was saved. The results were seen as a cluster of magenta bands (glycoprotein positive control gave magenta bands at around 40-80 kDa). The gel was subsequently stained with RAPIDstain

(provided with the kit) to view the non-glycosylated proteins and to enhance glycoprotein staining. The gel was initially washed three times in deionised water for 10 min each and then was developed with 25 ml RAPID stain for 30-40 min. Staining was monitored and when a suitable level of staining was seen, the gel was removed and washed in deionised water for 30 min on a shaker. The gel was again scanned and electronic image saved. Stained gels were stored in 3% acetic acid.

2.6 Molecular biology studies

2.6.1 DNA extraction

Genomic DNA of *Ureaplasma* was extracted using GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Gram-negative bacterial preparation procedure was followed and performed as per manufacturer recommendations. Bacterial cell pellet was obtained from 100 or 200 ml growth in USM by centrifuging for 1 hr. at 10,000 x g, and then removing and discarding the culture medium completely. The pellet was suspended thoroughly in 180 µl of lysis solution and 20 µl of RNase A solution was added and mixed. The mixture was incubated for 2 min at room temperature; and then 20 µl of the proteinase K solution was added the sample and mixed before incubating again for 30 min at 55ºC. A 200 µl of lysis solution C was added and mixed well by vortex for about 15 sec. to homogenise the mixture, and then re-incubated again for 10 min at 55ºC. The pre-assembled GenElute miniprep binding column was prepared by adding 500 µl of the column preparation solution to it and centrifuged at 12,000 x g for 1 min; and elute was discarded. 200 µl of ethanol (100%) was added to the lysate mixture and mixed thoroughly by vortexing for 10 sec to homogenise the mixture. The mixture was then transferred into the binding column using a wide bore pipette to avoid DNA shearing and centrifuged for 1 min at 6500 x g. The collection tube was discarded and replaced with a new 2 ml collection tube.

500 µl of washing solution 1 was added to the column and centrifuged at 6500 x g for 1 min. the elute was discarded and 500 µl of washing solution was added to the column and centrifuged for 3 min at 16,000 x g to dry the column. Elution was discarded and an additional centrifugation for 1 min at 16,000 x g was done to remove any residual ethanol from the column before eluting the DNA. The collection tube was discarded and replaced with a new 2 ml collection tube. DNA was eluted by adding 200 µl of the Elution solution directly onto the centre of the column and incubated for 5 min at room temperature before centrifuging at 6500 x g for 1 min. DNA yield was determined using spectrophotometer at 260 nm. DNA samples were stored at -20º C until needed.

DNA that was used for standard PCR experiments was prepared by centrifuging 300 µl of *Ureaplasma* growth at 13000 rpm for 20 min at 4º C. The supernatant was carefully removed and the cell pellet was resuspended in 50 µl of PCR water and then boiled for 10 min to lysis the cells and release the DNA. DNA samples were then stored at -20º C until needed.

2.6.2 Polymerase chain reaction (PCR)

PCR reactions were performed in 200 µl PCR tubes (Starlab, Milton Kenes) and run on a PCR cycler, the Prime Thermal Cycler (Bibby Scientific Ltd, Staffordshire, UK). PCR reagents were purchased from Promega. PCR reactions were prepared as shown in Table 2.6. All primers used in this study were synthesized by Invitrogen and are shown in Table 2.7.

Table 2.6 PCR ingredients for 20 µl and 50 µl reactions

Master mix ingredient	20 µl reaction	50 µl reaction
dH_2O	11.15 µl	27.9 µl
5X Taq buffer	4.0 µl	10 µl
$MgCl_2$ (25 mM)	1.2 µl	3 µl
dNTP	0.4 µl	1 µl
Forward primer	1 µl (5 pmol)	2.5 µl
Reverse primer	1 µl (5 pmol)	2.5 µl
Taq DNA polymerase	0.25 µl	0.625 µl
DNA template	1 µl	2.5 µl

PCR cycling parameters were common in all PCR reactions: denaturation at 95º C for 10 followed by 35-40 cycles [denaturation at 95º C for 30 seconds; annealing at temperature based on the pair primers used (see table 2.7) for 30 seconds; extension at 72º C 1min/1000bp], followed by a final extension at 72º C for 10 min. The reactions were then held at 10º C at the end of the PCR program. After each PCR experiment, PCR amplicons were run on agarose gel electrophoresis to confirm the results.

Table 2.7 Primers used in this study

No	Primer Name	Sequence (5` to 3`)	Tm (ºC)	PCR product (bp)	Reference
	Multiple Banded Antigen *UM-1*			403 (*U.p*) 448 (*U.u*)	(Teng *et al.*, 1994)
1	UMS-125	GTATTTGCAATCTTTATATTTTCG	54		
2	UMA-226	CAGCTGATGTAAGTGCAGCATTAAATTC			
3	rev MBA Uu1	AGCCCAATTCATAGGCTATTAAT	55.3		This study
4	rev MBA Uu2	GTTTTGTAGTTTCACCACTTCC	56.5		
	Gentamicin gene				
3	GentF	ACATGAATTACACGAGGGC	54.5	401	This study
4	GentR	GTTCTTCTTCTGACATAGTAG	54		
	pMT85 primers				
5	pMT85_143R	TTCCACACAATATACGACC	52.4		This study
6	pMT85_143R_bio	Biotin-TTTTCCACACAATATACGACC	52.4		This study
7	pMT85_195F	GCTATGACCTTGATTACGG	54.5		This study
8	pMT85_195R	CCGTAATCAAGGTCATAGC	54.5		This study
9	pMT85_195R_bio	Biotin-CCGTAATCAAGGTCATAGC	54.5		This study
10	pMT85_4780R	GAAAAATAATAAAGGAAGTGAGTC	54.2		This study
11	pMT85_346R	GGGACGACGACAGTATCGG	61		This study
12	start_uu_mCherry	TAGTTGAAGAAGTAATGGTAGCAC	57.6		This study
13	pMT85_56R	CCGTACTAGTGTGTCCAA	58		This study
	For circularisation:				
14	pMT85_195R	CCGTAATCAAGGTCATAGC	54.5	721	This study
15	pMT85_4294F	GATGCCAAGTTCACTTCCTAAG	58.4		This study
	For transposase:				
16	pMT85_195R	CCGTAATCAAGGTCATAGC	54.5	1190 (16+17) 419 (17+18)	This study
17	pMT85_3825F	CAACGAAACGAAAAGCCCTCAT	58.4		This study
18	pMT85_4244R	TTGTGCGCATCAGAAATAACGA	56.5		This study

Continued

Table 2.7 (*Continued*)

No	Primer Name	Sequence (5` to 3`)	Tm (ºC)	PCR product (bp)	Reference
	For Nested PCR- step1:				
19	pMT85_195R_bio	Biotin-CCGTAATCAAGGTCATAGC			This study
20	EcoR1-probe-tag	CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNGAATTC	45	Random	This study
21	EcoRV-probe-tag	CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNGATATC			This study
22	SpeI-probe-tag	CAGTTCAAGCTTGTCCAGGAATTCNNNNNNNATCAGT			This study
	For Nested PCR- step2:				
23	pMT85_143R	TTCCACACAATATACGACC	52	Random	This study
24	Toleman tag	CAGTTCAAGCTTGTCCAGG			This study
	For Mapping insertion site:				
25	pMT85_2552F	CGTAGTTAGGCCACCACTTC	57.99	556 (25+26)	This study
26	pMT85_2949R	GCGGTATCAGCTCACTCAAA	57.99		This study
27	pMT85_2747F	CGTCAGACCCCGTAGAAAAG	58.01	516 (27+28)	This study
28	pMT85_3263R	GCTTTCTCATAGCTCACGCT	58.07		This study
29	pMT85_1975F	AAGACAAATGCACGGTTTAGATT	57.29	600 (29+30)	This study
30	pMT85_2552R	CTTTTCTACGGGGTCTGACG	58.01		This study
31	pMT85_2150F	GAGGGTAAAAAGTGTTTATGCCA	57.28	530 (31+32)	This study
32	pMT85_2657R	TAGCTCTTGATCCGGCAAAC	57.98		This study
33	pMT85_2995F	GGACAGGTATCCGGTAAGCG	59.97	800 (33+34)	This study
34	pMT85_3795R	GCCATCACGTGTTCTGGGTA	60.04		This study
35	pMT85_3192F	TTTGCTGGCCTTTTGCTCAC	59.90	945 (35+36)	This study
36	pMT85_4137R	GCCGATAATTTCACGGTCGC	60.04		This study

Continued

Table 2.7 (*Continued*)

No	Primer Name	Sequence (5` to 3`)	Tm (ºC)	PCR product (bp)	Reference
For screening disrupted genes in *Ureaplasma* spp.					
37 38	pMT85_143R pMT85_195R	TTCCACACAATATACGACC CCGTAATCAAGGTCATAGC	52.4 54.5	Random size (combination of 37 or 38 with any of UU forward or revers primers, 39-54)	This study
39 40	UU390F UU390R	AGTATTCCCATTGCGACAA TATTTATTATCTTTTCTGGAGGTT	52.4	476	This study
41 42	UU187-end_F UU188_start_R	AGGTCACGATGTGTTGTTGCTGA CAAATATGGGCAACAGGAGCAG	58	600	This study
43 44	UU450_219F UU450_893R	TTGAATTGAACCCTCAGAGATCC ATTGCTTGATGGAAATGAATCCT	58	675	This study
45 46	UU520F UU520R	TCGGAGGGAGTTTGTCTCC TTTCGCAAAGGTGCAAACCA	58	730	This study
47 48	UU582_354F UU582_1230R	TTACCACGACCACTACGTCC TTATTGGCGTTGCACCAACAG	58	876	This study
49 50 51 52 53 54	per UU280F pre UU280R in UU280F inUU280R post UU280F post UU280R	GCATATGGTGTTGCTCATCG TTCATGACGTGACATACCGT ACGTCATGAAGAACGTGTTG AATACACCACCATCTGGTCC GTAGTGTTAATGCCAGGGGA AACGAACCTCTTTAGCTCGT	57.3 56.9 56.7 56.9 56.9 56.9	858 (49+50) 876 (51+52) 805 (53+54) 	This study
55 56 57 58 59	UU122 Uu 107F Up/Uu postUU122 UU pre UU122 Up pre UU122 UU122 Uu560R	GTGCAGTAATGTTAGGTCTAGG CCATAGTAAAAGGCCGAGC TATGTTTTAGCTGGTGCTGAAG TATGTTTAGCTGGCACTGAAG AACAAAAATAAAGTTGGGTGGCT	56.0 56.9 56.7 56.7 55.25		This study

2.6.2.1 PCR for speciation of *Ureaplasma* spp. and determination of serovars

PCR for separating the two groups of *Ureaplasma* spp. into *U. parvum* and *U. urealyticum* was used as described by (Teng *et al.*, 1994). Primers , UMS-125 and UMA226 (PCR UM-1) (Table 2.7), were used to amplify a DNA fragment within the *MBA* gene of *U. parvum* and *U. urealyticum* yielding different sizes of PCR product, 403 bp and 448 bp, respectively. *U. parvum* isolates were further discriminated into the four serovars (1, 3, 6, 14) by sequencing with the UM-1 primer set and aligning sequence results with the known sequences of MB antigen of the serovars (Kong *et al.*, 2000b). Differentiation of the four serovars was based on variations within two regions (between -54 to -56 and -81 to -83) of the MBA gene (Table 2.8). Diversity within MBA gene was also used in this study as an attempt to classify *U. urealyticum* isolates to known specific serovars. Furthermore, in an attempt to identify some unknown *U. urealyticum* clinical isolates, the promoter and MBA coding region were amplified using a set of primers (rev MBA Uu1 and rev MBA Uu2) designed based on conserved regions of the known genomic sequence of the prototype *U. urealyticum* serovars (Table 2.7).

Table 2.8 Nucleotide variations within MBA gene of *U. parvum*

Serovar	**Location of variation in nucleotides within MBA gene**					
	Region 1			Region 2		
	-84	-83	-82	-56	-55	-54
SV1	G	C	T	A	C	A
SV3	A	C	C	A	T	A
SV6	A	A	T	T	A	G
SV14	A	A	T	A	T	A

2.6.2.2 PCR for unknown Flanking DNA

This PCR protocol was used to map the insertion site of pMT85Gen plasmid in the genome of transformed *Ureaplasma* strains. This protocol is a modification of a method by O'Toole and Kolter (1998), which consisted of two consecutive PCR reactions. In the first PCR, a 50 µl PCR reaction for each sample was set up using a known plasmid-specific biotinylated primer, pMT85_195Rbio and one of three random primers: EcoRI-probe-tag, EcoRV-probe-tag or SpeI-probe-tag, (Table 2.7). The first round was run for 40 cycles under the conditions of 45ºC annealing and an extension time of 2 minute at 72º C. The biotinylated PCR products were immobilized on Dynabeads M280-streptavidin paramagnetic beads as per manufacturer instructions (Invitrogen, Life Technologies). Beads were initially washed three times in 2X wash buffer (2mM EDTA, 4M NaCl, 20mM Tris-HCl pH7.5), and then, 40 µl of the PCR product was mixed with 40 µl of Dynabeads (200 µg of beads) and incubated for a minimum of 15 minutes at room temperature. The beads with captured DNA were washed with 1X wash buffer (1mM EDTA, 2M NaCl, 10mM Tris-HCl pH7.5). 5 µl of beads with bound DNA fragments were then used as DNA template in the second round PCR reaction. This PCR reaction utilised a higher annealing temperature (Tm 52º C), an extension time of 2 minutes and 40 cycles. The primers used (Table 2.7) were a custom designed nested primer for the mini-transposon plasmid (pMT85_143R) and a flanking primer designed against the common 5` end of the random primers, named in the table as Toleman-tag. 5 µl PCR products were run on agarose gel electrophoresis to confirm amplification of plasmid-specific DNA fragment. Amplicons were then purified using Qiagen PCR clean up kit before being submitted to Eurofins for sequencing using the internal primers individually from the final nested amplification. Sequencing results were analysed by homology analysis comparing them against the genomic DNA of *U. parvum* serovar 3 and well as

the sequence of pMT85 to confirm and determine the accurate location of pMT85 in the genomic DNA.

2.6.3 Agarose gel electrophoresis

Following a PCR reaction, DNA from a PCR reaction was separated on an agarose gel by electrophoresis to determine the presence and size of the amplified DNA fragments. Agarose gels were prepared by dissolving a specific amount of molecular grade agarose powder (Invitrogen, UK) in a 1X Tris-Borate-EDTA buffer (TBE) (8.9 mM Tris, 8.9 mM Borate, 0.2 mM EDTA, pH 8.3) (MP Biomedicals, France). The percentage of agarose used was dependent on the expected size of the PCR product (0.7-1.5% w/v), 0.7% for DNA bands >2 kb, and 1.5% for DNA bands <500 bp. The agarose mixture was heated in a microwave oven until the agarose granules had entirely dissolved, and then it was cooled to approximately 60º C, before adding the ethidium bromide (MP Biomedicals, France) to a final concentration 1 µg/ml. The melted agarose was then immediately poured in a gel casting tray containing a comb and allowed to set at room temperature. After the gel was solidified, the casting tray with the gel was placed in the electrophoresis chamber, which was filled with the buffer TBE, and the comb was carefully removed. 3-5 µl of each PCR products (amplified DNA samples), including positive and negative controls, were loaded in each well. 5 µl of DNA ladder (Hyperladder IV, Bioline; or Kapa Universal DNA ladder, Kapa Biosystems, USA) was loaded alongside of samples and the DNA separated at 150 V for 30 min. DNA fragments were stained with ethidium bromide and visualised with a BioDoc-IT™ UV Tansilluminator (Ultra-Violet products, Cambridge) and image was saved as an electronic file on a memory card.

2.6.4 Purification of DNA from PCR product for sequencing

DNA fragments from PCR products were purified using a QIAquick PCR purification kit (Qiagen, UK). In which, five volumes of the binding buffer (PB) were added to one volume of PCR product (500 µl of buffer PB for every 100 µl of PCR product). Each sample mixture was then transferred into a QIAquick spin column that was placed on 2 ml collection tube and centrifuged at 13000 rpm for 1 min, and the flow-through was discarded. The spin column was placed back to the collection tube and 750 µl of Buffer PE was added to wash bound DNA. The column was then spun at 13000 rpm for 1 min, and the flow through was discarded. The spin column was again centrifuged for 1 min to remove any remaining ethanol from Buffer PE. The QIAquick column was transferred to a clean 1.5 ml microcentrifuge tube, and the purified DNA eluted with 50 µl of molecular grade water by centrifugation at 13000 rpm for 1 min. Assessment of quantity and purity of purified DNA was performed by measuring DNA and protein concentrations at wavelengths A260/280 nm respectively using a BIO-SPEC-1601 dual beam spectrophotometer (Shimadzu, Milton Keynes, UK) comparing absorbance against a cuvette containing the eluting buffer as a background control.

2.6.5 Sequencing PCR products

All DNA samples that were sent for sequencing were prepared by running 2 x 50µl PCR reactions for each sample and running 5 µl on an agarose gel to confirm the amplification of DNA fragment, followed by purification of the PCR product using a Miniprep kit (Qiagen). The DNA samples were then prepared for sequencing according to the company recommendations (Eurofins MWG). Samples were sent in tubes containing 15 µl of purified PCR DNA with 3 µl primer (10 µM).

Returned sequence results were analyzed by BLAST software to search sequence similarity and regions of locus similarity (NCBS http://www.ncbi.nlm.nih.gov/).

2.6.6 Purification of plasmid DNA using Miniprep kit

QIAprep spin miniprep kit (Qiagen, UK) was used to purify plasmid DNA from 5 ml growth of transformed *E. coli*. Plasmid DNA was screened by restriction digest to confirm successful ligation or investigate insert orientation, prior to scale up to obtain a higher yield of plasmid DNA that was used for *Ureaplasma* transformation. Bacterial cells (from 3-5 ml overnight growth) were harvested by centrifugation and pellet was resuspended in 250 µl Buffer P1 and then transferred to a 1.5 ml microcentrifuge tube. 250 µl of Buffer P2 was added and mixed by inverting the tube 4-6 times, and then, 350 µl of Buffer N3 was added and mixed immediately by inverting the tube 4-6 times. The tube was centrifuged at 13000 rpm for 10 min at 4º C. The clear supernatant was carefully taken and applied to the QIAprep spin column and centrifuged at 13000 rpm for 1 min, and the flow-through was discarded. The column was washed by adding 500 µl of Buffer PB and centrifuge again for 1 min, and the flow-through was discarded. The QIAprep spin column was washed again by adding 750 µl of Buffer PE and centrifuged at 13000 rpm for 1 min, and the flow-through was decanted. The column was subjected to additional centrifugation step for 1 min to remove any traces of the buffer. The QIAprep column was placed on a clean 1.5 ml microcentrifuge tube and 50 µl of molecular grade water was added to elute the plasmid DNA. Plasmid DNA samples were then stored at -20º C or directly subjected to restriction digestion by specific restriction enzyme.

2.6.7 Restriction digestion

Restriction enzyme digest was performed in a 0.5 ml microcentrifuge tube in a final volume of 20 µl. The enzyme reagents used in this study were obtained for the manufacturer, Promega. A list of all enzymes used in this study presented in Table 2.9. The reaction was set up by pipette mixing 16.8 µl (0.2-1.5 µg) of plasmid DNA, 2 µl of appropriate restriction enzyme buffer, and 0.2 µl (10 mg/ml) acetylated bovine serum albumin (BSA). And then, 1 µl (10 u/ µl) of the restriction enzyme was added and mixed gently by pipetting. When a ligation of a vector with compatible overhang ends was required, 1 µl shrimp alkaline phosphatase was added to the digest mix to prevent re-ligation of sticky-ends after digestion. The tube was then pulse centrifuged for few seconds and incubated at the optimum temperature for 1-4 hours. After incubation, 4 µl of loading buffer was added and digests were separated by agarose gel electrophoresis. Plasmid DNA fragments separated on agarose gel were then visualized and analyzed.

Table 2.9 Restriction endonucleases used in this study

Enzyme	Source	Recognition sequence	Cut site	Temperature
XbaI	*Xanthomonas badrii*	5'TCTAGA 3'AGATCT	5'---T CTAGA---3' 3'---AGATC T---5'	37° C
EcoRV	*Escherichia coli*	5'GATATC 3'CTATAG	5'---GAT ATC---3' 3'---CTA TAG---5'	37° C
SpeI	*Sphaerotilus natans*	5'ACTAGT 3'TGATCA	5'---A CTAGT---3' 3'---TGATC A---5'	37° C
HindIII	*Haemophilus influenzae*	5'AAGCTT 3'TTCGAA	5'---A AGCTT---3' 3'---TTCGA A---5'	37° C
BamHI	*Bacillus amyloliquefaiens*	5'GGATCC 3'CCTAGG	5'---G GATCC---3' 3'---CCTAG G---5'	37° C
EcoRI	*Escherichia coli*	5'GAATTC 3'CTTAAG	5'---G AATTC---3' 3'---CTTAA G---5'	37° C
BssHII	*Bacillus stearothermophilus* H3	5' GCGCGC 3' CGCGCG	5' ---G CGCGC--- 3' 3' ---CGCGC G--- 5'	50° C

(Promega, UK)

2.6.8 Ligation

In preparation, the linearized plasmid DNA (open vector) and the restricted DNA fragment (insert) were separated on 0.7-1% agarose gel stained with crystal violet (10 µg/ml), and visualised on a standard light box (to avoid formation of thymidine dimmers). The bands were then cut out with a clean scalpel and subjected to extraction and purification using QIAquick Gel Extraction Kit (Qiagen, UK) as described below. Purified linearized plasmid DNA and insert fragment DNA were then ligated using T4 DNA ligase reagents (Promega, UK). Ligation reactions were performed in 200 µl PCR tubes (Starlab, Milton Keynes, UK). The ligation was done by mixing 5 µl of insert fragment DNA, 1 µl of vector plasmid DNA, 2 µl of 10X ligase buffer, and 1 µl T4 DNA ligase (1U, Weiss units). The ligation mixture was incubated at 16º C overnight

2.6.9 Extracting DNA using QIAquick Gel Extraction Kit

The DNA band (excised from agarose gel) was weighed and 3 volumes of buffer QG were added per 100 mg of gel. The sample was incubated for 10 minutes at 50º C to dissolve the gel completely and then 1 ml of isopropanol was added to the DNA-gel solution. This solution was then pipetted into a QIAquick column and spun at 13,000 rpm for 1 minute. The sample was washed by adding 0.75 ml of buffer PE and spun again for 1 minute at 13,000 rpm. The DNA was eluted by adding 50 µl of molecular grade water to the center of the column and then subjected to centrifugation at 13,000 rpm for 1 minute.

2.6.10 Isolation of plasmid DNA using Midi kit

To scale up the yield of plasmid DNA, 100 ml growth of transformed *E. coli* was harvested by centrifugation at 6000 x g for 15 min. Plasmid DNA from the cell pellets was then isolates and purified using the QIAGEN Plasmid Midi kit (Qiagen, UK). The bacterial pellet was first re-suspended in 4 ml of Buffer P1 and mixed to lyse the cells. Then, 4 ml of Buffer P2 was added and mixed well by vigorously inverting the tube 4-6 times and incubated at room temperature for 5 min. 4 ml of chilled Buffer P3 was added to the previous mixture and immediately mixed by inverting 4-6 times, and incubated on ice for 15 min. The tube was centrifuged at 20000 x g for 30 min at 4º C. The supernatant containing plasmid DNA was promptly removed and transferred to another clean 50 ml tube and centrifuged again at 20000 x g for 15 min at 4º C. Meanwhile, QIAGEN-tip 100 was equilibrated by applying 4 ml of Buffer QBT and allowed to empty by gravity flow. After centrifugation, the clear supernatant containing plasmid DNA was removed and applied to the equilibrated QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was then washed by 2 x 10 ml Buffer QC that was allowed to go through the column by gravity flow. The plasmid DNA was eluted from the column by applying 5 ml of Buffer QF and collected in a clean 25 ml universal tube. The eluted plasmid DNA was then precipitated by adding 3.5 ml of room-temperature isopropanol, mixed and then centrifuged immediately at 15000 x g for 30 min at 4º C. The supernatant was carefully decanted, and the DNA pellet was washed by adding 2 ml of 70% ethanol without disturbing the pellet and centrifuged at 15000 x g for 10 min. The supernatant was carefully decanted and the pellet was allowed to air-dry for 5-10 min and then redissolved in 100 µl of molecular grade water. The plasmid DNA yield was determined using spectrophotometer at 260 nm and stored at -20º C till needed to be used for *Ureaplasma* transformation.

2.6.11 Pulsed field electrophoresis (PFGE)

PFGE in this study was performed according to protocols published by (Moser *et al.*, 2006; Xiao *et al.*, 2011a). *Ureaplasma* growth in 100 ml USM was harvested by centrifugation for 30 minutes at 10,000 x g. The supernatant removed and the pellet was washed 3 times in 1% PBS. The washed pellet was resuspended in 180 µl of Tris-EDTA buffer (10 mM Tris HCl, 1 mM EDTA, pH8). Agarose plugs (0.9%) were made by adding an equal volume of 1.8% agarose in TE. The plugs were then incubated overnight at 37º C in EC lysis buffer [6 mM Tris, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58 (Polyoxyethylene 20 Cetyl Ether; Sigma), 0.2% Sodium desoxycholate (Sigma), 0.5% sodium lauriylsarcosine (Sigma). The plugs were then overnight treated at 50º C with proteinase K in ESP buffer [1 mg/ml 2 mg/ml of lysozyme and 10 mg/ml of proteinase K (Sigma), 0.5 M EDTA, pH 9.2, 1% sodium lauroylsarcosine], then washed 4 times in TE buffer and stored at 4 ºC in TE buffer until needed for the restriction digestion. After equilibrating in 1X restriction buffer for 30 min, the buffer was removed and 1X buffer containing 40U BssHII (Promega, UK) was added and the plugs were incubated at 50 ºC for 2 h. Digested agarose plugs were positioned in 1% PFGE grade agarose gel (Invitrogen) and PFGE was run on a CHEF-DR III variable angle system (Bio-Rad, UK) using the following parameters: 200 V (6V/cm); temperature 14 ºC; initial switch 1 s; final switch 40 s; time 20h. DNA fragments were stained with ethidium bromide and visualised with a BioDoc-IT™ UV Tansilluminator (Ultra-Violet products, Cambridge) and image was saved as an electronic file on a memory card.

2.6.12 In Gel DNA/DNA hybridization

Ureaplasma genomic DNA was extracted from the *Ureaplasma* cells using GenElute bacterial genomic DNA kit as described above (section 2.6.1). 200 µl genomic DNA was subjected to digestion with HindIII; and digests were purified using QIAquick PCR purification kit (Qiagen, UK) as described above. The whole volume of purified HindIII digested genomic DNA sample (about 30 µl each sample), along with HindIII digested pMT85 plasmid as a positive control, were run on 0.7 % agarose gel stained with ethidium bromide for 4 h to separate DNA fragments. Separated DNA fragments were visualised and images were saved. The gel then was put between two filter papers and dried overnight at 50ºC; and then saved till needed.

To prepare for hybridization, the dried gel was re-hydrated again by soaking in deionised water for 5 min at room temperature. The gel then was denatured and neutralized subsequently by incubating in neutralising solution (0.5M NaOH and 1.5M NaCl) for 30 min at room temperature followed by another 30 min incubation at room temperature in neutralising solution (0.5M Tris-HCl, pH 7.5, 1.5M NaCl). The gel was transferred to a hybridisation tube, and 20 ml of pre-hybridisation solution [6X saline-sodium citrate buffer (SSC), 0.1% (w/v) polyvinylpyrrolidone 400, 0.1% (w/v) Ficoll, 0.1% (w/v) Ultra-high temperature (UHT) whole milk, 0.5 (v/v) SDS and 150 µg/ml denatured herring sperm DNA] was added and then incubated overnight at 65ºC in a hybridization oven (Techne, Barlow world scientific Ltd, Stone, UK) before being probe with gentamicin resistance gene ^{32}P dCTP labelled probe.

Labelling the DNA probe (gentamicin resistance gene) was performed using Prime-It II Random Primer Labelling kit and according to manufacturer recommendations (Startagen-Agilent Technologies). Gentamicin resistance gene (*aac-aphD*; 6'-aminoglycoside N-acetyltransferase) was first amplified by PCR using pMT85 plasmid

DNA, which carries the gene, as DNA template and primers set designed against the gene; Gent1 (forward) and Gent2 (Reverse) (401 bp amplicon, Tm=54°C, 35 cycles). After analysis on agarose gel, PCR amplicon was then purified using the QIAquick PCR purification kit before being labelled. To label the DNA probe, 16 µl of gentamicin resistance gene DNA (25ng), 8 µl of DNase-free water, and 10 µl of random oligonucleotide primers were add into the bottom of a screw-top eppendorf tube. The reaction tube was heated in a boiling water bath for 5 min and then centrifuged briefly at room temperature. Then, 10 µl of 5X dCTP primer buffer, 2.5 µl of [α-^{32}P], and 1 µl Klenow enzyme (5 U/µl) were added quickly to the tube and mixed thoroughly with the pipet tip. The reaction tube was incubated at 37ºC for 15 min. After incubation, the reaction was stopped by adding 2 µl stop mix. The labelled DNA probe was then purified by removing any unincorporated nucleotides using sephadex G-50 DNA grade column, illustra NICK Columns, (GE Healthcare, Amersham, UK). Then, 320 µl of 0.1 M Tris-HCl buffer, pH7.5, was added into the column and allowed to run through by gravity in a collection tube. The column was moved to a new collection tube, and additional 430 µl of Tris buffer was added. The run through liquid was collected and boiled for 6 min. The labelled probe, the gentamicin resistance gene ^{32}P dCTP labelled probe, was added directly to the gel to be probed in hybridisation tube, containing 20 ml pre-hybridization solution, and incubated overnight at 65ºC. The next day the gel was washed once with 100 ml of 2X SSC buffer for 30 min at 65ºC in a hybridisation oven. Then, it was washed 1-2 times with 0.1X SSC buffer for 30 min at 65ºC. The probed gel membrane was then wrapped in clingfilm and transfer to an X-ray cassette. DNA/DNA hybridisation reaction was visualised as an autoradiograph image on an X-ray film (Fujifilm, Tokyo, Japan) following an overnight exposure at - 80ºC. Autoradiograph images were scanned stored as TIFF files before being analysed.

2.7 Transformation of *Ureaplasma*

2.7.1 Bacterial strains

2.7.1.1 Escherichia coli

One Shot Top10 chemically competent *E. coli* cells (Invitrogen; Paisley, Scotland, UK), were used as per manufacturer's instructions. This bacteria has the genotype F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-.

2.7.1.2 *Ureaplasma* spp.

Three *U. parvum* strains for serovar 1 (DFK-1, O10, and HPA78), serovar 3 (HPA5, U6, and HPA56) and serovar 6 (HPA2, HPA61, HPA58) were investigated for the capacity to undergo transposon mutagenesis, and these strains were initially isolated from patients, but have been purified through sub-cloning and have also been characterised and used in previous publications (Beeton *et al.*, 2009b; Beeton *et al.*, 2012). Various ATCC strains of *U. urealyticum* representing serovar 2 (ATCC 27814), 4 (ATCC 27816), 8 (ATCC 27618), 9 (ATCC 33175), and 10 (ATCC 33699) were generously supplied by Dr. Janet Robertson, (University of Alberta, Canada), these strains as well as a clinical isolate, W11 (serovar 12) (Beeton *et al.*, 2009b), were investigated in parallel.

2.7.2 Expression constructs and the mini-transposon plasmid

The Tn*4001*-based mini-transposon plasmid, pMT85, has been previously described by Zimmerman and Herrmann (2005) and was generously provided by Prof. R. Herrmann (Heidelberg University, Germany). The gentamicin resistance gene was not codon-usage

optimised for *Ureaplasma* spp. expression. Expression constructs were generated by ligating various DNA fragments into the cloning vector pMT85. These constructs were made to try to create florescent *Ureaplasma* cells and also study the capability of *Ureaplasma* to express foreign genes. DNA insert constructs were synthesised, as required, by GenScript (GenScript, NJ, USA) and cloned into the vector pUC57, which contains ampicillin resistance gene as a selection marker. Upon arriving, the lyophilized plasmid DNA (pUC57) with a custom synthesised DNA insert was centrifuged at 6000 xg for 1 minute. DNA was the dissolved in 20 µl of sterilized molecular grade water by vortex mixing for 1 min. The plasmid DNA was then stored at -20 until needed. DNA inserts were excised from pUC57 (Using the restriction enzyme, XbaI) and ligated to the expression vector pMT85 (using T4 ligase). In this study, the pMT85 plasmid was the backbone vector for all DNA fragment inserts used to transform *Ureaplasma* spp., after transformation, validation and amplification in *E. coli*. All *mCherry* inserts used in this study are listed in Table 2.10.

2.7.3 Generating expression vector with different copy number of *mCherry*

Besides carrying a single copy of a variety of modified *mCherry* inserts, pMT85 was also engineered to carry more than one copy of the same DNA fragment under independent promoter expression. The aim of this experiment was to investigate the effect of copy number on expression of red fluorescent protein (RFP). A number of different constructs with one, two, three, or four copies of *mCherry* was generated, cloned and tested.

Table 2.10 The *mCherry* inserts used in this study

No	Insert name	Construct code	Description	Codon-usage	Gene length	Reference
1	Tandem *mCherry*	GRT	tandem copy of *mCherry*, with *U. parvum* SV3 EF-Tu promoter	Most frequent in *Ureaplasma* optimised UGA	1804bp	This thesis
2	Zimmerman	GRZ	Fused *mRFP1* with *M. pneumoniae* EF-Tu promoter as described by publisher	Non optimised	1011bp	(Zimmerman and Herrmann, 2005)
3	OPTmCherry3	GR-m	Optimised *mCherry* insert with His-tag ,*U. parvum* SV3 EF-Tu promoter and BamHI restriction site	Based on tRNA identified by Paralanov et al., (2012)	926bp	This thesis
4	uRFP-his	uRFP-his	*mCherry* with His-tag and *U. parvum* SV1 MBA promoter	Most frequent in *Ureaplasma* optimised	950bp	This thesis
5	Half tandem *mCherry*	GR-½Tn	½ tandem *mCherry* of the above construct (1) with minmal modification in the codon-usage	Most frequent in *Ureaplasma* optimised UGG	917bp	This thesis

2.7.3.1 Making expression vector with four-copy insert using tandem *mCherry* insert

A construct with 4 copies of *mCherry* insert was the first to be made by inserting two copies of optimised tandem *mCherry* insert into pMT85. First, pMT85 plasmid was cut with SpeI and dephosphorylated with SAP to prevent re-ligation, and then the linearized vector was used to receive the first copy of the optimised tandem *mCherry* insert in the SpeI site. The insert was cut with XbaI (which has a complementary overhang) so that following ligation the resultant plasmid could be cut with XbaI without removing the insert as the combined XbaI/SpeI ligation site is inactive for both restriction enzymes). Both linearised vector and the insert were initially purified using QIAquick Gel Extraction kit (see section 2.6.8) before ligation with T4 ligase. The pMT85 vector with one copy tandem *mCherry* was cloned into One Shot TOP10 *E. Coli* cells, and following confirmation of successful ligation by restriction of single colonies, the bacteria containing the plasmid was scaled up, extracted and purified using the QIAGEN Plasmid Midi kit (Qiagen, UK). Second, the pMT85_1Tn (with one copy tandem *mCherry*) was then cut again with XbaI (with SAP treatment), and another copy of tandem-*mCherry* was ligated into the single remaining XbaI site using T4 ligase. The correct construct with two copies tandem *mCherry* (total of 4 copies *mCherry*) was scaled up, extracted, and purified. After validation, both constructs (with 2 and 4 copies *mCherry*) were stored at -20º C until required for *Ureaplasma* transformation. Glycerol stocks of clonal *E. coli* containing confirmed expression vectors were also made and stored at -80 ºC.

2.7.3.2 Generating expression vector with different copy numbers using other inserts

In a similar way, other constructs with various numbers of DNA fragments were made. Inserts were ligated in the same restriction sites mention above, i.e. XbaI and SpeI sites.

Using this method, it was possible to generating expression vectors with one copy and two copies using ½ tandem *mCherry* fragment as well as expression vectors carrying one, two, three copies using OPT-*mCherry* insert. All these constructs were validated using restriction digestion analysis and/ or sequencing and then amplified, purified and stored at -80 ºC till needed.

2.7.4 Transformation of bacteria

Transformation of One Shot Top10 chemically competent *E. coli* cells was performed as per manufacturer's instructions using heat shock at 42°C and the plasmid containing bacteria selected with 15 mg/l gentamicin. Transformation of *Ureaplasma* spp. was carried out essentially as outlined for *M. mycoides* in King and Dybvig (1991) with some modifications. The key aspect of *U. parvum* growth is the conversion of urea to ammonium ions which increase the pH of the growth medium from pH =6.2 (yellow) to pH >9 (dark red). Three 96-well plates containing 10-fold serial dilutions of *Ureaplasma* (200 ml per well, titrated from Rows A-H) were set out the night before the experiment and all of the wells showing pH change consistent with the threshold of detection for phenol red indicator (pH =7) were pooled (total volume 10 ml) and utilised for transformation as these represent *U. parvum* in log phase growth. This 10 ml routinely gave titrations of $5x10^8$ CFU and were washed three times (centrifuged at 10000g for 20 min) with 1 ml of 1X Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen, Paisley, U.K.) at 4°C. The washed pellet was resuspended in a volume of 375 µl of 100 mM $CaCl_2$ and incubated on ice for 30 min, then a volume of 100 µl of bacterial cells containing 10 µg of yeast tRNA, 6 µg of pMT85 and 1 ml of 50% PEG-8000 were added at room temperature for 1 min. The transformation mixture was diluted by directly adding a volume of 5 ml of USM. Cells were allowed to recover in *Ureaplasma* selective medium (Mycoplasma experience plc, Surrey, UK) at 37°C for 3h and the growing cells

were pelleted by centrifugation at 3600g for 15 min at 4 C before being resuspended again in 1 ml USM. A volume of 20 µl cell suspension was diluted in USM in a 1/10 dilution series containing 128 mg/L gentamicin and incubated overnight. Control bacteria treated identically (except for the addition of pMT85) were run in parallel and no spontaneous gentamicin resistance was observed. When the first well of transformed bacteria turned the media red, the cells were plated out on *Ureaplasma* selective agar (Mycoplasma Experience ltd) and individual colonies were examined for presence of gentamicin resistance gene by PCR, prior to further characterisation. In some experiments a dilution series transformed bacteria were directly plated onto *Ureaplasma* selective agar plates containing 128 mg/L gentamicin to determine transformation efficiency relative to *Ureaplasma* selective plates without gentamicin. *Ureaplasma* cell were also transformed using pMT85 plasmid carrying different DNA fragments to investigate the expression of exogenous genes under specific parameters.

2.7.5 Screening of transformed bacteria

Successful transformation of gentamicin resistant bacterial clones was confirmed using PCR and primers (Table 2.7) designed against the gentamicin resistance gene (*aac-aphD*; 6'-aminoglycoside N-acetyltransferase); Gent1 (forward) and Gent2 (reverse) (401 bp amplicon, Tm=54°C, 35 cycles) using standard PCR methods amplified by Promega GoTaq green DNA polymerase. Mapping insertion site of pMT85Gen plasmid in the genome of transformed *Ureaplasma* strains was determined as previously mentioned in (section 2.6.2.2). *U. parvum* genes disrupted by transposon insertion into the genome were confirmed using primers against *Ureaplasma* genes: UU390 (hypothetical membrane protein); UU450 (hypothetical membrane protein); UU520 (hypothetical membrane protein); UU582 (RNA helicase); intergenic insertion between UU187 and

UU188. Amplifying the ends of the insertion site was performed using one of the above primers in combination with pMT85-specific primers (designed close to the 5' and 3' inverted repeat sequence) 195R (5'- CCGTAATCAAGGTCATAGC-3', Tm=54.5°C) or 3192F (5- TTTGCTGGCCTTTTGCTCAC-3', Tm=57°C) (Tables 2.6 and 2.7) at the lowest annealing temperature. Amplicons were purified using the Qiaquick PCR clean up kit (Qiagen, Manchester, U.K.) and submitted to Eurofins MWG Operon (Ebersberg, Germany) for sequencing. Primers specific to pMT85 (Table 2.6) were also used to determine if transposon insertion only utilised sequences between the inverted repeats (1-3437bp) or whether plasmid sequence containing the *tnp* transposase gene (3438-4820bp) were also present in the genome insertion. Figure 2.3 illustrates the way of mapping the insertion site of pMT85 plasmid and identifying disrupted genes in *U. parvum* genome.

Genomic DNA analysis was performed on a Genomic DNA extracted from gentamicin resistant transmutant strains using the GenElute Bacterial genomic DNA Kit (Sigma-Aldrich) as described above (see section 2.6.1). DNA from each preparation was either ethanol precipitated, resuspended in 10 µl of molecular grade water and utilised for Sanger sequencing of purified genomic DNA to determine the transposon insertion site using pMT85 primers 195R or 3192F, or digested with HindIII, separated on a 1% agarose gel and utilised for in gel radioactive probe hybridization experiments as previously described (section 2.6.12).

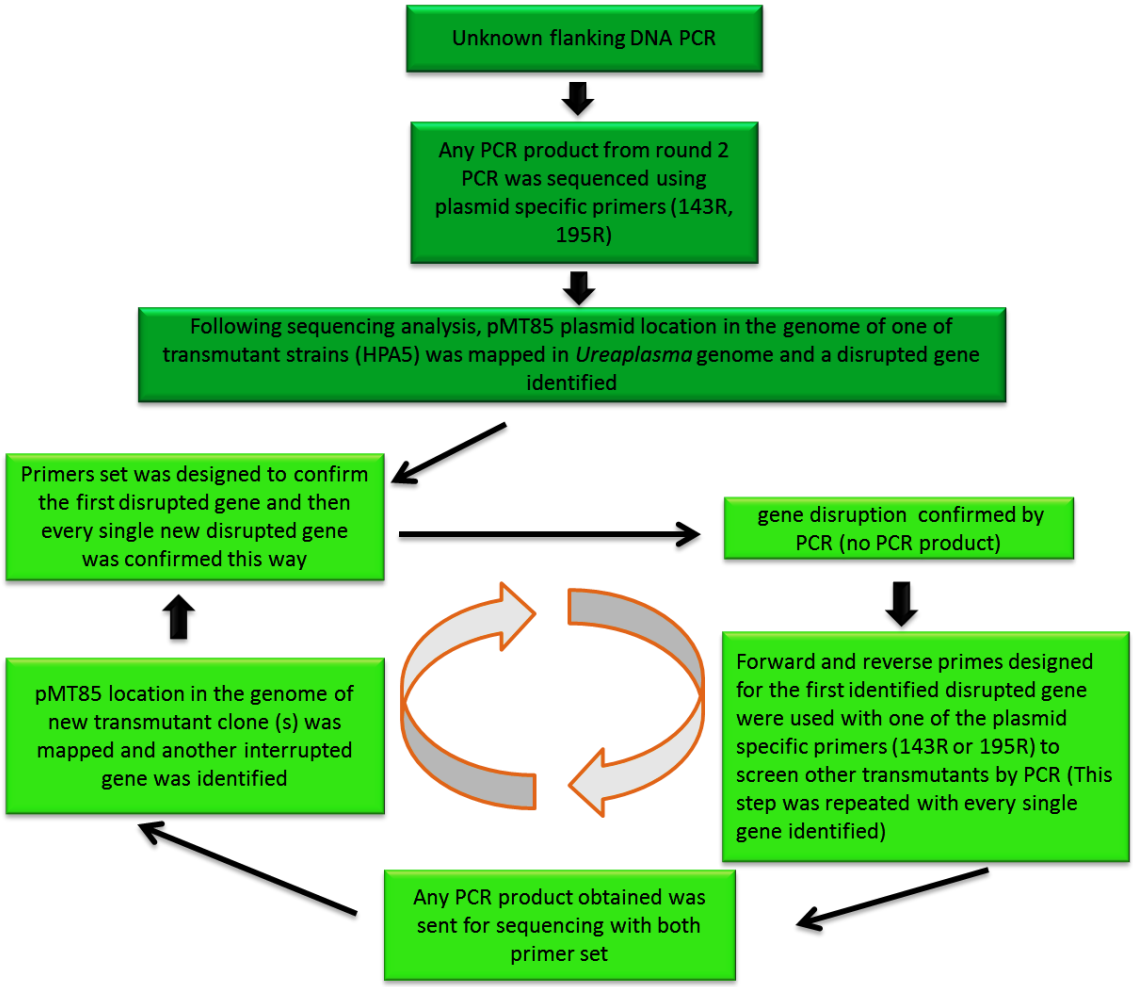

Figure 2.3 Schematic flow chart illustrates the way of mapping the insertion site of pMT85 plasmid and identifying disrupted genes in *Ureaplasma* genome. Following unknown flanking DNA PCR, the transposon insertion site was mapped in one of the transmutant strains. The first disrupted gene identified was confirmed using primer set designed to amplify open reading frames listed for the *U. parvum* ATCC serovar 3 strain 700970. Using standard PCR using both forward and reverse primers designed for the disrupted gene combined with plasmid specific primers (pMT85_143R or pMT85_195R), all other transmutant strains were screened and new pMT85 plasmid locations mapped and other disrupted genes identified. This process was repeated with every successful experiment.

Immunoblot analysis for strains under investigation was performed as detailed above (section 2.4). Blots were probed with either human high titre anti-*Ureaplasma* sera (Beeton *et al.,* 2012) or Virostat plc (Portland, ME) monoclonal anti-multiple banded antigen (MBA) antibody (clone 6525 or 6522). Bound human and mouse antibodies were detected with appropriate peroxidase-conjugated secondary antibodies from Jackson ImmunoResearch Europe Ltd (Newmarket, U.K.) and Pierce ECL detection reagent (Fisher Scientific, Loighborough, U.K.). In addition, other antibodies such as polyclonal anti-mCherry antibody, anti-His tag antibody, and anti-UU280 antisera (Table 2.3) were also used to investigate the expression of specific proteins by *Ureaplasma* spp. after transformation. Serum killing assay was also performed as detailed above (section 2.2.2), to screen any changes in serum susceptibility of transmutant strains relative to original strains.

2.7.6 Fluorescent microscopy

Fluorescent microscopy was used to view and analyse the expression of RFP by transmutant strains from both *E. coli* and *Ureaplasma* spp., under several parameters. Plates with growing colonies from transmutant strains were screened and visualized using a Leica DM IRE microscope (Leica, Germany) with a Hamamatsu ORCA-ER camera (Hamamatsu, Japan) and Imrovision Openlab 3 software (Improvision, UK) under objective power lenses 4X and 10X, and an appropriate cut-off filter was used for RFP. Images were captured and saved as TIFF files.

2.7.7 Qualification of red fluorescence from liquid cultures

Transmutant strains of *E. coli* harbouring constructs of *mCherry* or control strains (without *mCherry* insert) were grown overnight in LB medium supplemented with 15 mg/L gentamicin at 37 ºC. At an optical density (O.D) at 600 nm ($O.D_{600}$), the number of growing cells was adjusted to 1.0 to equalize the growth in all samples and a 100 µl of adjusted growth of each transmutant strain was transferred to a 96-well plate. In contrast, *Ureaplasma* transmutant strains carrying *mCherry* constructs were grown in USM supplemented with 128 mg/L gentamicin by titration in a 96-well plate at 37 ºC for 24 hr. Red fluorescence protein was then measured using a fluorometer, FLUOstar OPTIMA (BMG LABTECH GmbH, Germany) set at an excitation wavelength of 544 nm and emission detection at 590 nm.

2.8 Statistical analysis

Statistical analysis of data in this thesis was performed using Graphpad Prism software version 5.01 (Graphpad Software Inc). All serum killing assays data were expressed as mean and standard error of the mean (SEM). Significance, when needed, was taken as a *P* value < 0.05.

Chapter 3

The complement activity against *U. urealyticum*

3. The complement activity against *U. urealyticum*

3.1 Introduction

The bactericidal activity of normal human serum, which is mediated by complement system, plays an important role in the host defence against infection. Complement system is composed of more than 30 proteins circulating in serum and bound to cell surfaces. This system is activated via three defined pathways, the classical, the alternative, and the lectin pathways. Different initiating proteins in these pathways are able to recognise specific bacterial ligands and trigger the process of complement activation. The formed C3 convertases from these three complement pathways meet at the hydrolysis of the central C3 component and activate the common terminal pathway, which leads to the formation of membrane attack complex (MAC) (Walport, 2001b). Upon activation, the complement system either leads to direct cytolysis (particularly in Gram-negative bacteria) or bactericidal activity via opsonisation and phagocytosis, as is the case with Gram-positive bacteria (Fierer *et al.*, 1972; Joiner *et al.*, 1984).

Serum-resistant bacteria are believed to be more pathogenic as they are able to cause systemic infection. Many pathogens isolated from blood cultures have been found to be serum resistant (Joiner *et al.*, 1984). While all isolates of a species are not inherently serum-sensitive and serum-resistant, serum-resistant isolates for Gram-negative including *Escherichia, Klebsiella, Enterobacter, Pseudomonas, Salmonella,* and *Citrobacter* have been documented (Fierer *et al.*, 1972). The mechanism by which such strains had become resistant may vary from one species to another. For instance, one mechanism of serum-resistance in *E. coli* clinical isolates was found to be via higher O-antigen-substitution of lipopolysaccharide subunits in the outer membrane (Porat *et al.*, 1987). In general, many

pathogenic microbes have developed strategies to avoid complement killing and invade and cause diseases in humans; many of these evasion mechanisms are now well-defined as reviewed in (Celli and Finlay, 2002; Kraiczy and Wurzner, 2006; Lambris *et al.*, 2008).

Ureaplasma spp. and other members of the Mollicute class are unique among other types of bacteria in that they lack a classical bacterial cell wall that provides a protective coat to many bacteria. There are two human associated species within the genus *Ureaplasma*: *U. parvum* that consists of four serovars (SVs 1, 3, 6, and 14) and *U. urealyticum* that contains the remaining 10 serovars (SVs 2, 4, 5, 7-13). The first group is more frequently isolated from the urogenital tract of healthy men and women, but the second group is thought to be more pathogenic in male urethritis (Deguchi *et al.*, 2004; Waites *et al.*, 2005). The susceptibility of *U. parvum* to complement-mediated serum killing was investigated previously in my host laboratory (Beeton *et al.*, 2012). The results of that study showed variations in serum susceptibility among the four representative serovars, and complement killing was found to occur via the classical pathway (totally C1q dependent). However, complement activity against the other group of human ureaplasma is still unknown. Therefore, the aim of this first chapter of my thesis is to investigate the complement activity against *U. urealyticum.* In addition, characterisation and speciation of some unknown clinical isolates will be attempted

3.2 Results

3.2.1 Killing of *U. urealyticum* by seronegative human serum

The 10 standard serovars and 11 clinical isolates of *U. urealyticum* were investigated for their susceptibility to complement-mediated serum killing using 3 seronegative sera, two

were previously characterised against *U. parvum* (Beeton *et al.*, 2012) and one identified in this study (defined as an inability to detect separated *U. urealyticum* proteins by immunoblot). Complement killing assay was performed as described in section 2.2.2 using 50% NHuS. The results, summarised in table 3.1, showed that of all the 10 prototype *U. urealyticum* serovars, only three serovars, SV4, SV9, and SV13, were very sensitive to complement of all seronegative sera utilised (VxF1, VxF9, and VxM18). The growth titre reduction (fold-killing) ranged from > 100-fold to > 1000-fold killing reduction. SV5 and SV10 showed intermediate susceptibility to killing with VxF1 and VxM18 sera (< 100-fold killing) but were resistant to VxF9 (< 10-fold). Serum VxM18 also had intermediate killing activity against SV8 (killing fold reduction is < 100-fold). The remaining serovars were resistant to killing by all seronegative sera (>90% survival or <10-fold killing) (Figure 3.1). In parallel with known serovars, clinical isolates also demonstrated variations in susceptibility to complement killing using seronegative sera. HPA17, HPA24, HPA43, and HPA44 were killed by all sera, with a reduction in surviving titre of >1000-fold (i.e. fold killing) in most cases. While other clinical isolates (HPA3, HPA4, HPA6, HPA12, HPA20, HPA3, and W11) were resistant to complement killing (Figure 3.2).

As all sera used here were previously characterised using immunoblot as seronegative (i.e. there were no antibodies against *U. parvum*), the presence or absence of anti-*Ureaplasma* IgG antibodies against *U. urealyticum* was also investigated in this study using immunoblot analysis. Immunoblot analysis of all sera to whole cell lysates from all prototype and clinical *U. urealyticum* isolates confirmed the absence of any detectable anti-*Ureaplasma* antibodies (Figures 3.1 and 3.2). All negative blots were subsequently re-probed with seropositive serum to rule out any false negative results. Therefore, this

indicates that killing of serovars and clinical isolates by seronegative sera was not mediated by the presence of specific anti-*Ureaplasma* antibodies.

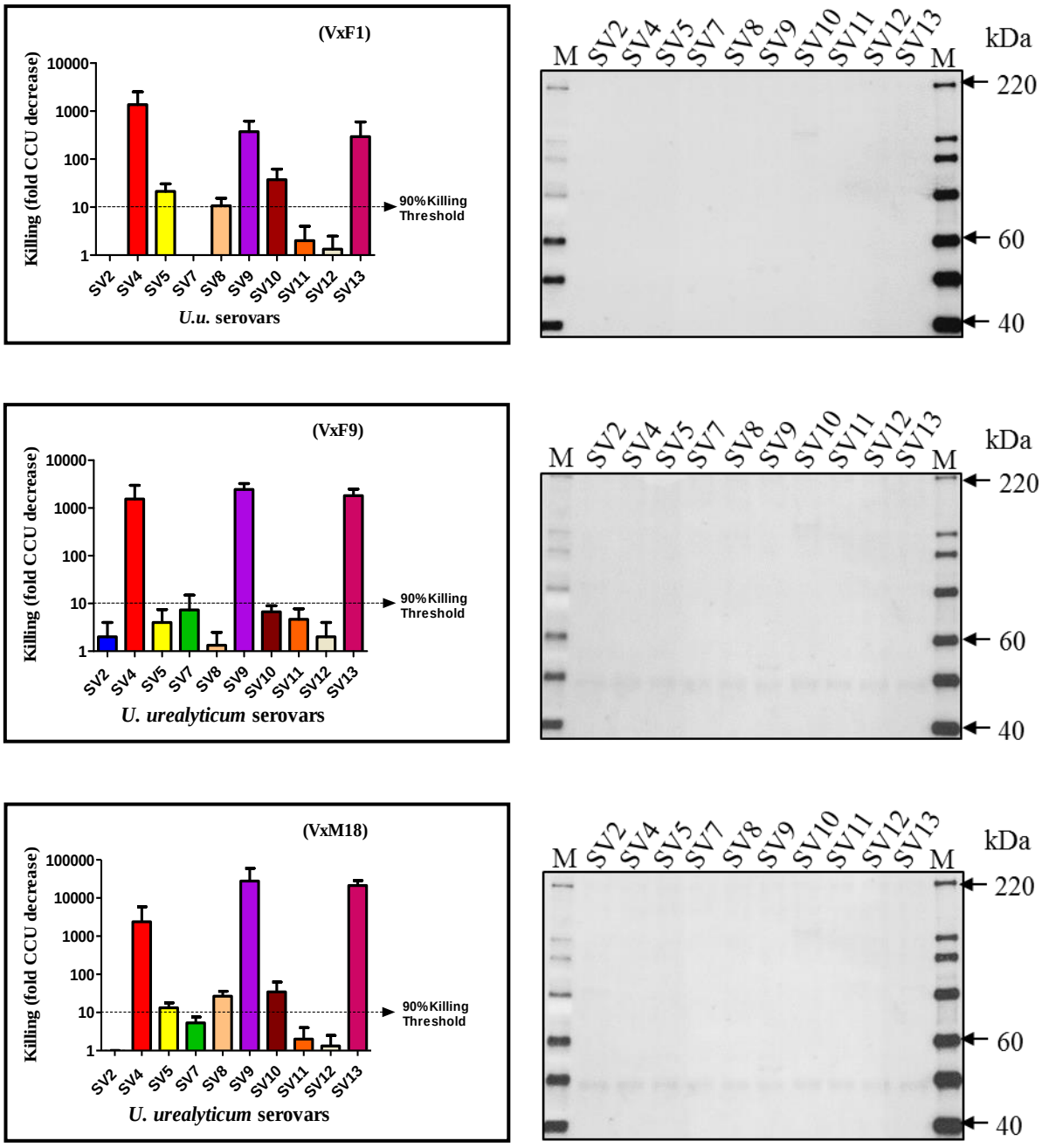

Figure 3.1 Complement killing and immunoblot analysis of *U. urealyticum serovars* using seronegative NHuS. On the left side: graphs show the bactericidal activity of 3 NHuS: VxF1, VxF9 & VxM18 against all known *U. urealyticum* serovars (2, 4, 5, 7-13). On the right side: immunoblot analysis of whole cell lysate of the serovars probed with 1:100 dilution of the matched NHuS on the left side of the image followed by secondary antibody (peroxidase-conjugated anti-human IgG). M: Molecular mass marker (kDa).

This immunoblot was serially probed with the three seronegative sera, but showed strong bands when subsequently probed with a seropositive serum.

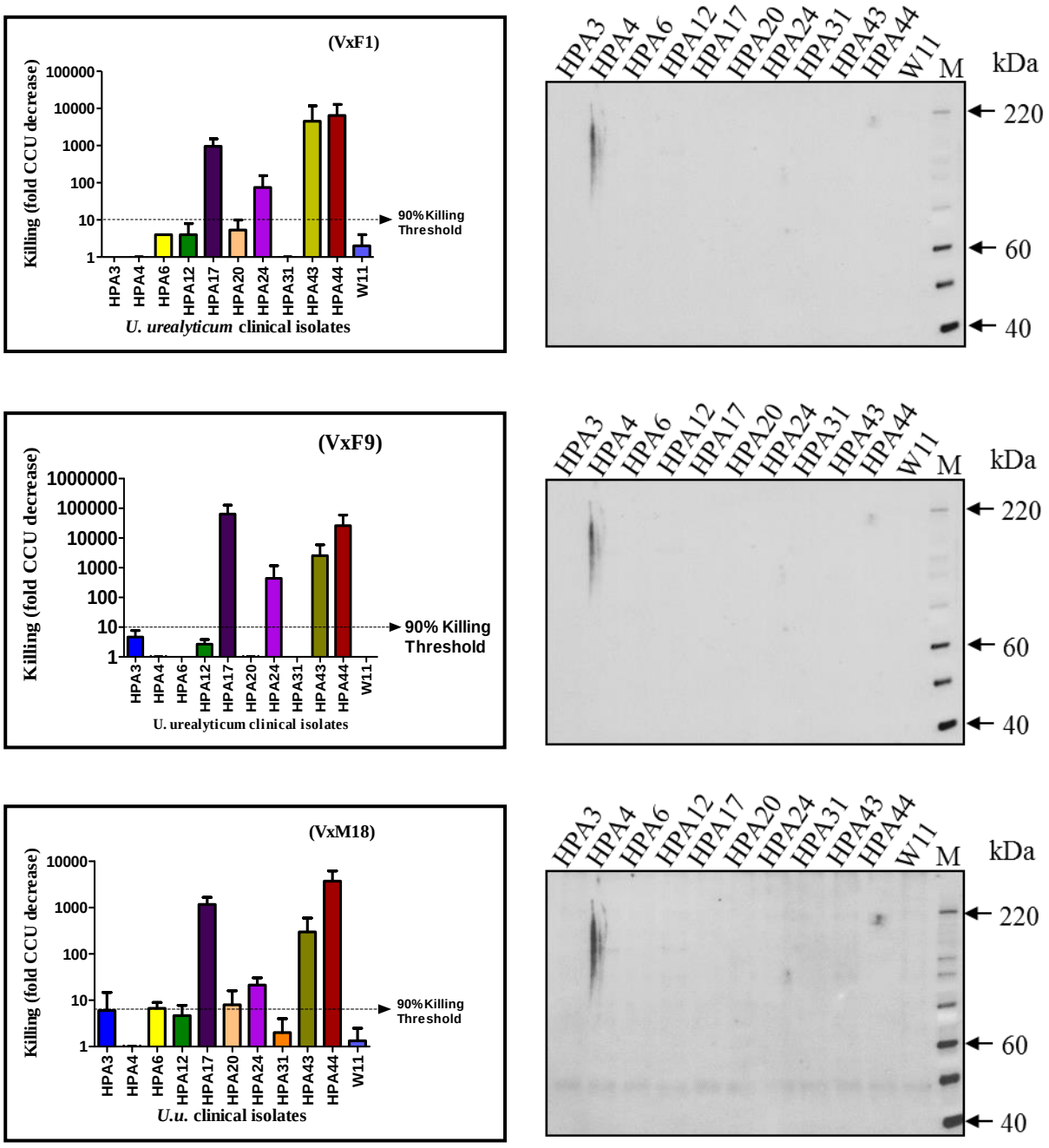

Figure 3.2 Complement killing and immunoblot analysis of 11 *U. urealyticum* clinical isolates using seronegative NHuS. On the left side, graphs show the bactericidal activity of 3 NHuS: VxF1, VxF9 & VxM18 against 11 clinical isolates of unknown serovars of *U. urealyticum* (W11 & HPA 3, 4, 6, 12, 17, 20, 24, 31, 43, & 44). On the right side, western blot analysis of whole cell lysate of the clinical isolates probed with 1:100 dilution of the matched NHuS on the left side of the image followed by secondary antibody (peroxidase-conjugated anti-human IgG).M; Molecular mass marker (kDa). This immunoblot was serially probed with the three seronegative sera, but showed strong bands when subsequently probed with a seropositive serum.

3.2.2 Killing of *U. urealyticum* by seropositive human serum

Serovars and clinical isolates were next challenged with normal human sera that were shown to have detectable anti-*Ureaplasma* antibodies that strongly recognised a large number of immunoreactive protein bands from separated *U. parvum* proteins (Beeton *et al.*, 2012). *U. urealyticum* was incubated with three different seropositive sera (VxF7, VxM11, and VxM12) to investigate if the presence of specific anti-*Ureaplasma* antibodies increased the susceptibility to complement attack. Figures 3.3 and 3.4 show that the bactericidal activity of seropositive sera had a greater degree of killing compared to seronegative sera. However, wide variations in susceptibility to complement killing among serovars and clinical isolates were still observed. Of the 10 *U. urealyticum* prototype serovars, three were completely resistant to complement-mediated killing from all seropositive sera in spite the presence of detectable anti-*Ureaplasma* antibodies. These serovars included SV2, SV11, and SV12. All other serovars (4, 5, 8, 9, 10, and 13) were susceptible to serum with killing higher than 1000-fold for the majority of them (Figure 3.3). One exception was noted, SV7 was sensitive to VxF7 and VxM11 but resistant to VxM12. No increased susceptibility for seronegative-susceptible strains SV4, SV9 and SV13 were observed, as they were almost completely killed by seronegative sera (Figure 3.1). However, complement-mediated killing of serovars 5, 7, 8 and 10 were increased by the presence of anti-*Ureaplasma* antibodies, while they were resistant to killing by seronegative sera.

Similarly, as shown in Figure 3.4, clinical isolates also showed variability to killing by seropositive sera. Some clinical isolates were resistant to complement-mediated killing even with the presence of anti-*Ureaplasma* antibodies: clinical isolates HPA3, HPA4, HPA6, HPA31, and W11 were resistant to killing by all seropositive sera used (less than

10-fold killing) and exhibited a similar susceptibility pattern to that of prototype SV2, SV11, and SV12. Some clinical isolates were more susceptible to a particular serum than other sera: HPA12, for example, showed intermediate susceptibility to sera VxF7 and VxM11 (> 100-fold killing) but was resistant to serum VxM12. Clinical isolates HPA43 and HPA44 were highly susceptible to serum killing to both seronegative (Figure 3.2) and seropositive (Figure 3.4) sera, while killing was increased for HPA17, HPA20, and HPA24 following incubation with seropositive sera relative to seronegative sera.

Immunoblot analysis using these seropositive sera showed immunoreactivity with a broad range of immunoreactive *Ureaplasma* proteins. This confirmed the presence of specific anti-*Ureaplasma* IgG antibodies (some recognising bands of the same molecular mass between isolates, others showing unique bands being recognised), as was previously shown for characterisation of *U. parvum* serum susceptibility (Beeton et al., 2012). Some serovars and isolates had several strong immunogenic bands whereas others have only few bands (Figures 3.3 and 3.4). Of all sera, VxF7 recognised a higher number of bands with different sizes for each serovar or isolate compared to the other two sera (VxM11 and VxM12). VxM11 seemed to recognise a common immunogenic protein band at about 50 kDa in both serovars and clinical isolates and another set of conserved double bands, with an apparent molecular mass just higher than 60 kDa, across all serovars. VxM12 recognised a few bands with low intensity among both serovars and clinical isolates.

The presence of immunoreactive protein bands and the intensity of the immunoreactivity showed no correlation to the susceptibility to serum killing. In fact, some serovars and clinical isolates were totally resistant to complement killing (e.g. SV2 and W11) despite the presence of a number of antigenic protein bands, which reacted strongly with antibodies (Figures 3.3 and 3.4). In contrast, others had few antigenic bands but showed

high susceptibility to the bactericidal activity of serum, (e.g. SV9 and HPA17) (Figures 3.3 and 3.4). The antigenic banding pattern detected by antibodies varied among serovars and clinical isolates as well as with the different seropositive sera used. However, some clinical isolates showed distinctive consistent banding patterns across the three sera (HPA20, HPA24, and HPA31); those clinical isolates had interestingly shown similar immunoblot banding pattern but had different serum susceptibility. HPA31 was totally serum-resistant while HPA20 and HPA24 were serum-sensitive. These observations indicate both antigenic diversity and conservation among serovars and clinical isolates.

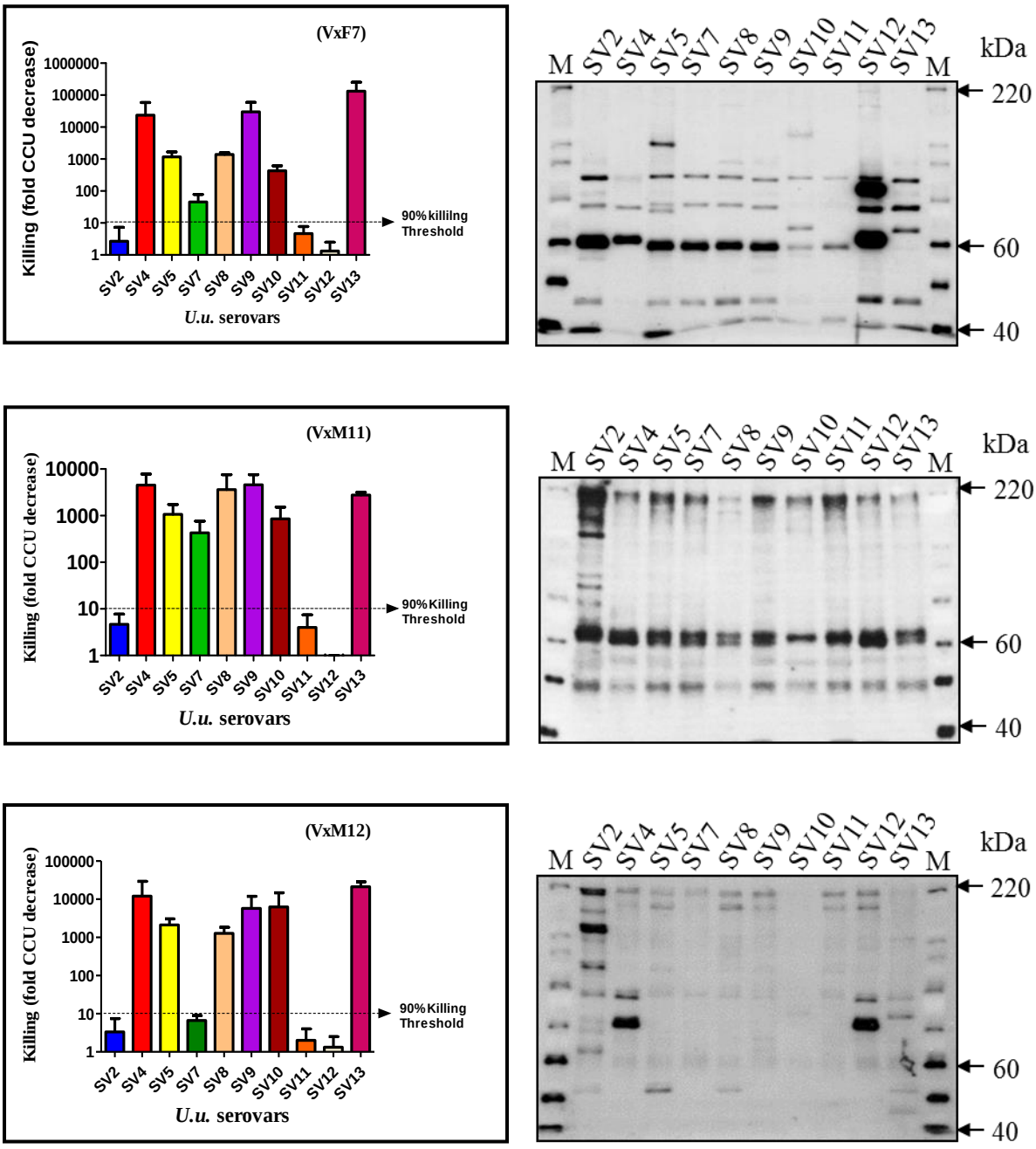

Figure 3.3 Complement killing and immunoblot analysis of *U. urealyticum serovars* using seropositive NHuS. On the left side: graphs show the bactericidal activity of 3 NHuS: VxF7, VxM11 & VxM12 (seropositive sera) against all known *U. urealyticum* serovars (2, 4, 5, 7-13). On the right side: immunoblot analysis of whole cell lysate of the serovars probed with 1:100 dilution of the matched NHuS on the left side of the image followed by secondary antibody (peroxidase-conjugated anti-human IgG). M: Molecular mass marker (kDa).

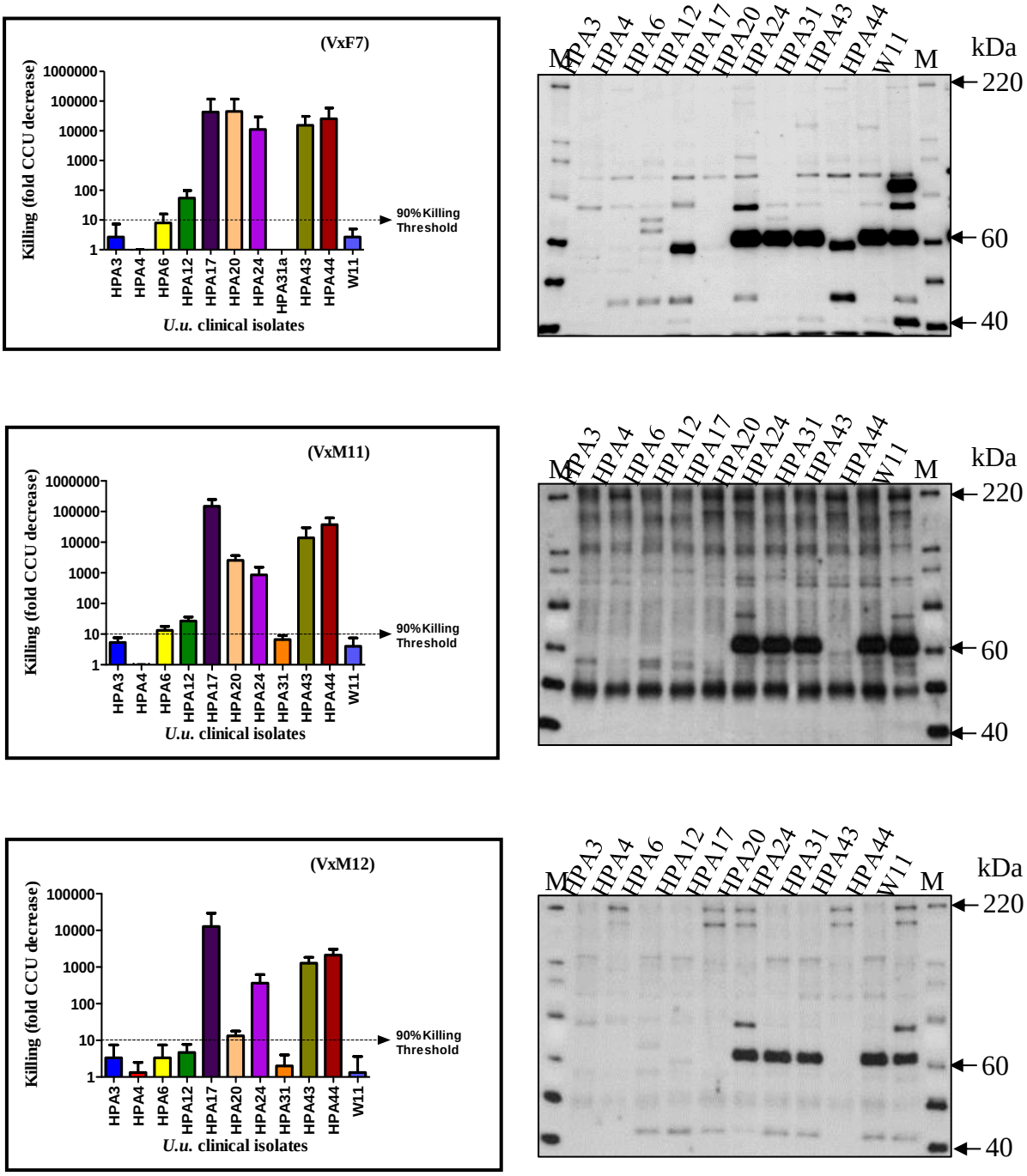

Figure 3.4 Complement killing and immunoblot analysis of *U. urealyticum* clinical isolates using seropositive NHuS. On the left side, graphs show the bactericidal activity of 3 NHuS: VxF7, VxM11 & VxM12 (seropositive sera) against 11 clinical isolates of unknown serovars of *U. urealyticum* (W11 & HPA 3, 4, 6, 12, 17, 20, 24, 31, 43, & 44). On the right side, western blot analysis of whole cell lysate of the clinical isolates probed with 1:100 dilution of the matched NHuS on the left side of the image followed by secondary antibody (peroxidase-conjugated anti-human IgG).M; Molecular mass marker (kDa).

3.2.3 Segregation of *U. urealyticum* based on serum susceptibility

U. urealyticum revealed a wide variation of susceptibility to complement-mediated serum killing, ranging from antibody-independent bactericidal activity to complete bactericidal resistance despite the presence of anti-*Ureaplasma* antibodies in the serum as observed by immunoblot analysis (Tables 3.1 and 3.2). According to their serum susceptibility, *U. urealyticum* serovars and clinical isolates can be clustered into three groups (Table 3.3): Killed by all NHuS (antibody-independent bactericidal activity), killed by seropositive sera only (antibody-mediated bactericidal activity) and not killed by any sera (complete bactericidal resistance). The first group contained serovars 4, 9, and 13 as well as the clinical isolates HPA17, HPA24, HPA43, and HPA44. These were shown to be the most serum-sensitive representative of *U. urealyticum*. The second group consisted of SV5, SV7, SV8 and SV10, with the clinical isolates HPA12 and HPA20. The final group composed of SV2, SV11 and SV12 as well as the clinical isolates HPA3, HPA4, HPA6, HPA31, and W11, and this group represent the first reported strains of serum-resistant *U. urealyticum*. Although the unknown clinical isolates were grouped with known serovars based on that category, the next step is to try to identify which serovars these isolates belong to, to see if serum-susceptibility is correlated to serovar.

Table 3.1 Susceptibility of *U. urealyticum* serovars to killing by normal human serum

Serovars	Susceptibility to serum killing						Presence of immune-reactive bands (kDa) detected by NHuS					
	Seronegative sera			Seropositive sera								
	VxF1	**VxF9**	**VxM18**	**VxF7**	**VxM11**	**VxM12**	**VxF1**	**VxF9**	**VxM18**	**VxF7**	**VxM11**	**VxM12**
SV2	R	R	R	R	R	R	-	-	-	90, 72, 60 & 56	220, 120, 80, 82, 84, 61& 50	220, 200, 122, 90, 72, 61 & 50
SV4	S	S	S	S	S	S	-	-	-	90, 72, 61	61& 50	220, 200
SV5	S	R	R	S	S	S	-	-	-	120, 90, 72, 71, 60 &56	61& 50	220, 200, 90, 72 & 50
SV7	R	R	R	S	S	R	-	-	-	90, 72, 60 &56	61& 50	220 & 72
SV8	S	R	S	S	S	S	-	-	-	90, 72, 60 &56	61& 50	220, 200 & 50
SV9	S	S	S	S	S	S	-	-	-	90, 72, 60 &56	61& 50	220, 200
SV10	S	R	S	S	S	S	-	-	-	122, 90,63, & 60	61& 50	80
SV11	R	R	R	R	R	R	-	-	-	90 & 60	61& 50	220, 200 & 90
SV12	R	R	R	R	R	R	-	-	-	90, 82, 72, 60 & 56	61& 50	220, 200, 90, 80 & 60
SV13	S	S	S	S	S	S	-	-	-	90, 72, 63 & 56	61& 50	220, 120, 95, 90 & 50

R = resistant to complement killing; S = Sensitive to complement killing.
- = absence of detestable antibodies against *U. urealyticum*
+ = presence of detectable antibodies against *U. urealyticum*

Table 3.2 Susceptibility of *U. urealyticum* clinical isolates to killing by normal human serum

Clinical isolates	Susceptibility to serum killing						Presence of immune-reactive bands (kDa) detected by NHuS					
	Seronegative sera			Seropositive sera								
	VxF1	VxF9	VxM18	VxF7	VxM11	VxM12	VxF1	VxF9	VxM18	VxF7	VxM11	VxM12
HPA3	R	R	R	R	R	R	-	-	-	90 & 72	220, 200, 120, 100, 72, 58 & 50	121, 90, 72 & 50
HPA4	R	R	R	R	R	R	-	-	-	90, 72 & 45	220, 200, 120, 100, 58 & 50	220, 200, 90 & 50
HPA6	R	R	R	R	R	R	-	-	-	90, 72, 62, 63 & 45	220, 200, 120, 100, 55 & 50	121, 90, 50 & 43
HPA12	R	R	R	S	S	R	-	-	-	90, 72, 59 & 45	220, 200, 120, 100, 58 & 50	121, 90, 50 & 43
HPA17	S	S	S	S	S	S	-	-	-	90	220, 200, 120, 100 & 50	220, 200, 90, 50 & 43
HPA20	R	R	S	S	S	S	-	-	-	100, 90, 72, 60 & 45	220, 200, 120, 100, 72, 60, 58 & 50	220, 200, 90, 72, 60, 50 & 43
HPA24	S	S	S	S	S	S	-	-	-	72 & 60	220, 200, 120, 100, 60, 58 & 50	121, 90, 60, 50 & 43
HPA31	R	R	R	R	R	R	-	-	-	90, 72 & 60	220, 200, 120, 100, 60, 58 & 50	121, 90, 60, 50 & 43
HPA43	S	S	S	S	S	S	-	-	-	90, 72, 59 & 45	220, 200, 120, 100 & 50	220, 200, 90, 50 & 43
HPA44	S	S	S	S	S	S	-	-	-	90, 72 & 60	220, 200, 120, 100, 60, 58 & 50	121, 90, 60, 50 & 43
W11	R	R	R	R	R	R	-	-	-	90, 85, 72, 60, 45 & 41	220, 200, 120, 100, 72, 60, 58 & 50	220, 200, 90, 72, 60, 50 & 43

R = resistant to complement killing; S = Sensitive to complement killing.
- = absence of detestable antibodies against *U. urealyticum*
+ = presence of detectable antibodies against *U. urealyticum*

Table 3.3 Segregation of *U. urealyticum* based on serum susceptibility

	Group I	**Group II**	**Group III**
	Killed by all sera in the absence or presence of antibodies	***Antibody-mediated killing*** ⇧	***Resistant to all sera even in the presence of antibodies***
Serovars	SV4, SV9, and SV13	SV5, SV7, SV8 and SV10	SV2, SV11 and, SV12
Clinical isolates	HPA17, HPA24, HPA43, and HPA44	HPA12 and HPA20	HPA3, HPA4, HPA6, HPA31, and W11

⇧= some serovars and clinical isolates within this group showed low susceptibility to complement-mediated serum killing by some seronegative sera (SV5, SV8, SV10 & HPA20) and others were resistant to killing by one of seropositive sera (SV7 & HPA12).

3.2.4 Determination of *U. urealyticum* serovars and clinical isolates by variation in MBA serovar type and apparent molecular mass.

Determination of serovars for *U. urealyticum* is not as easy as it is for *U. parvum*. Historically the serovars were determined by growth inhibition zones around paper circles soaked in various rabbit anti-sera (Shepard and Lunceford, 1978). More recently, sequence variation within the promoter and coding region for the MBA have validated the separation of isolates into SV1, 3, 6 and 14 for *U. parvum* (Beeton *et al.*, 2009b; Kong *et al.*, 1999b; Teng *et al.*, 1994). However, the sequence variation is not as distinct for *U. urealyticum*. Only 3 polymorphisms in the promoter region separate SV7/11 from SV2/5/8/9 and SV4/10/12/13 (Figure 3.5), while predicted amino acid sequence of the entire open reading frame for SV7 and 11 are identical as are SV4, 12 and 13 and SV2, 5 and 8. Only SV9 and SV10 have unique MBA amino acid sequence (Figure 3.5). The promoter and MBA coding region were amplified using a set of primers designed based on conserved regions of the known genomic sequence of the prototype *U. urealyticum* serovars. The sequences of the amplicons were then compared by nucleotide blast for the closest homology in the NCBI database. Based on alignment of sequence results, all 11 unknown clinical isolates were assigned to known *U. urealyticum* serovars as presented in Table (3.4). Clinical isolates HPA3 and HPA43 were defined as SV9; HPA4, HPA6 and HPA12 were grouped with SV4/12/13 cluster; and HPA17 and HPA20 were assigned to SV2/5/8. HPA24, HPA31 and HPA44 were defined as SV10, of which HPA24 and HPA31 were further confirmed with anti-MBA monoclonal antibody generated specifically for SV10 (Figures 3.6). The clinical isolate W11 was assigned as SV12 by both total genome sequencing and MBA gene homology.

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Sv11        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv7         MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv9         MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv4         MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv12        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
sv13        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv10        MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv2         MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv5         MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
Sv8         MKLLKNKKFWAITLGVTLVGAGVVAVAASCSSSNVKSKLSSQLVKSKDEKSFYAVYDIEN
            ************************************************************

Sv11        FDDLTENDKKALNETEFNVAITSVENKTENATIKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv7         FDDLTENDKKALNETEFNVAITSVENKTENATIKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv9         FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv4         FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv12        FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
sv13        FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv10        FDDLNENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv2         FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv5         FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
Sv8         FDDLTENDKKALNEAEFNVAITSAENKTENATTKGHLLNKKIYVKLPREPKAKEQLTIIN
            ****.*********:********.******** ****************************
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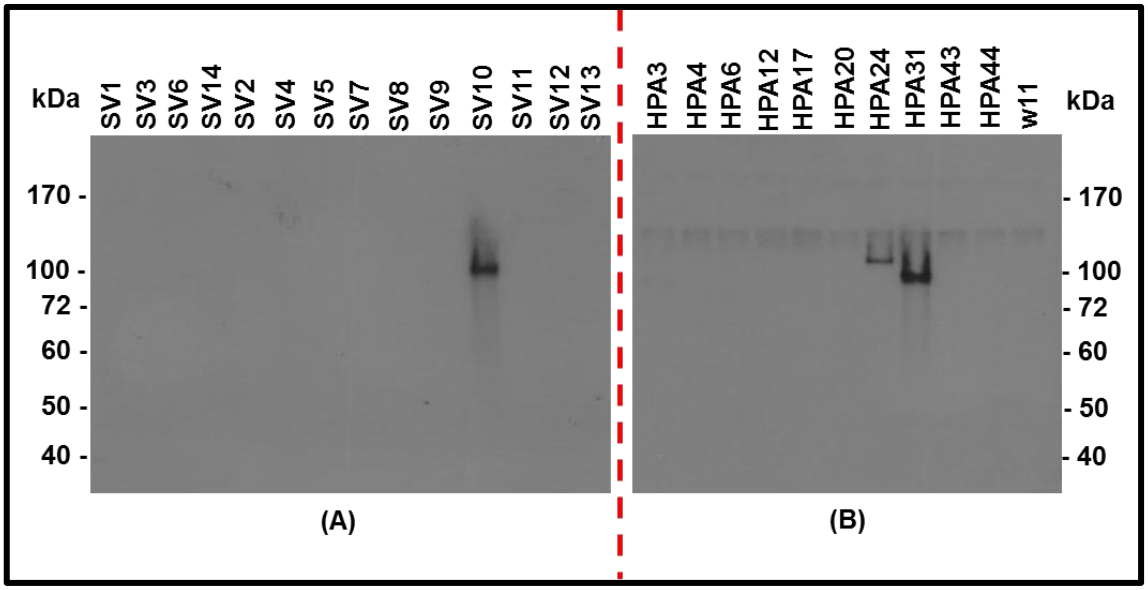
Figure 3.5 Amino acid polymorphisms within the promoter region of the multiple-banded antigen (MBA) of *U. urealyticum* serovars. Multiple sequence alignment results indicated that the 10 serovars of *U. urealyticum* can be separated into three groups: SV7/11, SV2/5/8/9 and SV4/10/12/13.



Figures 3.6 Immunoblot analysis of total cell lysates of *Ureaplasma* spp. probed with anti-SV10 monoclonal antibody (MAb 8A1.2; Watson et al. 1990). A) The SV10 specific antibody detected only SV10 among all 14 known serovars. B) The same antibody recognized two unknown clinical isolates of *U. urealyticum* HPA24 and HPA31 as SV10. Molecular mass weight indicated in kilo Daltons.

Table 3.4 Summary of sequencing results shows the assigning of the 11 unknown *U. urealyticum* clinical isolates to known serovars based on nucleotide homology in MBA gene.

Clinical isolate	Defining repeat sequence	Serovar	Primer
HPA3	No repeats- MBA short	Closest SV9 (92% aa & 96% nt) than SV7/SV11	revMBA Uu2
HPA4	PEKPGNGTTS	SV4/SV12/SV13	revMBA Uu1
HPA6	PEKPGNGTTS	SV4/SV12/SV13	revMBA Uu1
HPA12	PEKPGNGTTS	SV4/SV12/SV13	revMBA Uu1
HPA17	No repeats	Closest SV2 (92% aa & 96% nt) than SV12 (90% aa)	revMBA Uu2
HPA20	PEKPGNGTTS	SV4/SV12/SV13	revMBA Uu1
HPA24	PGSGSTTQ	SV10	revMBA Uu2
HPA31	PGSGSTTQ	SV10	revMBA Uu2
HPA43	KKPETGSTEGGSTEG	SV9	revMBA Uu2
HPA44	No repeats	Closest SV10 (92% aa), but this is the only annotated gene in the genebank for *U. urealyticum*	revMBA Uu1
W11 (1)	No repeats	Closest SV12 (98% aa)	revMBA Uu2

(1): W11 was also defined as SV12 by full genome sequence homology.

3.2.5 Correlation between serum sensitivity and serovar type

Previously, Beeton et al (2012) had identified that all SV3 types examined were sensitive to killing by seronegative sera. Similarly, using my prototype strains, I found that SV4, 9 and 13 were sensitive to seronegative sera. Examination of the typing results for my clinical strains (Table 3.4) found that HPA43 was also a SV9, and it was also sensitive to killing by seronegative sera, which supports this hypothesis. However, it is not possible to separate serum sensitive SV4 and 13 from serum resistant SV12 by the MBA typing method. It is possible that the resistant clinical isolates W11, HPA4 and 6 were SV12, but given the lack of variation in MBA sequence between SV4, 12 and 13, it is not possible for the MBA to be the mediator of differential serum susceptibility. Furthermore, HPA24 and 31 were found to be SV10, but HPA24 was serum sensitive, HPA31 was serum resistant and prototype SV10 was intermediate in its serum sensitivity. Therefore, I conclude that serovar type for *U. urealyticum* does not differentiate the serum sensitivity of isolates.

3.2.6 Correlation between serum sensitivity and mass of the MBA

The purpose of this experiment was to investigate differences in the MBA repeats among the 14 standard serovars of *Ureaplasma* spp., using monoclonal anti-MBA antibodies, and correlate such changes with serum susceptibility among serovars. Immunoblot analysis of whole cell lysate of all 14 serovars showed variations in the MBA banding pattern among serovars. The MBA band sizes ranged from less than 40 kDa to larger than 80 kDa (Figure 3.7). The amino acid sequence shows complete conservation of the N-terminal 63 a.a. residues for *U. urealyticum* and only 6 polymorphisms in the first 141 amino acid residues (Figure 3.5). The repeat units that separate MBA into specific

serovars begin at 146 amino acid residues. The region of short repeat sequences determined the varying mass of the MBA by having greater or fewer relative numbers of repeats (Chang-tai *et al.*, 2011; Zheng *et al.*, 1995). Therefore, serovars with a large MBA band have greater number of tandem repeat units, while lower MBA bands contain fewer tandem repeat units. In addition, some isolates were observed to have more than one MBA with different sizes. Correlating these findings with complement killing results, which were obtained in this study as well as from the previous Beeton *et al* (2012) *U. parvum* study, showed no clear relationship between the MBA protein size and susceptibility of *Ureaplasma* to complement-mediated serum killing. While the seronegative-sensitive serovars SV3, SV4, SV9 and SV13 all have the smallest MBA isoforms, suggesting that smaller MBA correlate to high serum sensitivity, the totally serum resistant strains SV2, SV11, and SV12, did not have the largest MBA with masses between sizes 60-72 kDa (Figure 3.7.A and Table 3.3). Those prototype strains with the largest MBA, SV5, 10 and 14, had intermediate susceptibility that was increased by the presence of anti-*Ureaplasma* antibodies in seropositive serum indicating that the MBA serves as a target for antibody-mediated complement activation. In a similar way, the clinical isolates did show any correlation between the size of MBA and their susceptibility to complement killing. This observation was clearly presented in two clinical isolates (HPA24 and HPA31) that had a large MBA size but different serum susceptibility. HPA24 was very sensitive to complement-mediated serum killing, while HPA31 was serum-resistant (Figure 3.7.B and Table 3.3). Failure to correlate MBA size to seronegative sensitivity was also shown for HPA17, which was sensitive but had an intermediate size of MBA.

To investigate such a correlation further, several clinical isolates of SV3 of *U. parvum* that have different serum susceptibility were compared with one another based on MBA

sizes, which were correlated to their sensitivity to NHuS. Beeton et al (2012) had previously published that strains HPA5, 7, 8 and 9 were all sensitive to seronegative sera killing, while I have found strains HPA56, HPA57, UHW4 and UHW5 to be sensitive to seropositive sera killing, but resistant to seronegative sera killing (detailed in Chapter 4). Two conclusions can be drawn from this: 1) SV3 isolates are not all inherently sensitive to seronegative sera as previously reported and 2) Comparison of MBA mass in Figure 3.8 shows no difference in MBA molecular mass between serum sensitive and serum resistant isolates.

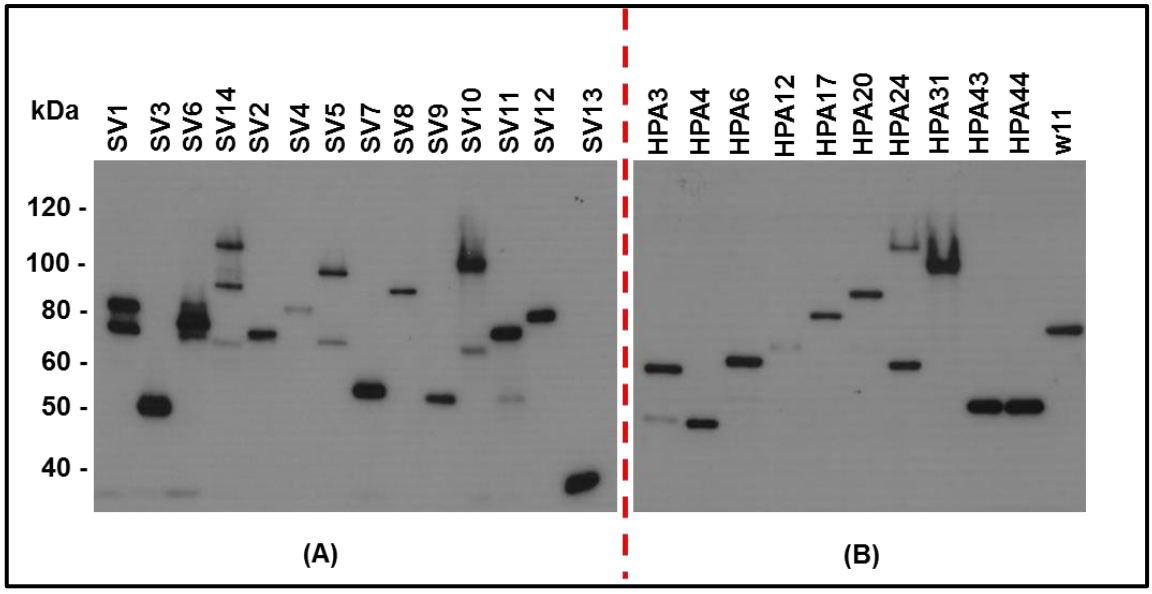


Figure 3.7 Immunoblot analysis of whole-cell lysate from *Ureaplasma* spp. probed with anti-MBA antibody (6522). The blot shows variations in MBA expression among the 14 serovars (A) and 11 *U. Urealyticum* clinical isolates. Some serovars and clinical isolates expressed only one variant of MBA; while others expressed more than one variant. The MBA band size varied from just less than 40 kDa to larger than 100 kDa.

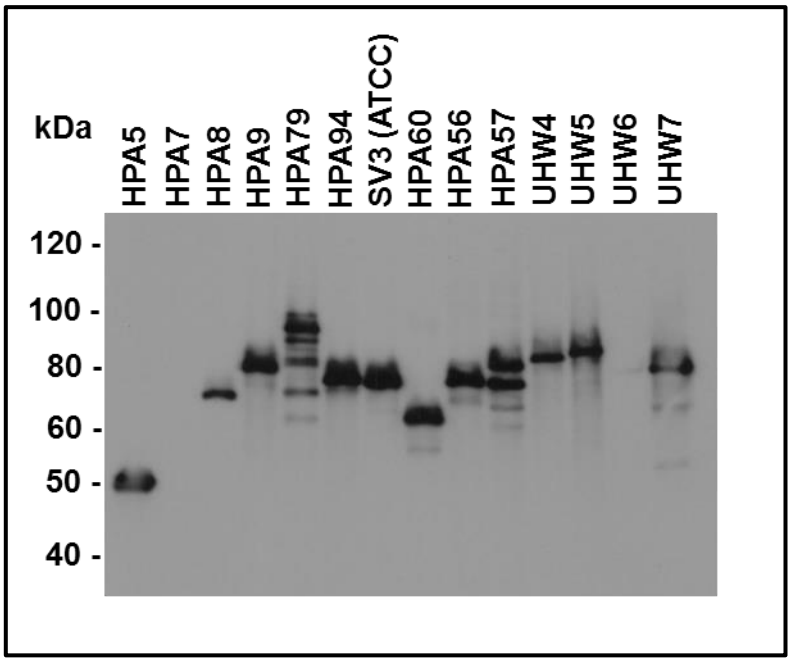


Figure 3.8 Immunoblot analysis of total-cell protein of a number of *U. parvum* SV3 strains probed with anti-MBA monoclonal antibody, SV3 specific, (MAb 10C6.6). The blot shows variations in MBA expression among SV3 isolates and strains with varying sizes. Some of them expressed only one variant of MBA; while others expressed more than one variant with different intensity. One strain appeared as MBA-negative clone (HPA7), while another one (UHW6) showed weak reactivity. The MBA band size varied from 50 kDa to 100 kDa.

3.3 Discussion

Complement-mediated serum killing is one of the effector arms of the innate immunity (Walport, 2001a). Many pathogenic bacteria have been shown to be serum-sensitive. However, within these bacterial species, serum-resistant strains have also been shown to emerge as a way of avoiding clearance by the immune defences. Serum-resistant isolates were proven to be more invasive and pathogenic than the serum-sensitive strains (Fierer *et al.*, 1972; Joiner *et al.*, 1984). The complement system, as one of the initial innate immune barriers that mediate microbial removal, has been targeted by various pathogens strategies to prevent or avoid the complement activation, and hence, the subsequent innate and acquired immune responses. Gram-negative and Gram positive pathogenic bacteria have been known to avoid the complement system activation by several mechanisms, many of them shared across species (Zipfel *et al.*, 2007). Although the complement system cannot directly lyse Gram-positive bacteria, it is able to opsonize and label them to be recognized by the other arsenals of immune system. In turn, Gram-positive bacteria have evolved several ways to evade such activity. A typical example of complement system evasion by Gram-positive bacteria has been reported for *S. aureus*. This pathogen produces and expresses a number of proteins that prevent the recognition and inhibit the activation of the classical, lectin, and alternative pathways. Of these proteins, two surface-expressed proteins bind to and disrupt IgG function and prevent the antibody-dependent activation of the classical pathway: staphylococcal protein A (SpA) and staphylococcal immunoglobulin-binding protein (Sbi). The latter surface protein, Sbi, is also believed to impair binding of C1q and hence the activation of the classical pathway (Atkins *et al.*, 2008). In addition, Sbi can prevent the activation of alternative pathway via binding C3, factor H (FH) and factor H-related proteins (Haupt *et al.*, 2008). On the other hand, many Gram-negative bacteria are able to regulate and inhibit

complement activation by binding complement regulators; an example of this is *P. aeruginosa* which produces a protein termed pseudomonas CRASP-1 that binds host plasminogen and factor H and prevents the activation of the alternative pathway (Zipfel *et al.*, 2007). In addition, EF-Tu of this pathogen has also reported to be a binging protein for FH and plasminogen (Kunert *et al.*, 2007).

In this chapter I have presented an extension to the previously published work from our laboratory, which investigated the effect of the bactericidal activity of normal human sera, from 12 healthy people, upon *U. parvum* (Beeton *et al.*, 2012). In this study, I investigated the complement killing activity against the other important species of human *Ureaplasma*, *U. urealyticum*, using 5 previously characterised sera and one new seronegative serum from a healthy individual. The 10 standard serovars and 11 clinical isolates were assessed for their susceptibility to complement killing using 50 % normal human serum (with serological status defined by immunoblot absence or presence of detectable anti-*Ureaplasma* antibodies).

The study by Beeton and colleagues (2012) demonstrated variations in susceptibility to complement among the four representative serovars of *U. parvum* (SV1, SV3, SV6 and SV14), which is in agreement with my findings in this study. *U. urealyticum* serovars and clinical isolates also showed wide variations in susceptibility to complement killing. The results presented here indicated that a similar pattern of susceptibility between the two species could be seen. For example, with *U. parvum*, it was found that SV3 was very susceptible to killing by all sera used even in the absence of anti-*Ureaplasma* IgG or IgM antibodies. Killing of SV3 was found to be C1q-dependent; therefore, mediated by the classical complement pathway. Whether this was mediated through the direct binding to C1q or via low affinity anti-bacterial carbohydrate IgM that is not detectable by

immunoblot remains to be determined. Similarly in this study, SV4, SV9 and SV13 were found to be highly sensitive to serum killing; and the mechanism of killing by complement appeared to be via complement activation, independent of the presence of detectable anti-*Ureaplasma* antibodies.

Additionally, antibody-mediated killing among *U. parvum* was also documented. SV1, SV6, and SV14 were found to be killed by serum only with the presence of detectable anti-*Ureaplasma* IgG specific antibodies (presumably via the classical pathway of the complement). Although I have not investigated the role of each independent complement pathway in the killing of *U. urealyticum* serum-sensitive strains, the results obtained here suggest a similar mechanism of killing. Some *U. urealyticum* serovars and isolates presented a comparable susceptibility pattern; SV5, SV7, SV8, and SV10, for instance, were killed by complement in the presence of specific anti-*Ureaplasma* IgG antibodies, which indicates that their complement killing requires activation of the classical pathway by binding the C1q to antibody bound to *Ureaplasma* surface protein(s). Complement-mediated serum killing in other bacteria has been extensively studied. In Gram-negative bacteria, it was found that their killing is mainly derived by the classical pathway, which, in most cases, requires binding of C1q to antibody for the activation. However, antibody-independent binding of C1q to Gram-negative bacterial cell surface components has also been demonstrated (Alberti *et al.*, 1993; Taylor, 1983). Beeton and colleagues have studied the role of the three complement activation pathways, the classical, the alternative and the lectin (CP, AP, and LP), all together and separately, in complement activation and killing of *U. parvum*. These pathways were investigated by depleting calcium, removing C1q or MBL to inactivate AP, CP or LP, respectively (Beeton *et al.*, 2012). The authors concluded that complement killing of *U. parvum* was mediated by the CP (C1q-dependent) and that AP and LP had no role in the killing.

In contrast to the previous study by Beeton et al., (2012), I found some strains that were completely resistant to killing by seropositive sera that was bactericidal to all other strains tested. I found serum resistance among prototype *U. urealyticum* SV2, SV11 and SV12 strains that were totally resistant to serum bactericidal activity even in the presence of specific anti-*Ureaplasma* IgG antibodies. This observation was also seen among some clinical isolates (HPA3, HPA4, HPA6, HPA31, and W11). Evolving resistant strains among *U. urealyticum* can be strong evidence that these tiny bacteria species is also able to manipulate the immune defences and develop sophisticated strategies to survive the killing mediated by the complement activation. Several potential mechanisms could be involved in such resistance. A wide array of evasion mechanisms identified in other bacteria to avoid complement killing could also be utilised by *Ureaplasma*. An excellent review on mechanisms used by microbial pathogens to elude the complement lethal activity was published by (Lambris *et al.*, 2008).

In my study I have attempted to test a postulated mechanism of serum resistance that could be utilised by *Ureaplasma* spp. to overcome complement activation. This hypothesis was the correlation between serum resistance and MBA size. In the closely related species, *M. pulmonis*, it was found that the variable surface antigens (Vsa), which is one of the virulence factors (adhesin molecule), plays a direct role in serum susceptibility (Simmons *et al.*, 2004; Simmons and Dybvig, 2003). These antigens have a C-terminal variable region that consists of tandem repeats that vary in length from one strain to another. Strains that produce a VsaA protein with 40 tandem repeats were found to be highly serum-resistant compared to that with short tandem repeats. However, the repeat unit of VsaA was found to contain a glycosylation site that resulted in contributing to the glycocalyx of the bacteria as a physical shield against complement attack (Bolland *et al.*, 2012). Similarly, the multiple banded antigens (MBA) is a major surface protein

expressed by all *Ureaplasma* spp. proposed as one of the virulence factors for these microorganism. This antigen also has a C-terminal region composed of multiple tandem repeat units. It has been found that alterations in the copy number of the tandem repeats of that region were responsible for size variations in the MBA (Zheng et al., 1995). However, the role of modulations in the C-terminal regions in *Ureaplasma* pathogenesis and susceptibility to complement killing has not been defined yet. I hypothesized that a similar role for serum resistance found with VsaA may exist with the MBA (i.e. serovars with long tandem repeats would be serum-resistant and those with short repeats would be sensitive). Upon testing my hypothesis, I found that such correlation did not exist among the species of *Ureaplasma*, as some small-size MBA *Ureaplasma* strains were serum-resistant and vice versa. This may be related to the fact that the repeat units for *Ureaplasma* are not predicted to be glycosylated.

The next chapter of the thesis will focus on investigating some other potential mechanisms that lead to serum resistance and avoiding host immune responses among *Ureaplasma*.

Chapter 4

Monitoring and determining immunogenic proteins and their stability under immunological pressure

4. Monitoring and determining immunogenic proteins and their stability under immunological pressure

4.1 Introduction

The capacity to escape a host immune response is an adaptive strategy employed by several microbial pathogens. A variety of bacterial species have developed different sophisticated strategies to avoid the bactericidal activity of normal human serum via the complement system and survive in their host milieu. For instance, some bacteria use molecular mimicry, altering their outer membranes, to resemble host tissues in order to avoid complement activation (Janulczyk *et al.*, 2000; Rokita *et al.*, 1998; van den Berg *et al.*, 1996; Wurzner, 1999). Other bacterial species express proteins that are able to bind host-derived complement regulators such as factor H and factor H-like protein 1 (FHL-1) to survive the potentially lethal attack of complement system (China *et al.*, 1993; Kraiczy *et al.*, 2001; Neeleman *et al.*, 1999; Pérez-Caballero *et al.*, 2000). In a similar way, members of the genus *Mycoplasma*, which is closely related to *Ureaplasma*, have also been shown to alter their surface antigens and manipulate the host immune defences. Both antigenic and phase variations are believed to be mechanisms by which *Mycoplasma* overcomes host immune responses and establish chorionic infections (Calcutt *et al.*, 1999; Citti *et al.*, 2010; Razin *et al.*, 1998).

The species of *Ureaplasma* are opportunistic pathogens that colonise the mucosal membranes of the urogenital tract of humans and are associated with several medical problems such as non-gonococcal urethritis, infertility, pregnancy complications, chorioamnionitis and bronchopulmonary dysplasia in premature babies (Yu *et al.*, 2005). Little is known about the pathogenesis of *Ureaplasma* spp. and their interactions with

their human host immune system. Members of the *Ureaplasma* genus have also been shown to use such strategies to manipulate the immune defences by switching their surface antigens. A number of studies have demonstrated phase and antigenic variations in a major surface antigen of *Ureaplasma*, the multiple-banded antigen (MBA), both *in vivo* and *in vitro* (Dando *et al.*, 2012; Monecke *et al.*, 2003; Robinson *et al.*, 2013; Zheng *et al.*, 1994; Zimmerman *et al.*, 2011; Zimmerman *et al.*, 2009). Therefore, it was suggested as one of the virulence factors that play a pivotal role in the pathogenesis of these species. However, the exact role that this antigen plays in the pathogenesis of human *Ureaplasma*, especially serum resistance, is still not fully understood. Furthermore, observations of changes in other immunoproteins have not been reported yet.

This chapter is divided into two parts: the first part is an *in vitro* study, which aims to induce serum-resistance of known serum-sensitive strains through repeated sublytic challenges by seropositive or seronegative human sera. Changes in *Ureaplasma* antigenicity (size and number of bands detected by Western blotting) as a result of immunological pressure will then be determined and a candidate protein(s) will be identified and studied. Subsequently, mechanisms of serum resistance and avoidance of complement killing among human *Ureaplasma* will also be identified. The second part of the chapter is the examination of serially collected isolates from endotracheal secretions obtained from preterm babies, who are intubated for ventilated-assisted breathing, to examine phenotypic and antigenic differences that evolve *in vivo* over a period of time.

4.2 Part 1 results: the *in vitro* investigation

4.2.1 Generation of serum-resistant strains following serial challenge with seropositive serum

Generation of serum-resistant (SR) strains from serum-sensitive (SS) parental strains was accomplished by subjecting serum-sensitive *Ureaplasma* clinical isolates to repeated immunological pressure *in vitro*, until resistant strains emerged, using a modified complement killing assay as described in section 2.2.3. Two serum-resistant strains, SR-DFK1 (SV1) and SR-HPA2 (SV6) were created from serum-sensitive patients (SS-DFK1 and SS-HPA2) following serial challenge with a high titre seropositive serum. Induced resistance was accomplished with several other strains of the same serovars, SV3 and some *U. urealyticum* strains. However, these two strains were initially chosen for in depth investigation of underlying mechanism of serum resistance. The parent strains were initially sensitive to seropositive sera but resistant to seronegative sera, and therefore repeated serum challenge was performed using seropositive sera. Resistant variants, SR-DFK1 and SR-HPA2, had emerged after 5 and 4 repeated challenges, respectively, and they became completely resistant to seropositive sera compared to their parent strains. Altered serum sensitivity was confirmed by the complement killing assay, in triplicate, on the induced serum-resistant clones compared with parental strains (Figure 4.1). Matched sensitive parental strains and resistant progeny were further investigated using immunoblotting and proteomic analyses to identify and study any alterations in immunogenic proteins and determine possible mechanisms of serum resistance.

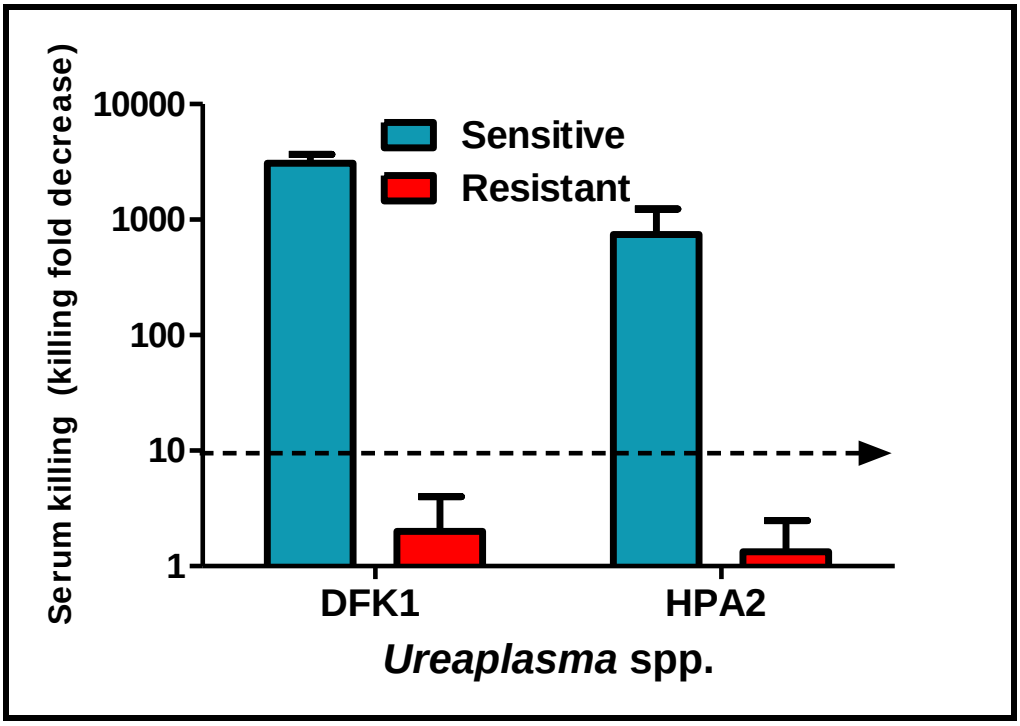


Figure 4.1 Serum susceptibility of the induced serum-resistant strains, SR-DFK1 and SR-HPA2, (red bars) compared with their parent strains (blue bars), DFK1 (SV1) and HPA2 (SV6). The black dotted arrow indicates the 90% killing threshold.

4.2.2 Alterations in immunogenic proteins following challenge with seropositive serum

Immunoblot analysis was performed using the seropositive serum used to generate the resistant strains (VxF7) and anti-MBA monoclonal antibodies to identify any changes in immunogenic proteins in the induced serum resistant strains compared to the parent strains. The results showed significant variations in immunoreactive protein bands between the serum-resistant strains and parents, as detected by Western blotting (Figure 4.2). The high titre seropositive serum detected a loss of a single band at approximately 90 kDa and gain of two new bands at about 81 and 41 kDa in the SR-DFK1 (SV1) compared to the parent strain (Figure 4.2.A). In contrast, the other induced resistant strain, SR-HPA2, did not show any loss of immunogenic proteins but expressed an immunogenic protein at about 41 kDa (similar to that seen with SR-DFK1) (Figure 4.2.C). Anti-MBA monoclonal antibody, 2G9 (specific for SV1 and SV6) detected and confirmed changes in MBA in only one of the induced resistant strains, SR-DFK1. The lost band (90 kDa) and the new emerging band (81 kDa) in DFK1 strains were detected with the anti-MBA monoclonal antibody and confirmed as MBA bands with difference in size (Figure 4.2.B-1). HPA2 strains did not show any changes in MBA bands and both of them (SS-HPA2 and SR-HPA2) expressed MBA bands of the same size (about 80 kDa) when detected by anti-MBA monoclonal antibody (Figure 4.2.B-2). The new expressed protein band (41 kDa), in both serum-resistant strains (SR-DFK1 and SR-HPA2) was not detected by anti-MBA monoclonal antibody (Figure 4.2.B-1 and B-2). This indicated that these resistant strains expressed a new immunogenic protein, consistently co-incident with induced resistance and was a strong candidate to play a role in direct evasion of complement killing.

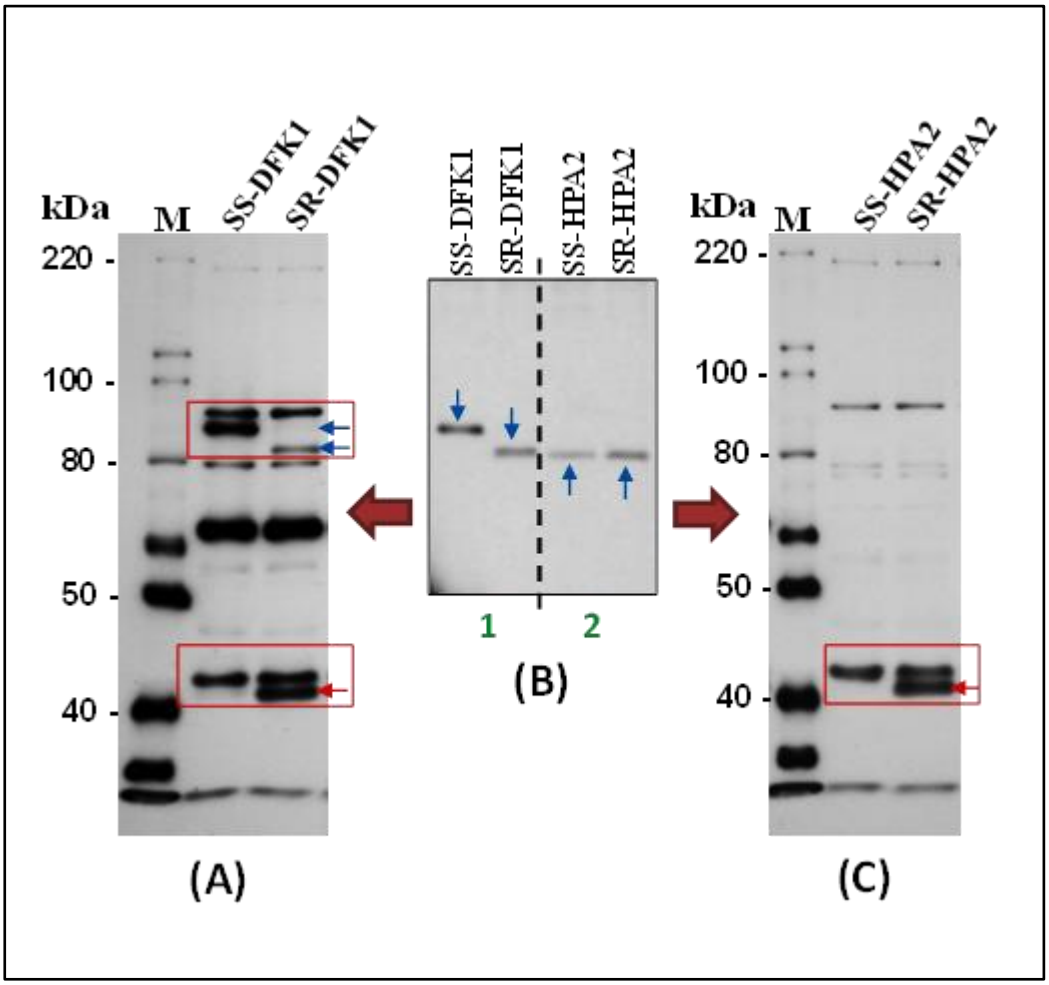


Figure 4.2 Immunoblot analysis of total cell lysate from two induced serum-resistant strains, SR-DFK1 and SR-HPA2, compared to their serum-sensitive parents, SS-DFK1 (SV1) and SS-HPA2 (SV6). **Blots A** and **C** were probed with the high titre seropositive serum (VxF7). In blot A, blue arrows indicate loss and gaining of immunogenic protein bands in the resistant strain, SR-DFK1, whereas red arrows in blots A and C indicate new immunogenic protein bands expressed only in the resistant strains, (SR-DFK1 and SR,HPA2). **Blot B** probed with anti-MBA monoclonal antibody (2G9); blue arrows in **B-1** indicate and confirmed changes in MBA bands (change in size) in SV1 resistant strain, whereas no change in SV6 strains. M= molecular mass marker.

4.2.3 Generation of serum-resistant strains following serial challenge with seronegative serum

To further investigate serum resistance and alterations in immunogenicity of *Ureaplasma*, two representative seronegative-sensitive ureaplasmas (SV9 and HPA5 (SV3)) were also challenged with seronegative NHuS. These particular strains were different from the above challenged isolates as they were readily killed by both seronegative and seropositive sera. SV9 was indicated as one of the serum-sensitive strains identified among *U. urealyticum*, as presented in chapter 3 of this thesis. On the other hand, HPA5 (SV3) was found to be the most sensitive strain to complement-mediated serum killing among *U. parvum* (Beeton *et al.*, 2012). The results showed that following 9 serial challenges with seropositive serum, the serum-sensitive SV9 had become resistant to seronegative sera killing. However, this resultant strain, SR-SV9, was also found to additionally be resistant to seropositive sera as well (Figure 4.3) as it was totally resistant to serum killing. On the other hand, no serum-resistant strains were developed from the serum-sensitive HPA5 after 14 consecutive challenges (Figure 4.3). Both SR-SV9 and serum-attacked HPA5 (SA-HPA5) were also investigated by western blotting to observe any significant differences in immunogenic proteins as compared to their respective parental strains.

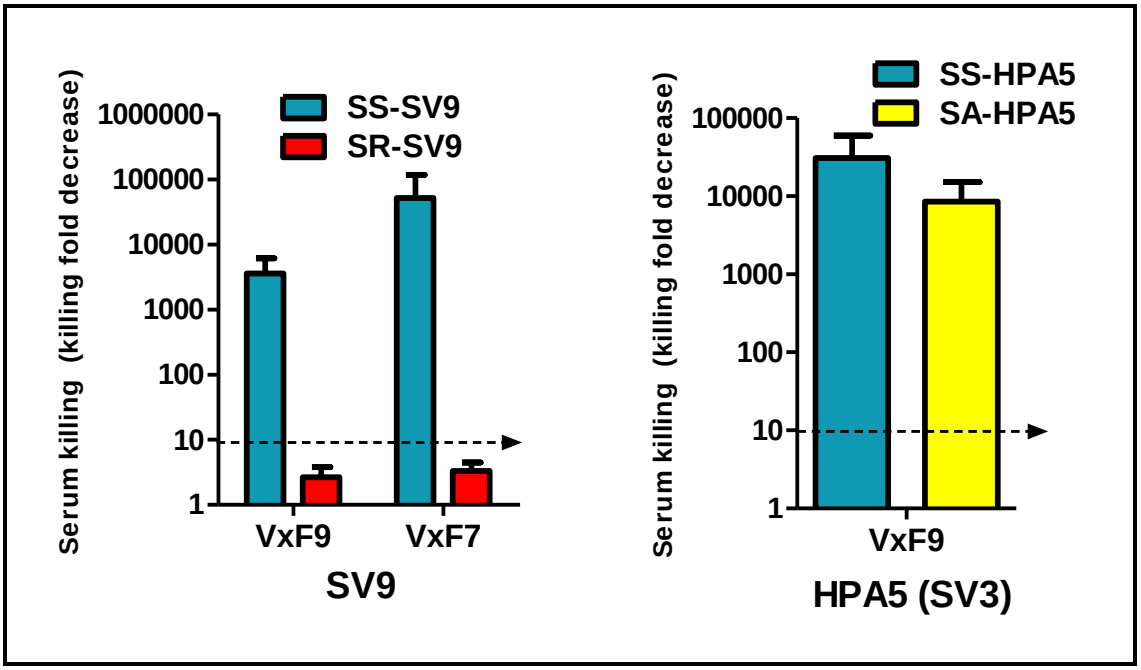


Figure 4.3 Serum susceptibility of the induced serum-resistant SV9 strain, SR-SV9 (red bars) and serum-attacked sensitive HPA5 (SV3) strain, SA-HPA5 (yellow bar) compared to their serum-sensitive parental strains (blue bars). The graphs show clear difference between SV9 isolates, whereas the attacked HPA5 remained sensitive to complement killing. VxF9 = seronegative serum and VxF7 = seropositive serum. Black dotted arrows indicate the 90% killing threshold.

4.2.4 Alterations in immunogenic proteins following challenge with seronegative sera

Immunoblot analysis of total cell proteins of SR-SV9 using monoclonal anti-MBA antibody (6522) detected a single band of the multiple banded antigen (MBA) at about 90 kDa with a significant size variation compared to that of the parent strain (SS-SV9), with an MBA size of approximately 50 kDa (Figure 4.4.B-2). Probing the same blot with seropositive serum clearly showed a new immunogenic protein band at about 41 kDa in SR-SV9 compared to the parent strain (SS-SV9) (Figure 4.4.C). This protein band was exactly the same as the ones seen in both SR-HPA2 and SR-DFK1 above, and was coincident with developing serum resistance. In the repeatedly serum attacked HPA5 strain (SA-HPA5) monoclonal anti-MBA antibody (10C6.6, SV3 specific) detected a minor size variation in MBA band (a small decrease of about 1-2 kDa) compared to the MBA band (about 50 kDa) of the parent strain, SS-HPA5 (Figure 4.4.B-1). However, no alterations in other immunogenic proteins were detected when probed with seropositive serum (Figure 4.4. A).

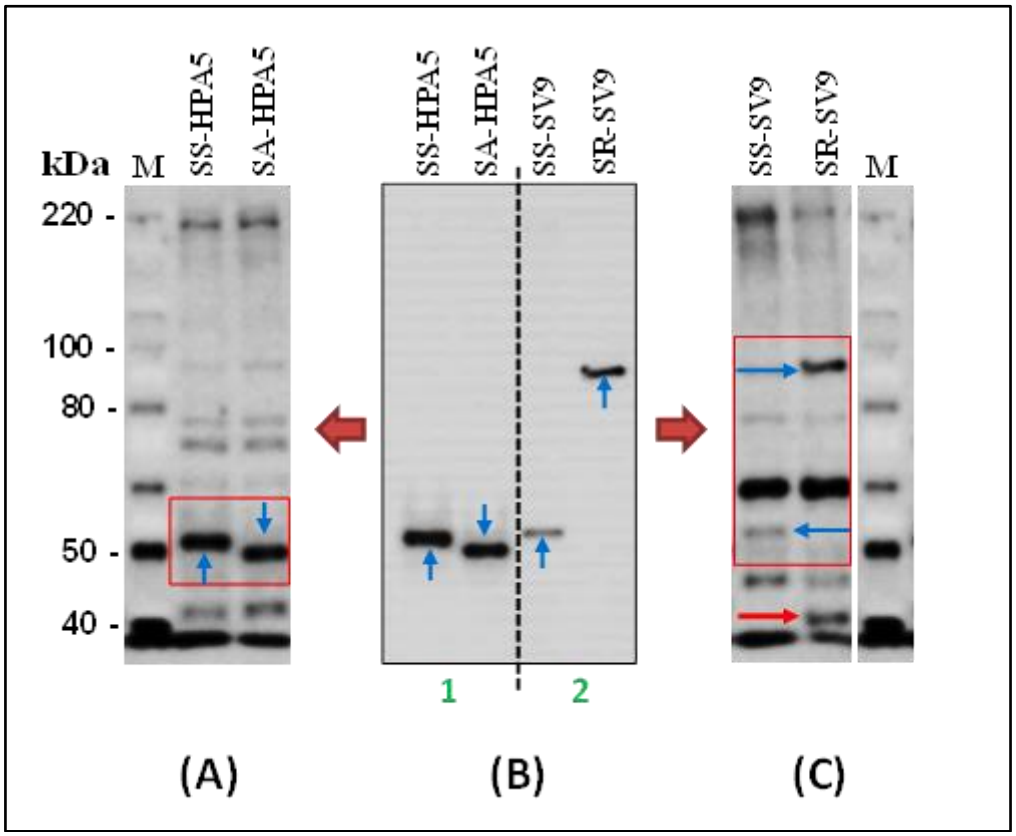


Figure 4.4 Immunoblot analysis of total cell lysate from induced serum-resistant strain, SR-SV9 and serum-attacked none resistant strain, SA-HPA5, compared to their serum-sensitive parents, SS-HPA5 (SV3) and SS-SV9. Blots **A** and **C** probed with seropositive serum; blot **(B)** probed with monoclonal anti-MBA antibodies, 10C6.6 **(B-1)** and 6522 **(B-2)**, respectively. Blue arrows indicate size variations in MBA surface protein. Red arrow indicates expression of new immunogenic protein (41 kDa) in SR-SV9 strain. M= molecular mass marker.

4.2.5 Observation of changes in banding patterns of selected *Ureaplasma* isolates following challenge with seropositive serum

Selected isolates of *Ureaplasma* were also serially challenged with seropositive serum. The purpose of this work was to generate serum resistant strains but one of the strains was already completely resistant to seropositive sera (*U. Urealyticum* serovar 12 isolate, W11) and it was included to see if repeated exposure of the strain to sera induced alteration to the MBA size. Two *U. parvum* serovar 3 isolates, HPA56 and HPA57, were included to see if this serovar was capable of induced serum resistance as SV3 HPA5 was not, although these strains were different in that they were resistant to seronegative sera. Additionally, a clinical isolate (*U. urealyticum* isolate, Ply128M) was included as serial isolates obtained (see section 4.8) did not show any alteration in MBA size *in vivo*. Therefore, it was included to see if alteration in size could be induced *in vitro*.

Following repeated challenge with NHuS, the results showed that the MBA changed only for one of the tested strains, HPA57, co-incident with induction of serum resistance (Figure 4.5-A). Furthermore, this isolate appeared to undergo selection from two isoforms of MBA (90 and 80 kDa band) to the single higher MBA isoform. This alteration had occurred by or before 3 challenges with seropositive serum and remained persistent after 7 serum challenges. Expression of MBA in all other strains remained unchanged after 3 and 7 challenges (Figure 4.5-A). Interestingly, all isolates had expressed a new 41 kDa protein band (Figure 4.5-B) similar to that expressed by all induced serum-resistant strains (discussed earlier in this chapter) and the endogenously serum-resistant clinical isolate, W11 (SV12). Following 7 consecutive challenges, all serum-sensitive isolates became resistant to serum killing by seropositive serum. W11 remained serum-resistant and did not show any alterations in immunogenic proteins.

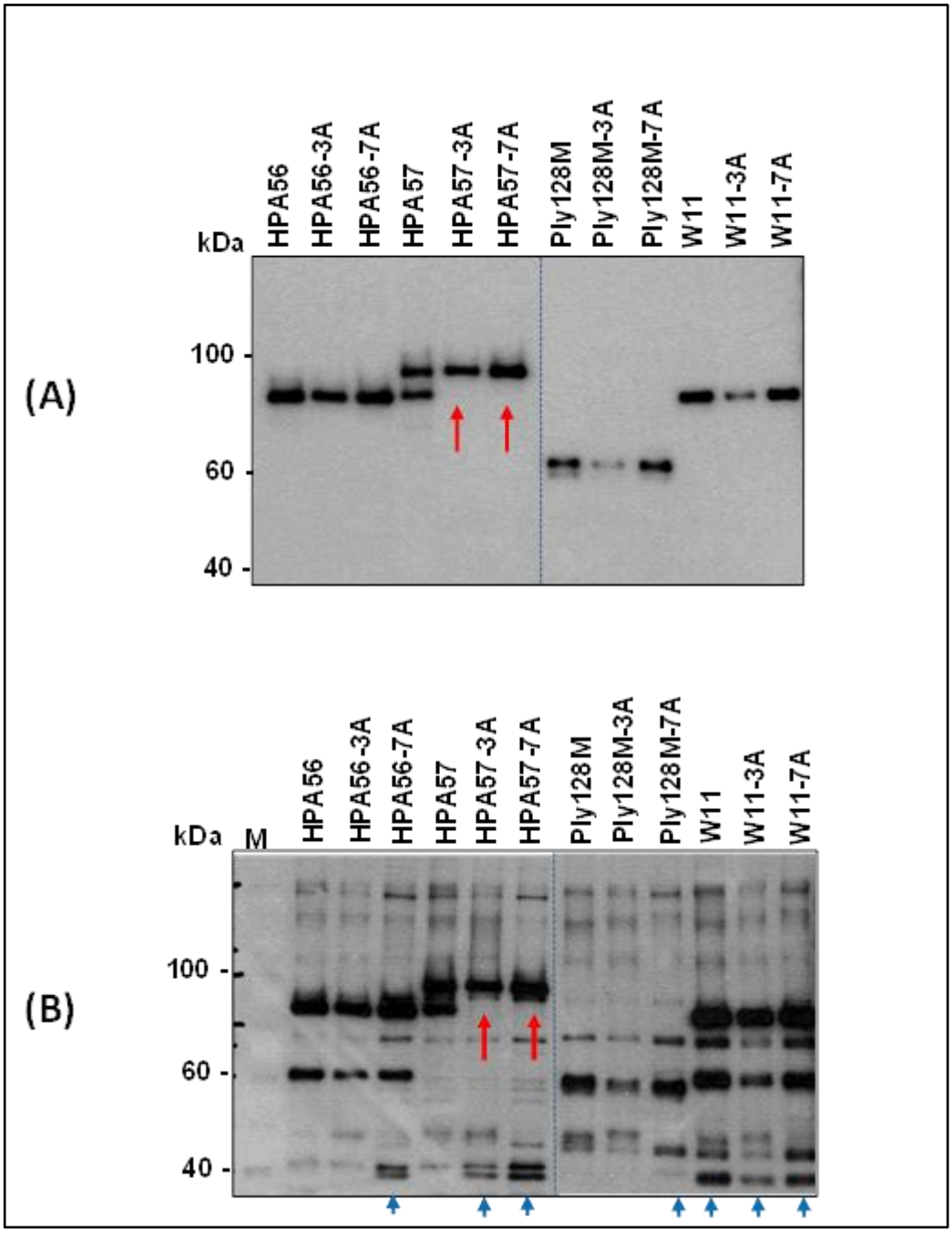


Figure 4.5 Immunoblot analysis of *Ureaplasma* total cell lysate from selected *Ureaplasma* isolates serially challenged with seropositive serum. The upper blot (A) probed with monoclonal anti-MBA antibody (6522); the lower blot (B) probed with seropositive serum. Arrows indicate differentially expressed proteins relative to parent strains (loss of immunogenic protein bands (MBA; red arrows) and new immunogenic protein bands (41 kDa band; blue arrows). The numbers 3A and 7A indicate 3 and 7 serial challenges, respectively. M= molecular-weight size marker.

4.2.6 Identification of differentially expressed proteins by proteomic analysis

Total cell proteins were extracted from both serum-resistant strains (SR-DFK1) and SR-HPA2) and their serum-sensitive parental strains (SS-DFK1 and SS-HPA2) and separated by 2-DE. Subsequently, the separated protein spots were visualised with Colloidal Coomassie blue stain and analysed. A total of 8 and 20 differentially expressed protein spots from DFK1 and HPA2 strains, respectively, were selected and excised from stained gels, as shown in Figure 4.6 and then sent for protein identification by mass spectrometry. Consequently, 3 of 8 protein spots of DFK1 strains and 9 of 20 protein spots of HPA2 were identified using MALDI TOF/TOF mass spectrometric analysis (Tables 4.1 and 4.2). These proteins were identified in the annotated open reading frames (ORFs) of the genome of *U. parvum* serovar 3 (strain ATCC 27815/ 27/ NCTC 11736) as well as in the secured genomic database access from the J. Craig Venter Institute (JCVI) for the genomes of *U. parvum* serovar 1 (strain ATCC 27813) and *U. parvum* serovar 6 (strain ATCC 27818).

The proteomic analysis indicated that all serum-resistant strains (SR-DFK1 and SR-HPA2) had translation elongation factor Tu protein with altered isoelectric points (appeared as chain of protein spots with same molecular mass size but different *pI*) as compared to parental strains (spots A3 & A5 for SV1 strains and spots B6, B7, B15 & B16 for SV6) (Figure 4.6.A and 4.6.B). In addition, over-expression of 5`-nucleotidase, lipoprotein e (P4) Family in SR-HPA2 (SV6) was observed (spot B10, Figure 4.6.B). Alongside with these deferentially expressed proteins, common immunogenic proteins, among susceptible and resistant strains, were also identified in both. Conserved hypothetical protein-UPA6_A0338 (spot B9), inorganic diphosphatase (spot B3) and methylenetetrahydrofolate dehydrogenase (spot B19) for SV6 (Figure 4.6.A) were seen

with SV1 strains, while translation elongation factor G (spot A1) was detected among SV6 strains (Figure 4.6.B).

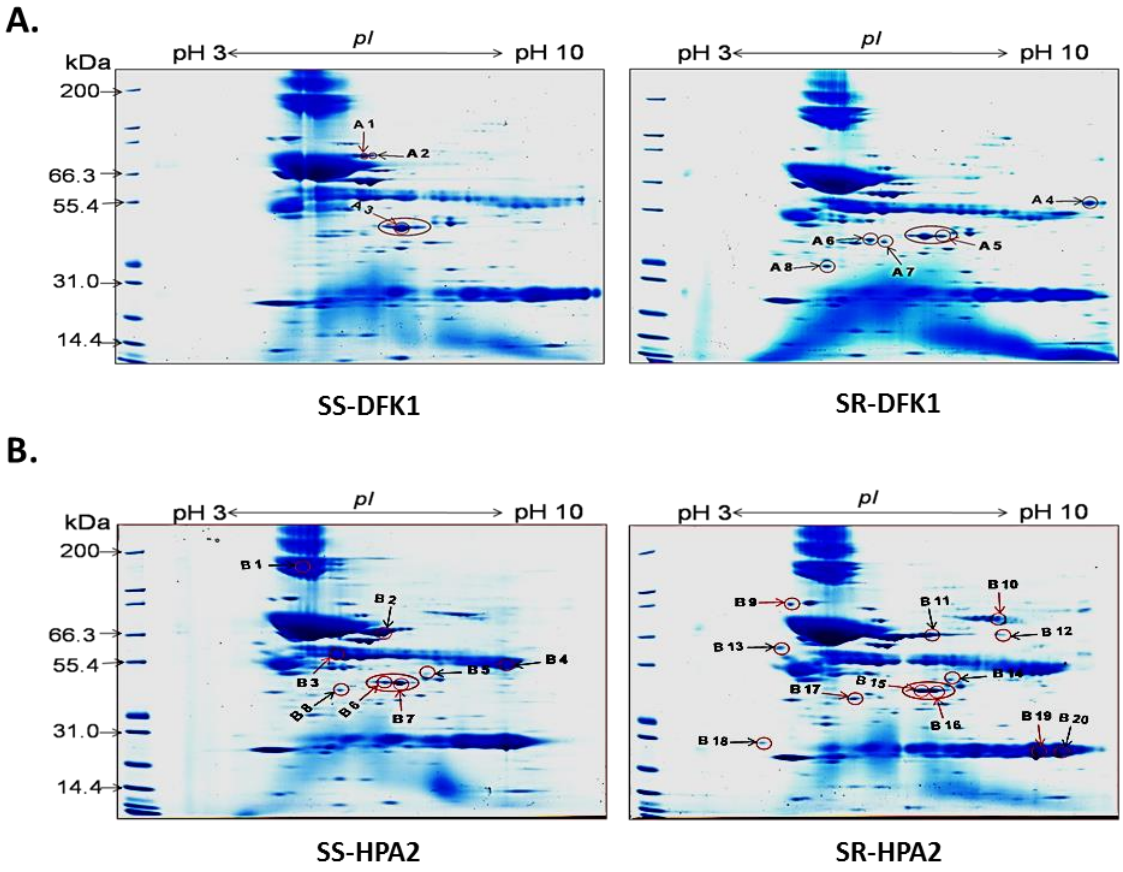


Figure 4.6 Two-dimensional electrophoretic analysis of total cell protein extract from induced serum-resistant *Ureaplasma* strains compared to serum-sensitive parents. A: SR-KFK1 and SS-DFK1 (SV1); B: SR-HPA2 and SS-HPA2 (SV6). Proteins were separated on a IPG strip (3-10NL) followed by electrophoresis on a vertical NuPAGE 4-12% Bis-Tris Zoom gel (1mm) and Colloidal Coomassie blue-stained. A and B indicate selected spots sent for identification by tandem mass spectrometry (red circles). Red arrows refer to successfully identified spots and Black arrows indicate unidentified spots. Representative 2-D gels selected from three repeated experiments.

Table 4.1 Proteins putatively identified from *U. parvum* SV6 (strain HPA2) using tandem mass spectrometry

Spot no. on 2-D gel	ID category*	Accession no.	Protein ID compared to SV6 (strain ATCC-27818)	Mascot score	e value**
B3	3	UPA6_A0317	Inorganic diphosphatase (ppa) [3.6.1.1]	35	0.22
B6	1	UPA6_A0567	Translation elongation factor Tu (tuf)	245	1.9e-022
B7	1	UPA6_A0571	Translation elongation factor Tu (tuf)	344	2.4e-032
B9	2	UPA6_A0338	Conserved hypothetical protein	103	3e-008
B10	3	UPA6_A0571	5`-nucleotidase, lipoprotein e (P4) family	36	0.16
B15	1	UPA6_A0567	Translation elongation factor Tu (tuf)	303	3e-028
B16	1	UPA6_A0567	Translation elongation factor Tu (tuf)	296	1.5e-027
B17	1	UPA6_A0547	Cytosol aminopeptidase family protein	324	2.4e-030
B19	3	UPA6_A0340	Methylenetetrahydrofolate dehydrogenase	34	0.25

* ID category: Cat 1 (2 or more peptides with e<0.05) and best publication quality data; Cat 2 (one peptide with e<0.0001) which is publication quality with spectra shown; Cat 3 is not publishable but may indicate an ID (one or more peptides with overall E value p <0.05 but p>0.0001 (multiply all the values). ** expectation value (probability that the assignment is a random event).

Table 4.2 Proteins putatively identified from *U. parvum* SV1 (strainDFK1) using tandem mass spectrometry

Spot no. on 2-D gel	ID category*	Accession no.	Protein ID compared to SV1 (strain ATCC-27813)	Mascot score	e value
A1	1	UPA1_G0171	Translation elongation factor G	180	6.1e-016
A3	1	UPA1_G0170	Translation elongation factor Tu (tuf)	368	9.6e-035
A5	1	UPA1_G0170	Translation elongation factor Tu (tuf)	276	1.5e-025

* ID category: Cat 1 (2 or more peptides with e<0.05) and best publication quality data; Cat 2 (one peptide with e<0.0001) which is publication quality with spectra shown; Cat 3 is not publishable but may indicate an ID (one or more peptides with overall E value p <0.05 but p>0.0001 (multiply all the values). ** expectation value (probability that the assignment is a random event).

4.2.7 Identification of the novel 41 kDa protein using 1-D gel- based proteomic method

As it was difficult to identify the altered immunogenic proteins via 2-DE-based proteomics, an effort to identify these proteins directly from matched 1-DE bands stained with Colloidal Coomassie blue was achieved. Following extraction and separation of total cell proteins from DFK1 strains (SR-DFK1 and SS-DFK1) using 1-DE, separated proteins on gels were then stained, visualised and analysed. Protein spots from the 90 and 80 kDa bands, previously identified as MBA protein using monoclonal anti-MBA antibody, from both SS-DFK1 and SR-DFK1 respectively and the novel 41 kDa band from SR-DFK1 (Figure 4.7) were excised from the gel and sent for MALDI TOF/TOF mass spectrometric identification. The aforementioned protein bands were differentially expressed bands detected by immunoblot analysis (Figure 4.2). The MBA bands (90 kDa and 80 kDa) were indicated as a change in size of MBA protein, but the unidentified 41 kDa protein was newly expressed in all resistant strains observed in this study.

Following MS identification, protein spots from 1-DE Colloidal Coomassie blue stained gels were identified in the genome of *U. parvum*serovar 1 strain ATCC-27813 (Table 4.3). Both 90 kDa and 80 kDa bands were identified as a multiple banded antigen (MBA), and confirmed the previous identification obtained using monoclonal anti-MBA antibodies. The candidate potential protein that may mediate serum resistance, the 41 kDa band was also identified using this approach as UU280 protein of *Ureaplasma*, an endo-1, 4-beta-glucanase. Further experiments were required to confirm the identity of this protein, UU280, and to further investigate the possible role of this protein in serum resistance as the 41 kDa was a unique protein expressed in all serum-resistant strains seen so far.

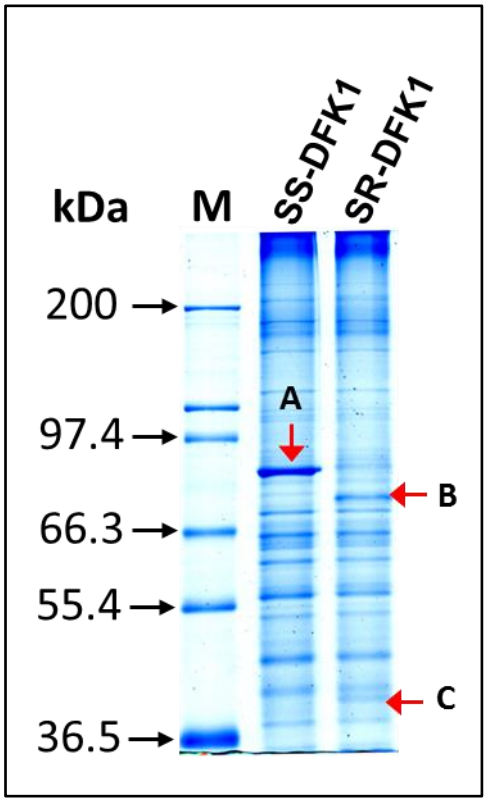


Figure 4.7 Visualisation of 1D protein profile of serum resistant and serum sensitive DFK1 strains. proteins separated by 1-DE. Proteins were extracted from both induced serum-resistant DFK1 (SR-DFK1) and their serum- sensitive parent strain DFK1 (SS-DFK1; SV1) and then separated on 7.5% SDS-PAGE and stained with Colloidal Coomassie blue stain. Red arrows indicate bands with variation in protein expression: (A) 90 kDa band, (B) 80 kDa and (C) 41 kDa, from which protein spots were excised and processed for protein identification using mass spectrometry. M= molecular weight marker, Mark12.

Table 4.3 Proteins putatively identified from *U. parvum* SV1 (strain DFK1) using tandem mass spectrometry

Band no. on 1-DEG	ID category*	Accession no.	Protein ID compared to SV1 strain ATCC-27813 proteins	Mascot score	e value**
A	1	UPA1_B0001	multiple banded antigen	465	3.50e-11
B	1	UPA1_B0001	multiple banded antigen	496	6.70e-15
C	1	UPA1_G0515	UU280 (endo-1,4-beta-glucanase)	186	2.40e-13

* ID category: Cat 1 (2 or more peptides with e<0.05) and best publication quality data; Cat 2 (one peptide with e<0.0001) which is publication quality with spectra shown; Cat 3 is not publishable but may indicate an ID (one or more peptides with overall E value p <0.05 but p>0.0001 (multiply all the values). ** expectation value (probability that the assignment is a random event).

4.2.8 Immunoblot analysis of proteins separated by 2-DE

Whole cell protein lysates separated using 2-DE from DFK1 (SV1), HPA2 (SV6), SV9, and HPA5 (SV3) were also analyzed using western blotting detected by seropositive serum. Immunoblot analysis was done to investigate immunoproteins that showed differences in expression by 1-DE western blotting. 2-DE immunoblot analysis revealed several immunogenic protein spots in the 20-220 kDa regions with a wide range of isoelectric points (Figure 4.8 and 4.9). Although the results revealed some clear differences in immunoproteins between the resistant and sensitive strains, it was not possible to see such differences in matched gels stained with Colloidal Coomassie blue stain in order to identify them, particularly between strains of both SV1 and SV6. For example, there was a significant difference in immunogenic proteins between SR-DFK1 compared to SS-DFK1; a chain of immunogenic proteins with similar molecular mass size and different range of protein charges, seen in SS-DFK1, was completely lost in SR-DFK1 (Figure 4.8). However, this line of protein spots was not detected by Colloidal Coomassie blue stain (Figure 4.6). The only exception (visualized by both immunoblot and Colloidal blue stain) was the EF-Tu protein isoforms for DFK1 and HPA2 strains. This protein was shown to be immunogenic and the isoforms differentially expressed between serum-sensitive and serum-resistant strains (Figures 4.6 and 4.8). SV9 strains also revealed a significant difference in EF-Tu isoforms identified by immunoblot ((Figure 4.9). On the other hand, no differences in EF-Tu isoforms observed by immunoblotting between SA-HPA5 and the parent strain (SS-HPA) were observed (Figure 4.9).

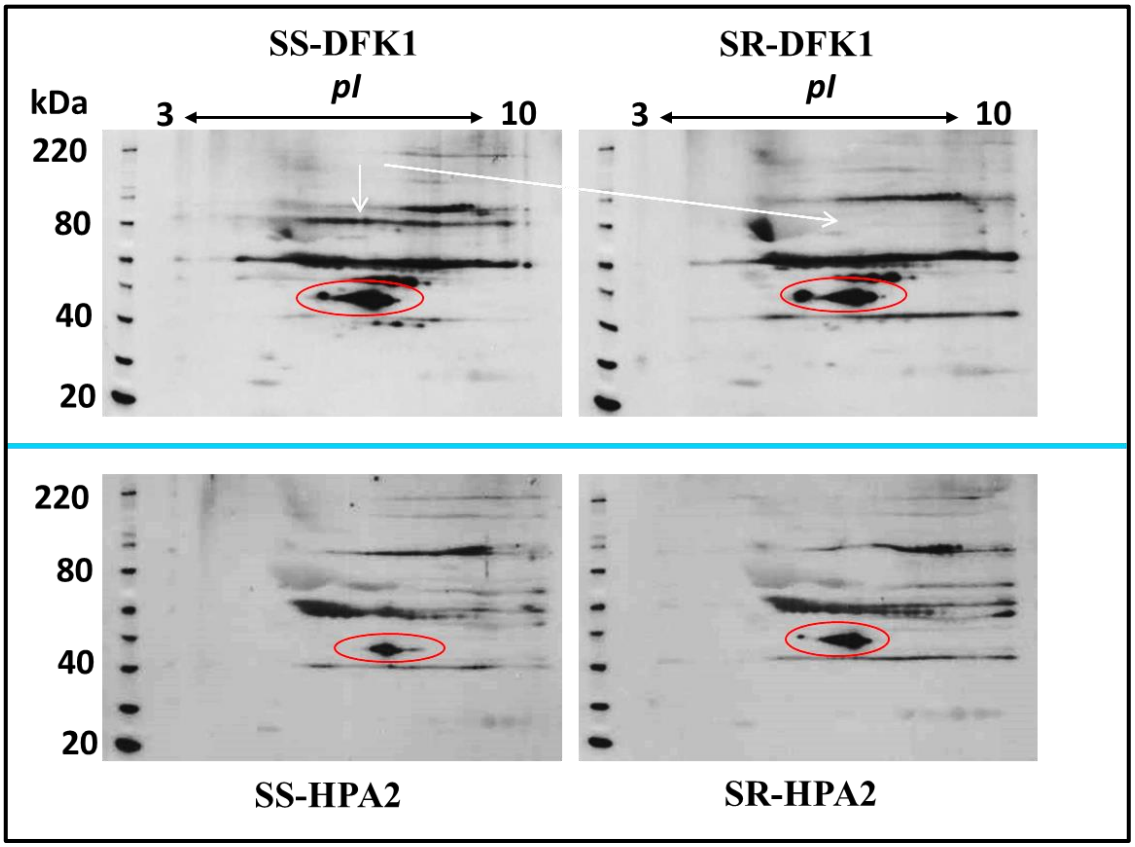


Figure 4.8 Two-dimensional immunoblot analysis of total cell proteins from serum-sensitive *Ureaplasma* isolates (DFK1 and HPA2) compared to their serum-challenged strains. Separated proteins (65 µg) from serum-resistant strains and their serum-sensitive parental strains were detected using a high-titre seropositive serum. White arrows indicate loss of immunoproteins with wide range of isoelectric points (*pI* 5-10) and the same molecular mass size (80 kDa) from SR-KFK1 clone. Red circles refer to EF-Tu protein isoforms with variations in isoelectric points between serum-sensitive and serum-resistant of both SV1 and SV6 strains. On the left side of blots kDa indicates molecular mass marker size. Representative immunoblots selected from three repeated experiments.

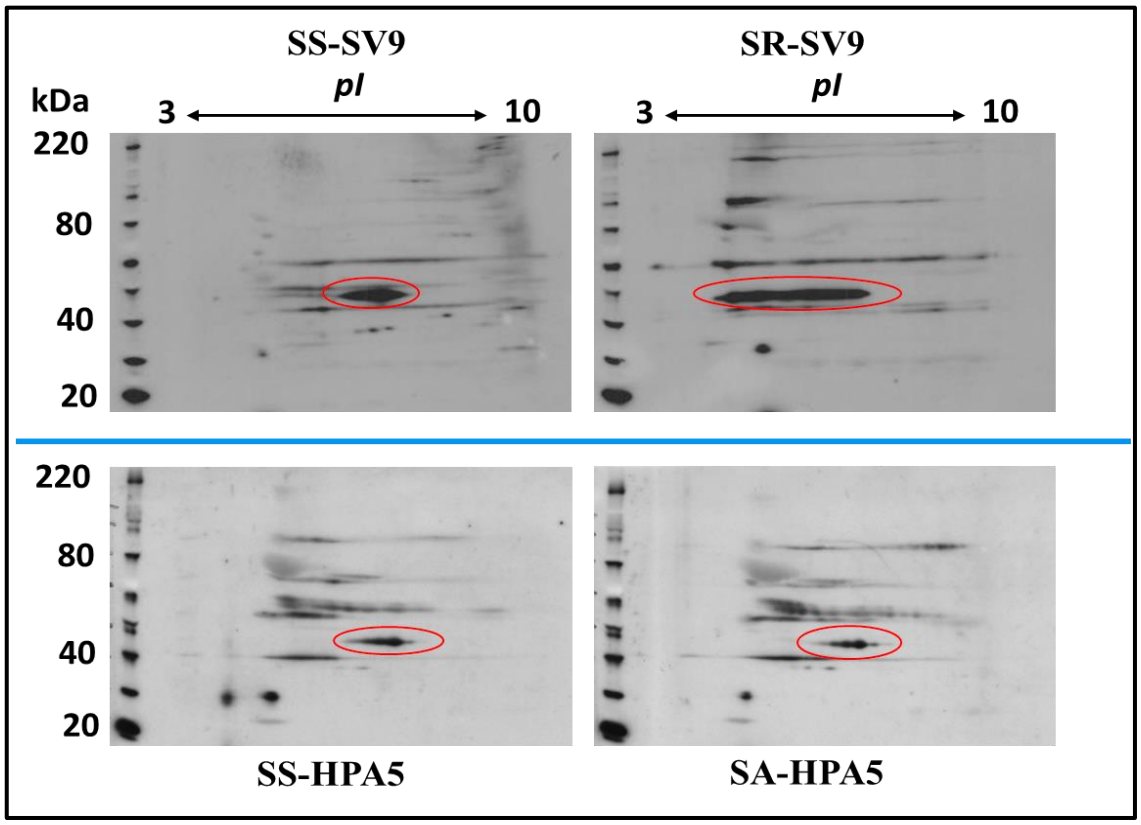


Figure 4.9 Two-dimensional immunoblot analysis of total cell proteins from serum-sensitive *Ureaplasma* isolates (SV9 and HPA5) compared to their serum-challenged strains. Separated proteins (65 µg) from one serum-resistant strain (SR-SV9) and one serum-challenged non- resistant strain (SA-HPA5) and their serum-sensitive parental strains were detected using a high-titre seropositive. Red circles refer to EF-Tu protein isoforms, with significant variations in isoelectric points between SS-SV9 and SR-SV9 strains. No differences in EF-Tu or any other immunoproteins were observed with HPA5 strains. On the left side of blots kDa indicates molecular mass marker size. Representative immunoblots selected from three repeated experiments.

4.2.9 Alteration in EF-Tu associated with serum resistance in *Ureaplasma*

Although differences in EF-Tu isoforms were identified by tandem mass spectrometry analysis of Colloidal blue-stained protein spots matching the characteristics of those observed by immunoblot with seropositive human serum, these results were also confirmed through 2-DE immunoblot analysis using a monoclonal anti-EF-Tu (raised against the N-terminus of *E.coli* EF-Tu). The results showed a consistent change in EF-Tu charge among all serum-resistant strains, when compared to parental strains. All resistant strains had more acidic *pI* isoform species of the EF-Tu protein (Figure 4.10). This alteration was only seen with serum-resistant strains that emerged from serum-sensitive strains after being serially put under immunological pressure via serial challenge with NHuS *in vitro*. However, such change was not seen with the HPA5 strain that was also subjected to numerous consecutive challenges but remained sensitive (Figure 4.11).

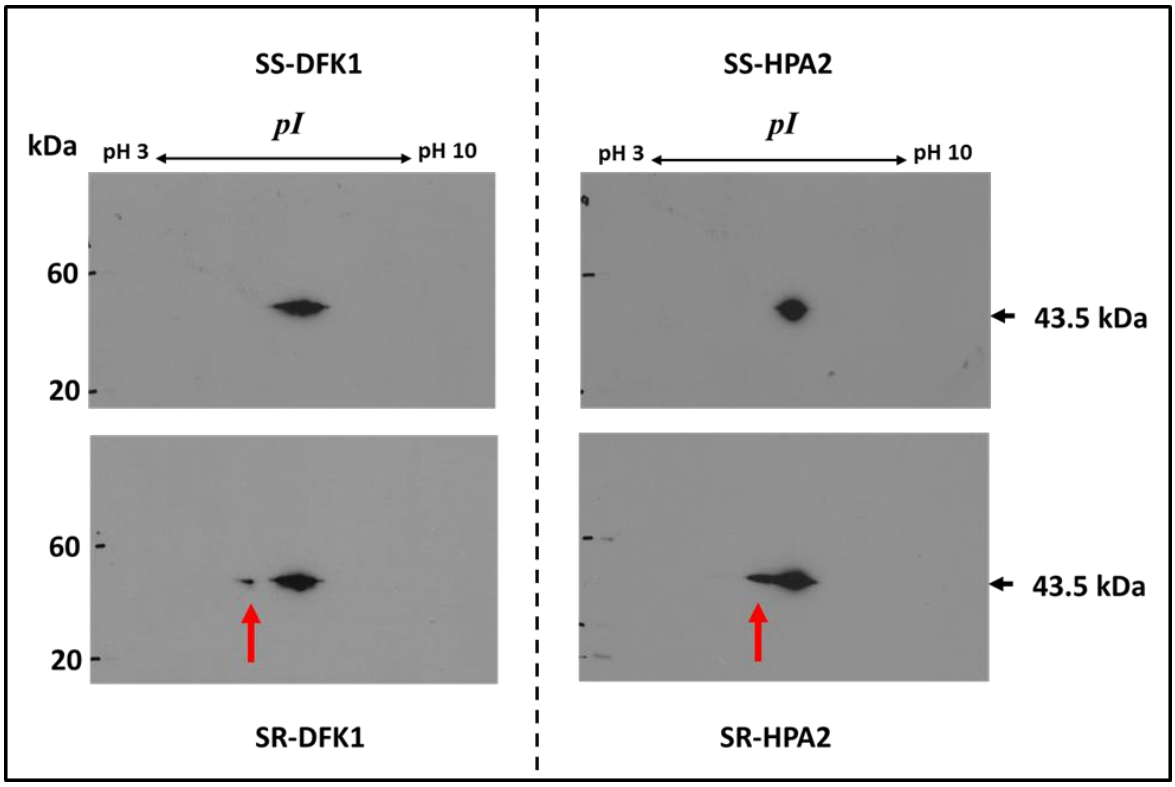


Figure 4.10 Altered expression of EF-Tu in *Ureaplasma* isolates (DFK1 and HPA2) following challenges with NHuS. Total cell protein extract separated by 2-DE on an IPG strip followed by vertical NuPAGE 4-12% Bis-Tris gel. EF-Tu was detected using anti-elongation factor Tu antibody. The blots show changes in the EF-Tu protein charge, a novel acidic *pI* isoform (red arrow), in the serum-resistant strains (SR-DFK1 and SR-HPA) compared to parent strains (SS-DFK1 and SS-HPA2). Representative immunoblots selected from three repeated experiments.

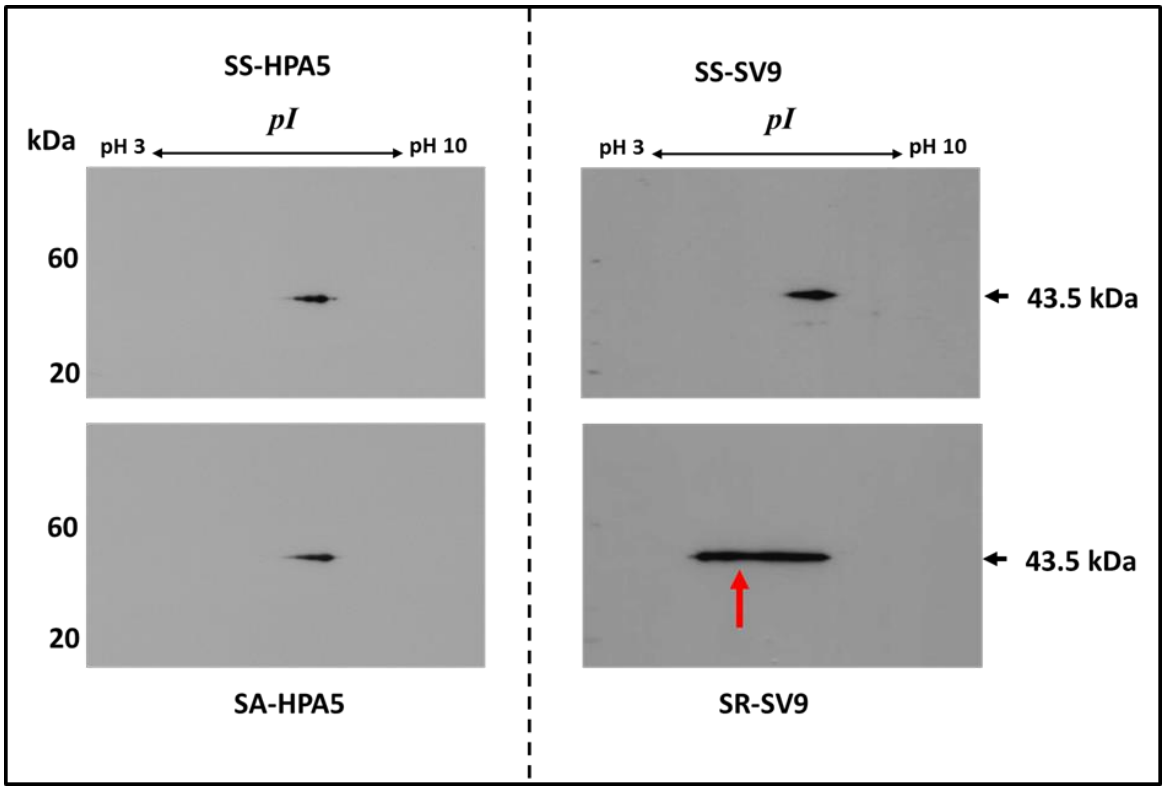


Figure 4.11 Altered expression of EF-Tu in *Ureaplasma* isolates (HPA5 and SV9) following challenges with NHuS. Total cell protein extract separated by 2-DE on an IPG strip followed by vertical NuPAGE 4-12% Bis-Tris gel. EF-Tu was detected using anti-elongation factor Tu antibody. The blot on the right-hand side shows a significant shift in *pI* (a novel acidic isoform (red arrow) in the induced serum-resistant strain of SV9 (SR-SV9) compare to parent strain (SS-SV9). EF-Tu remained similar in HPA5 strains (SA-HPA5 and SS-HPA5) as seen in the left-hand side blot. Representative immunoblots from three repeated experiments.

4.2.10 Investigation of glycosylation in total-cell proteins

Total cell proteins from both serum-resistant and serum-sensitive *Ureaplasma* strains were extracted and separated by SDS-PAGE and then subjected to glycoprotein staining. This experiment was performed to investigate any glycosylation in proteins from serum-sensitive and serum-resistant *Ureaplasma* strains and to try to link any of these glycosylation processes to induced serum resistance in ureaplasmas. In *M. pulmonis,* the repeat unit of VsaA, which was shown to mediate serum resistance, was also found to contain a glycosylation site that resulted in contributing to the glycocalyx of the bacteria as a physical shield against complement attack (Bolland *et al.*, 2012). Therefore, this experiment was conducted to examine the possibility of glycosylation of the repeat unit of MBA as well. As it can be seen in Figure 4.12A, the results showed no clear evidence of glycosylated proteins within the total cell proteins as no apparent stained proteins were visualized with glycoprotein stain compared to positive control bands of glycoproteins, which appeared as magenta bands. All proteins were also visualized with Colloidal Coomassie blue stain where variations in MBA could be seen among serum-sensitive and serum resistant emerged strains (Figure 4.12B).

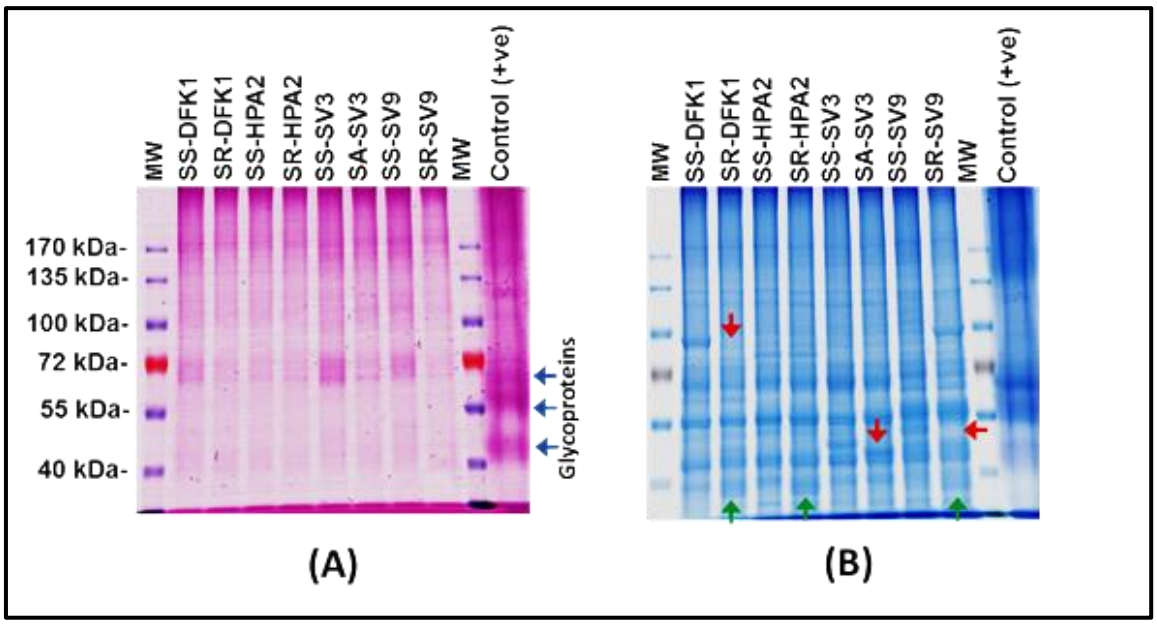


Figure 4.12 Detection of glycoproteins in total cell proteins from *Ureaplasma* species. Serum-resistant or serum-attacked non-resistant clones were compared to their serum-sensitive parental strains. Proteins were extracted with 1% SDS and separated on 7.5% SDS-PAGE gels. Gels were first stained with glycoprotein stain (A) to visualise glycosylated proteins and then subsequently stained with Colloidal Coomassie stain, RAPIDstain, (B) to visualise other non-glycosylated proteins and enhance staining of glycoproteins. Glycoproteins appear as magenta bands as seen in positive control bands (blue arrows). Red arrows indicate variations in MBA and green arrows refer to the 41kDa protein expressed by serum-resistant strains. MW= molecular size marker.

4.2.11 Investigation of UU280 protein as the putative 41 kDa induced protein

Following the successfully identification of the 41 kDa protein as UU280 protein using proteomic analysis this protein was further investigated to confirm its role in serum resistance among the species of *Ureaplasma*. In fact, the successfully developed method in this thesis for *Ureaplasma* transformation was also utilised successfully to deliver an exogenous copy the UU280 gene to some known serum-sensitive strains and investigate its expression and role in avoiding complement killing. In preparation, the UU280 gene insert was first synthesised and cloned to the vector plasmid, pMT85, and which was then cloned into *E. coli* strains, where it was amplified and validated. The pMT85-UU280 construct was used to transform serum-sensitive *Ureaplasma* strains under study. In parallel with that work, a polyclonal anti-UU280 antibody was also produced against UU280 KLH-peptide conjugate (the UU280 hapten consisted of 23 amino acids (322-343): `N`- CPQRYLHAPIGVATVKAAFD). Anti-UU280 polyclonal antibody was first screened and tested by dot blotting with the UU280 peptide to validate it for immunoblot analysis. A strong reactivity was observed on the first three 1:10 dilutions of UU280 protein using both polyclonal anti-UU280 antisera generated, but no reactivity was noted using seropositive serum.

Following a successful delivery of pMT85-UU280 construct, which was carrying UU280 gene insert tagged with His-tag, expression of UU280 was investigated in both *E. coli* and *Ureaplasma* by immunoblot analysis using anti-His tag antibody, anti-UU280 antisera and seropositive serum. The results showed that UU280 protein was successfully expressed by the two *E. coli* strains (carrying UU280 insert with forward and reverse orientations), as it was successfully detected by both anti-his tag antibody (strong reactivity) and by anti-UU280 specific antibody (low intensity); however, seropositive

serum, which detects the 41 kDa protein, did not detect this exogenous UU280 protein expressed in *E. coli* (Figure 4.13.A, B &C).

This finding and dot blot analysis indicated that IgG antibodies against ureaplasmal UU280 protein were not present in that seropositive normal human serum and what was recognised as a 41 kDa protein by NHuS in all induced serum-resistant strains is probably not UU280 protein. Furthermore, the pMT85-UU280 plasmid construct was also delivered into *Ureaplasma* serum-sensitive strains successfully, as was confirmed by the presence of gentamicin resistant gene marker using PCR. However, expression of UU280 protein could not be detected by any of the antibodies used (Figure 4.14.A, B & C). Anti-his tag antibody did not recognise any His-tag and seropositive serum did not detect any 41 kDa immunogenic protein in all of transformed strains. Additionally, anti-UU280 antisera did not react with any band with a similar molecular weight size of that of 41 kDa band. Instead, anti-UU280 antibody recognised a common immunogenic protein band with different size (higher than 41 kDa) expressed by all serum-sensitive and serum-resistant *Ureaplasma* strains, which might be endogenous UU280 protein. Besides and more importantly, all *Ureaplasma* strains cloned with pMT85 carrying UU280 gene did not gain resistance to NHuS and remained serum- sensitive similar to the parental strains following a successful transformation (see section 5.2.3).

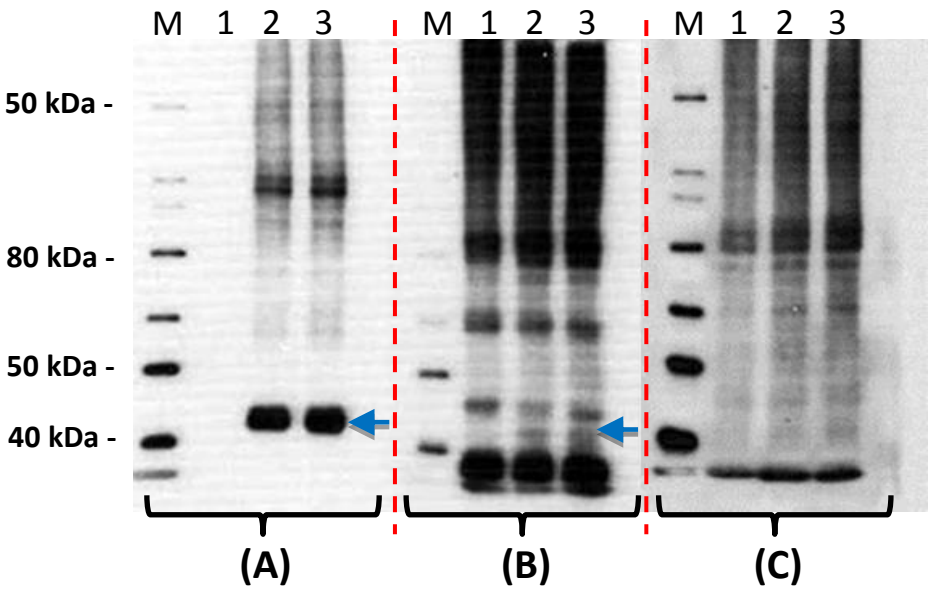


Figure 4.13 Expression of UU280 protein by transformed *E. coli* strains. Two transmutant strains carrying UU280 gene (Lanes 2 and 3) with forward and reverse orientation, respectively, were compared to another transformed strain carrying an empty pMT85 vector (Lane 1) as a negative control. Total cell protein lysate (10 µg) separated on 1-DE SDS-PAGE gel and blotted on nitrocellulose membrane. UU280 protein was detected by both anti-His tag antibody **(A)** and anti-UU280 polyclonal antibody **(B)** but not with a high titre seropositive serum **(C)**. Blue arrow indicated UU280 bands. M= molecular weight marker

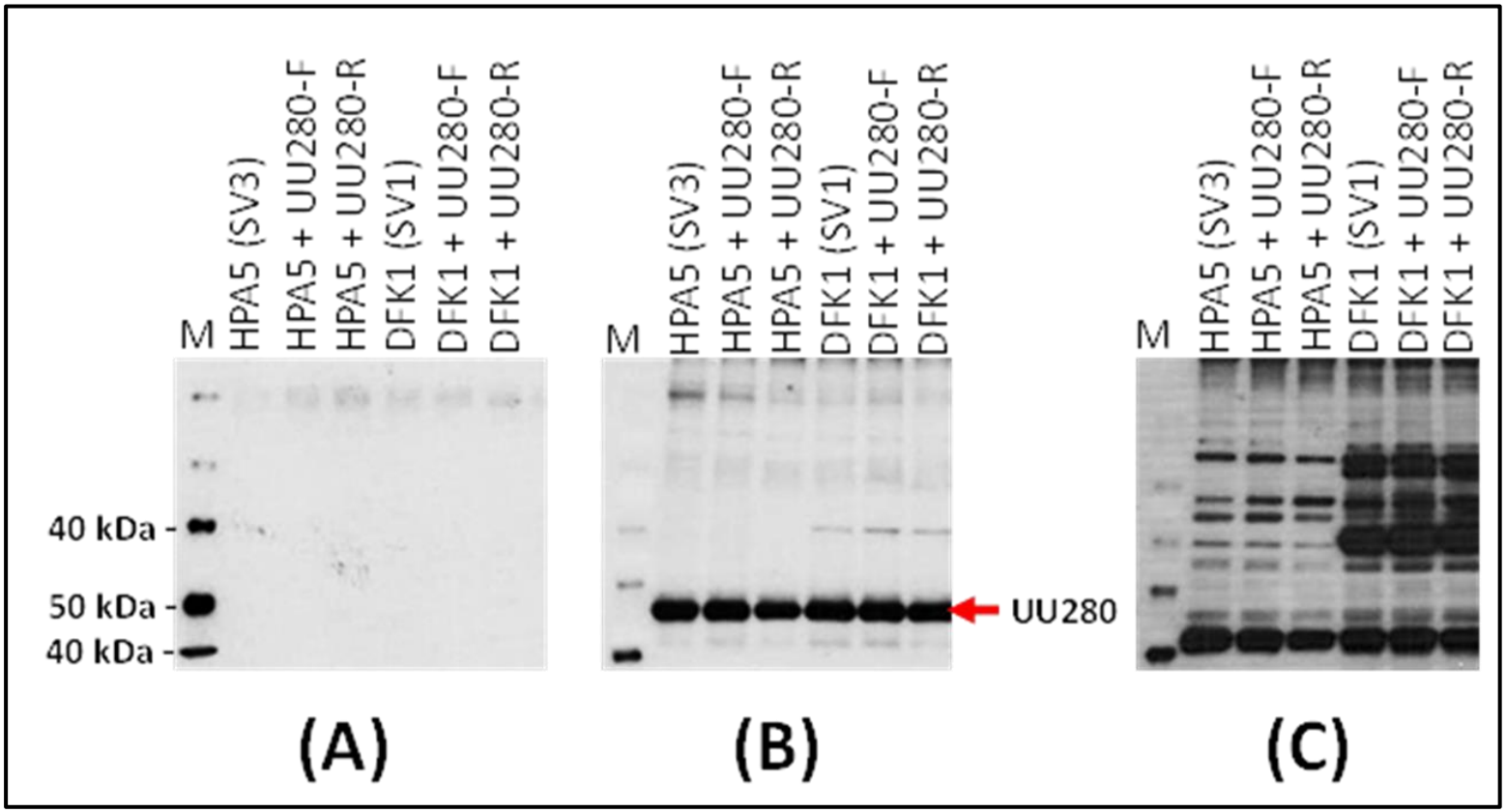


Figure 4.14 Investigating expression of UU280 protein by transformed *Ureaplasma* strains compared to untransformed parental strains (HPA5 and DFK1). Whole cell proteins extracted and separated on SDS-PAGE gels (7.5%) and then transferred into nitrocellulose membranes. UU280 protein was not detected with anti-His tag antibody (A), but was detected as 46 kDa band by anti-UU280 antisera **(B)** in all *Ureaplasma* strains (expressed by all isolates). Re-probing blot **(A)** with seropositive serum **(C)** revealed several common immunogenic protein bands with a common intense band of about 41 kDa combined with a higher common band with lower reactivity in all *Ureaplasma* strains. M= molecular weight marker.

The development of the transposon mutagenesis method for *Ureaplasma* spp. has provided another alternative and powerful tool to knock out, identify and study gene function(s) for *Ureaplasma*. Subsequent screening of all transformed strains for disrupted genes, after transposon mutagenesis as detailed in chapter 5, showed that one of the transmutant strains generated from a naturally serum-resistant isolate, W11 (SV12), had lost the 41 kDa protein band, which is a similar protein band to that observed in all induced serum-resistant strains. The loss of that protein was associated with altered susceptibility to complement killing as the transmutant strain became sensitive to complement-mediated serum killing. In fact this provided another alternative way to accurately identify and confirm the involvement of that candidate 41 kDa protein in serum resistance. Based on this observation, the role of the "UU280 protein", as identified by the proteomic approach, in serum resistance was re-examined again in the transmutant strain of W11. To achieve this, primer sets designed from *U. parvum* open reading frames to screen the UU280 gene region (forward primer pre-UU280 and revers primer post-UU280) were used to check the integrity of UU280 gene in the transmutant W11 strain. These primers failed to identify the gene disruption for the only transmutant strain of *U. urealyticum* (W11; SV12) indicating the integrity of the UU280 gene and ruling out its involvement in the phenotypic alteration seen in this particular strain following transformation.

Alternatively, sequencing and mapping the integration site of pMT85 mini-transposon in the genome of W11 transmutant strain had eventually revealed the identity of the disrupted gene responsible for that phenotypic change. The gene was identified as a hypothetical protein, UUR10_0137, in the genome of *U. urealyticum* serovar 10 (ATCC strain 33699). Therefore, an another potential gene that could control the expression of the 41 kDa protein is now available for further investigation, in similar way to that

applied to UU280 gene, to evaluate its role and function in mechanism of serum resistance among *Ureaplasma* spp.

4.3 Part 2 results: the *in vivo* investigation

4.3.1 Effect of immunological pressure *in vivo* on the immunogenicity of *Ureaplasma* species

As the previous part of this chapter focused on investigating the effect of immunological pressure on *Ureaplasma in vitro*, the aim of this part was to investigate the outcome of such immunological challenge *in vivo*. To achieve this goal endotracheal aspirate samples were collected from 57 intubated premature babies from Deriford Hospital in Plymouth and 10 of these patients (17.5%) were ureaplasma-positive as detected by culture. Following up samples from all ureaplasma-positive preterm babies were then serially collected and detected for the presence of *Ureaplasma* for further investigation. Of all collected samples, only the first and last *Ureaplasma* clinical isolates from the previously mentioned patients (20 clinical isolates) were investigated. In addition, other clinical isolates were also included in the study: 4 isolates were serially collected for two premature babies in the previous study (O1/O10 and W4/W11), two single samples collected from two premature babies at the University Hospital of Wales (UHW4; and UHW5) and 2 isolates from couples from France who were believed to have passed infection to each other (UUf1 and UUg1).

All these clinical isolates of *Ureaplasma* spp. were typed by PCR using the UM-1 primer set (Teng *et al.*, 1994) to differentiate the two *Ureaplasma* species and confirm that the first and last isolates from a patient were identical. Furthermore, all *U. parvum* isolates were then assigned, to one of the four known serovars (SVs 1, 3, 6, and 14) by

sequencing the UM-1 PCR product, while *U. urealyticum* isolates were grouped into one of the three clusters of *U. urealyticum* serovars based on nucleotide polymorphism in MBA gene (SVs 7/11, 2/5/8/9 and 4/10/12/13) as discussed in Chapter 3 of this thesis (Table 4.4). Monitoring changes in immunogenicity *in vivo* among the first and last or other studied clinical isolates was performed using immunoblot analysis. In addition, susceptibility to complement was investigated using the complement killing assay. Table 4.4 shows a summary of all clinical isolates used in this study.

Table 4.4 *Ureaplasma* clinical isolates investigated in this study

No.	Clinical isolate	Time period between samples	Source of isolates	Serovar ID	Patient source
1	UUf1	Single isolates	France	*U. urealyticum* (SV4,10,12 or 13)	Adult sexual partners
2	UUg1				
3	W4	3 weeks	University Hospital of Wales	*U. urealyticum* (SV4,10, 12 or 13)	Premature baby
4	W11				
5	01	3 weeks	University Hospital of Wales	*U. parvum* SV1	Premature baby
6	010				
7	UHW4	Single isolates	University Hospital of Wales	*U. parvum* SV3	Neighbouring Unrelated premature babies
8	UHW5				
9	Ply101B	2 weeks	Deriford Hospital Plymouth	*U. parvum* SV3	Premature baby
10	Ply101D				
11	Ply108B	1 week	Deriford Hospital Plymouth	*U. parvum* SV1	Premature baby
14	Ply108C				
15	Ply128A	34 days	Deriford Hospital Plymouth	*U. urealyticum* (SV2, 5, 8 or 9)	Premature baby
16	Ply128M				
17	Ply130B	3 weeks	Plymouth Hospital	*U. parvum* SV6	Twin premature baby
18	Ply130G				
19	Ply131B	17 days	Plymouth Hospital	*U. urealyticum* (SV2, 5, 8 or 9)	Premature baby
20	Ply131F				
21	Ply140A	1 week	Plymouth Hospital	*U. parvum* SV3	Premature baby
22	Ply140B				
23	Ply141A	2 weeks	Plymouth Hospital	*U. parvum* SV3	Premature baby
24	Ply141F				
25	Ply144A	2 days	Plymouth Hospital	*U. parvum* SV3	Premature baby
26	Ply144B				
27	Ply152A	1 month	Plymouth Hospital	*U. urealyticum* (SV2, 5, 8 or 9)	Premature baby
28	Ply152J				
29	Ply157A	10 days	Plymouth Hospital	*U. parvum* SV3	Premature baby
30	Ply157C				

4.3.2 Changes in antigenicity following immunological pressure *in vivo*

Monitoring alterations in *Ureaplasma* immunogenic proteins following immunological pressure *in vivo* were achieved by western blotting using monoclonal anti-MBA antibodies and seropositive human serum. Comparing the immunogenic profile of the first and last *Ureaplasma* clinical isolates showed that 7 Plymouth isolates had variations in the MBA which in most cases was a shift in MBA size. These isolates included Ply128A/128M, Ply130B/130G, Ply131B/131F, Ply140A/140B, Ply144A/144F, Ply152A/152J and Ply157A/157C (Figure 4.15.A). Ply130B/130G and Ply144A/144F also showed expression of new variants of MBA bands with low intensity. Besides these changes in the MBA some of those isolates (Ply144A/144F, 152A/152J and 157A/157C) also showed alterations in other immunogenic proteins (Figure 4.17.B). Ply101B/C and Ply108B/108C showed no changes in MBA but there were variations in other immunoreactive proteins detected by seropositive serum. In contrast, Ply128A/128M, Ply130B/130G, Ply131B/131F and Ply140A/140B had differences in MBA but not in other surface immunogenic proteins (Figures 4.15.A&B). Ply141A/141F showed no changes in immunogenicity at all.

Other clinical isolates also had variations in MBA and/or immunogenic proteins. While isolates O1/O10 had only a change in MBA, W4 compared to W11 had a change in other immunoreactive proteins. W11expressed new immunogenic protein at about 41 kDa but showed identical MBA bands (Figures 4.16.A&B). Single samples collected from neighbourhood premature babies, UHW4 and UHW5, showed variations in MBA as well as some other immuno-antigens. In contrast UUf1 and UUg1 which were collected from sexual partners were identical and no differences were observed (Figures 4.16.A&B). The MBA size in all these clinical isolates ranged from between 40 kDa to 120 kDa.

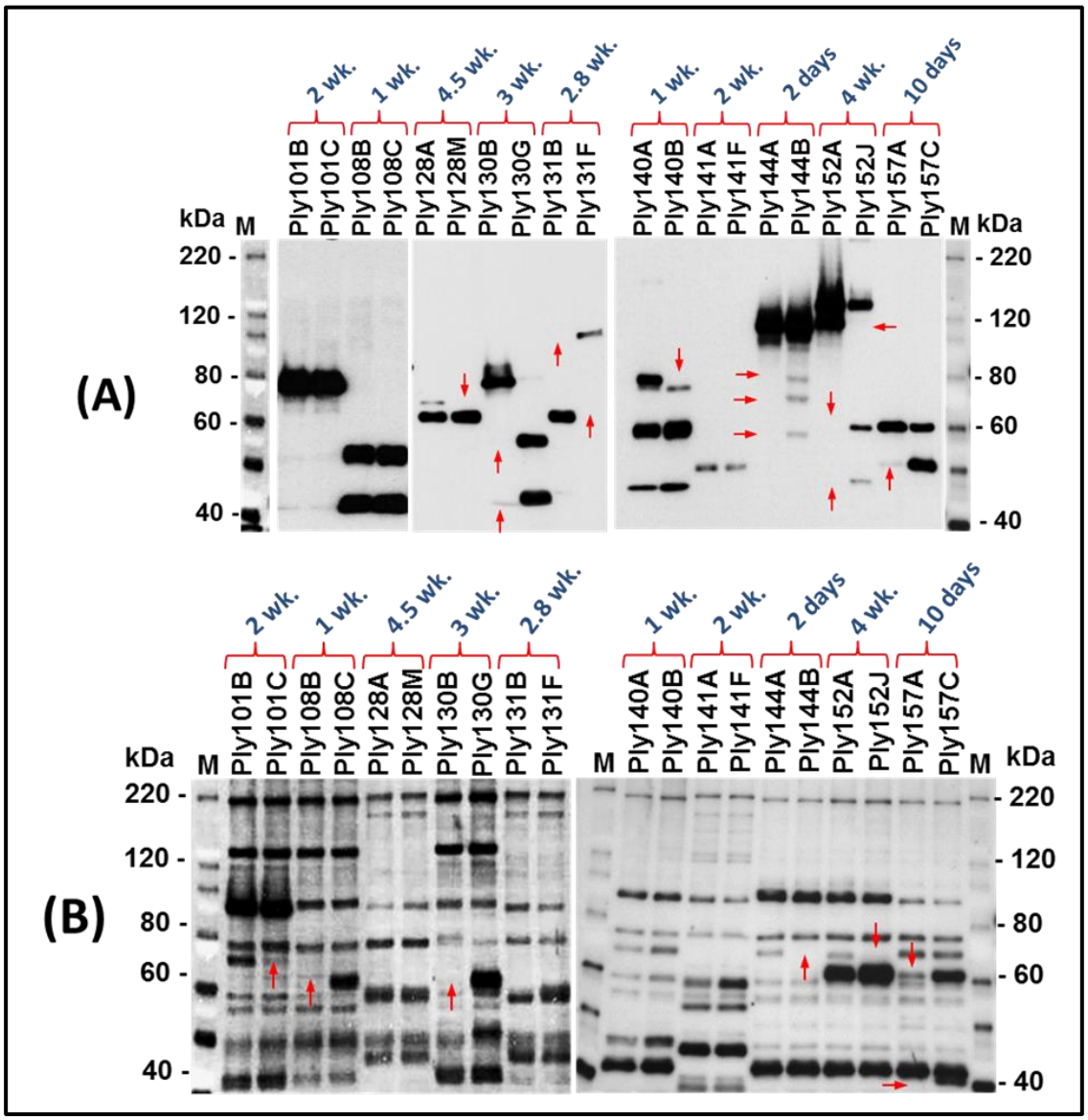


Figure 4.15 Variations in *Ureaplasma* immunogenicity following immunological pressure *in vivo* (Plymouth isolates). Immunoblot analysis of total cell protein of *Ureaplasma* isolates serially collected from intubated immature babies. First and last sample collected from each premature baby were run in parallel. The blots were first probed with monoclonal anti-MBA antibody (A) and then subsequently reprobed with seropositive NHuS (B). Time periods between isolates is indicted in days or weeks. Red arrows indicate changes in MBA recognised by monoclonal antibody (A), and variation in other immunodominant antigens identified detected by NHuS (B). M: molecular weight marker.

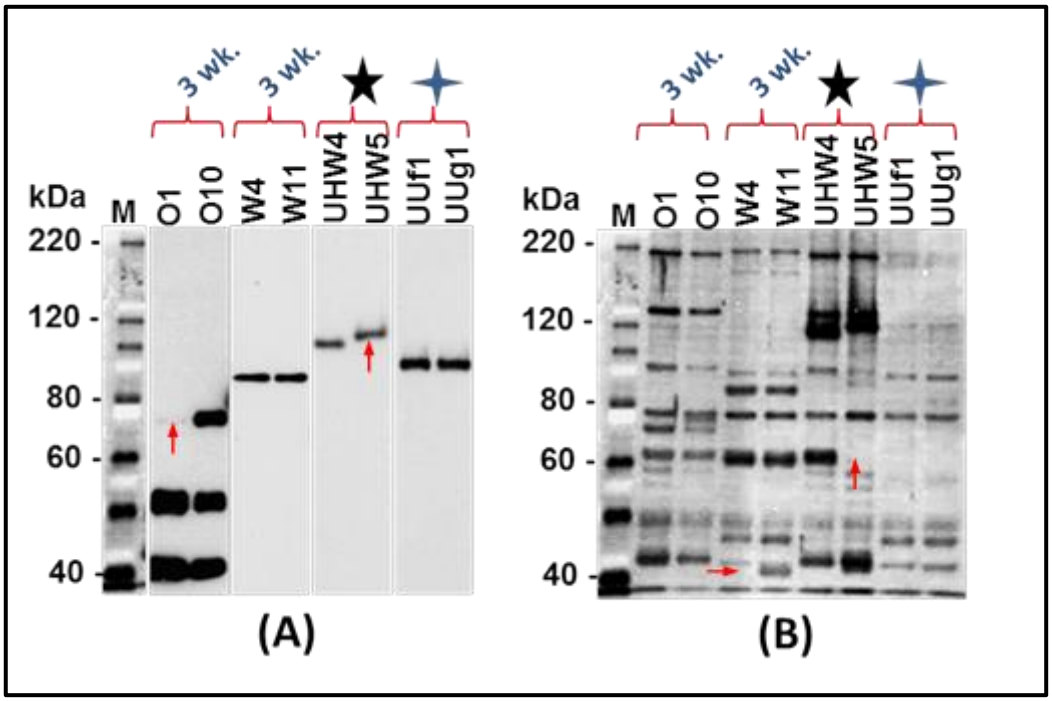


Figure 4.16 Variations in *Ureaplasma* immunogenicity following immunological pressure *in vivo* (UHW and Other isolates). Total cell protein was extracted from Ureaplasma and run on 1-DE and analysed by western blotting. The blots were first probed with monoclonal anti-MBA antibody (A) and then subsequently reprobed with seropositive NHuS (B). Time periods between isolates is indicated in days or weeks. Red arrows indicate changes in MBA recognised by monoclonal antibody (A), and variation in other immunogenic proteins detected by NHuS (B). Black stars: single samples from neighbouring babies; blue stars: single samples from couples. M= molecular weight marker.

4.3.3 Effect of immunological challenge *in vivo* on susceptibility of *Ureaplasma* to serum killing

After monitoring and reporting changes in immunogenicity among *Ureaplasma* clinical isolates collected serially from patients, it was worthwhile to investigate any changes in another phenotypic trait which is susceptibility of these clinical isolates to complement-mediated serum killing. Serum killing was performed by the complement killing assay described previously in chapter 2 using both seronegative and seropositive sera. The results obtained from screening and comparing first and last Plymouth clinical isolates collected from premature babies as well some other clinical isolates showed no significant differences in complement susceptibility when compared to each other (Figure 4.17.A&B). Exceptions were the isolates Ply130B/Ply130G and W4/W11 that showed differences in complement susceptibility. The first isolates (Ply130B and W4) were sensitive to killing by seropositive serum compared to the last collected isolates (Ply130G and W11) which had become serum-resistant (Figure 4.18.B). However, all of them were resistant to seronegative serum (Figure 4.18.A).

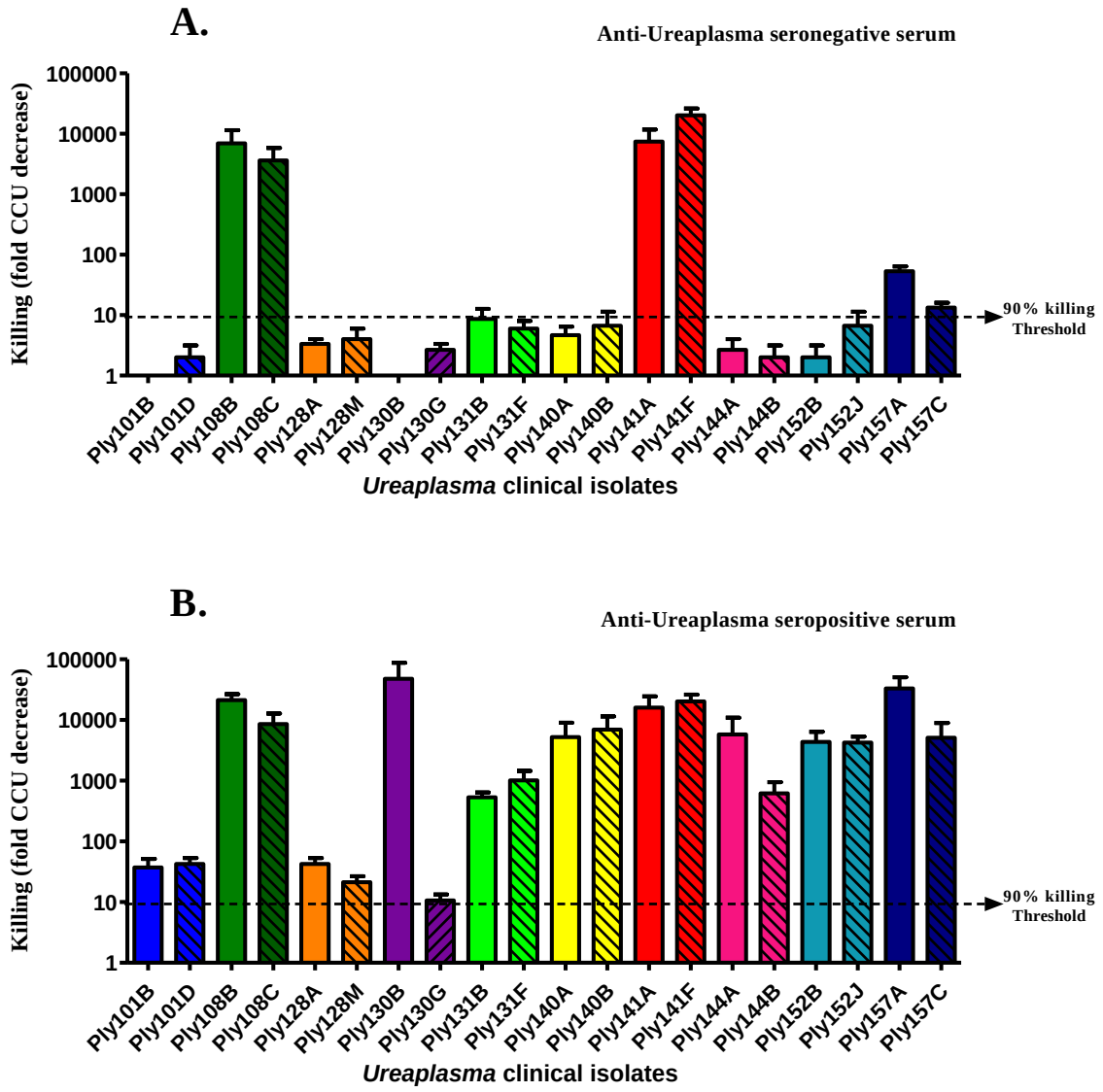


Figure 4.17 Complement susceptibility of *Ureaplasma* clinical isolates collected serially from premature babies at Deriford Hospital Plymouth . First (solid bars) and last (striped bars) isolates were compared with each other to monitor changes in serum susceptibility after a direct contact over a period of time with the developing immune system of baby (*in vivo*). Graphs A and B show susceptibility to complement from seronegative and seropositive sera, respectively.

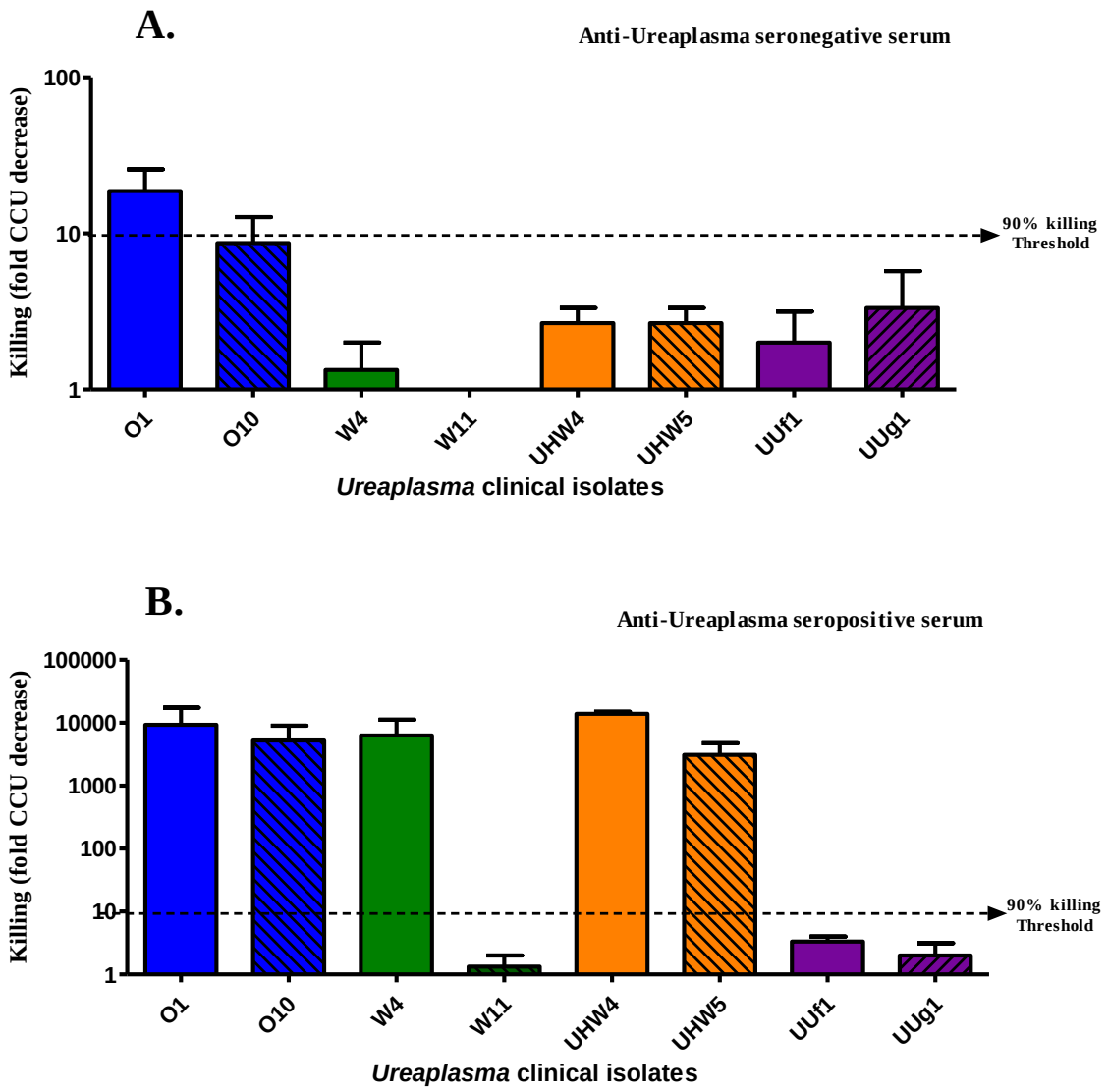


Figure 4.18 Complement susceptibility of *Ureaplasma* clinical isolates collected from premature babies and adults. First (solid-bars) and last (striped-bars) isolates were compared with each other to monitor changes in serum susceptibility. Isolates O1/O10 and W4/W11 were collected from the same baby after a direct contact over a period of time with the developing immune system (*in vivo*). Isolates UHW4/UHW5 were collected from unrelated neonates in adjacent cots whereas isolates UUf1 and UUg1 were isolated from sexual partners. Graphs A and B show susceptibility to complement from seronegative and seropositive sera, respectively.

4.4 Discussion

Despite being faced with an extremely powerful complex immune system of their host that is ultimately bacteriocidal successful pathogenic microorganisms have in turn developed sophisticated and effective approaches that allow them to adapt to the host milieu, resist killing and evade immune responses. Many of these strategies have recently been characterised and studied intensely, and a number of comprehensive reviews have provided some information on ways pathogenic microorganisms overcome the immune system of their own host, maintain survival and establish colonisation (Bernet *et al.*, 2003; Celli and Finlay, 2002; Laarman *et al.*, 2010; Lambris *et al.*, 2008; Sansonetti and Di Santo, 2007; Wurzner, 1999; Zipfel *et al.*, 2013; Zipfel *et al.*, 2007). Antigenic variation is one of these mechanisms that have been extensively investigated in several pathogens as a strategy utilised for avoiding immune defences (Deitsch *et al.*, 2009). There are two mechanisms that govern the variation of microbial antigenicity: genetic and epigenetic mechanisms. The first mechanisms include mutation and recombination that occur in the DNA sequence of a gene (encodes for immunogenic protein) or its regulators. The subsequent event of this alteration is a change in the expression level of a gene or the amino acid sequence of the gene product. The second mechanisms do not alter the basic DNA sequence of a gene, hence the term epigenetic, but leads to alterations in a gene expression by other ways such as DNA methylation (Casadesus and Low, 2006; Deitsch *et al.*, 2009; van der Woude and Baumler, 2004).

In the closely-related species to *Ureaplasma*, *Mycoplasma*, alteration of the expression of surface membrane proteins, as a way to manipulate the immune responses is a common tactic that have been demonstrated and investigated in a number of their members and the

molecular mechanisms by which this antigenic variety occurs have been well-defined (Citti *et al.,* 2010). Phase variation in mycoplasma surface lipoproteins is believed to occur as a result of spontaneous mutations in sites inclined to DNA slippage by nucleotide insertions/deletions in simple sequence repeats or recombination of a DNA sequence (Citti *et al.,* 2010). *Ureaplasma* spp. has also been shown to alter expression of one of the surface membrane proteins MBA to adapt to harsh environments both *in vitro* and *in vivo* and the mechanisms that control such alterations are thought to be similar to that utilised by *Mycoplasma species* (Zimmerman *et al.,* 2009). The main aim of this chapter was to monitor the stability of *Ureaplasma* immunogenic proteins following challenges with NHuS (*in vitro*) and direct interaction with the host immune system (*in vivo*). The work has also attempted to address and understand the role of *Ureaplasma* antigenic variation as a mechanism of serum resistance and manipulation of the complement system. Observations of changes in immunogenic proteins of several *Ureaplasma* strains following serial challenges with NHuS (*in vitro*) as well as direct interaction with the immune system of a number of premature babies (*in vivo*) have indicated the importance of such mechanisms among *Ureaplasma* in avoiding and adapting to harsh environments.

Several interesting findings were observed in this study that are worth further investigations as there was insufficient time to investigate them completely. Serum-resistant strains emerged following exposure of *Ureaplasma* to a repeated immunological pressure *in vitro* using NHuS. Those strains were the main focus of investigation in this study as they revealed potential ability among these tiny bacteria to overcome the complement-mediated serum killing. Significant changes in some immunogenic proteins that coincided with the acquisition of serum resistance were seen in all induced serum-resistant strains when compared with their parental strains. Herein, this chapter seeks to

highlight, identify and study the role of some putative proteins that showed alterations in expression in avoiding the complement-mediated serum killing. Of these antigenic variable proteins, three interesting proteins were identified and investigated. These immunogenic proteins included the multiple banded antigen (MBA), the elongation factor Tu (EF-Tu) and a putative 41 kDa new expressed antigen that, using a proteomic approach, was identified as UU280 protein (endo-1, 4-beta-glucanase), but later and following excluding the involvement of UU280 protein and an alternative candidate protein was identified in the transmutant strain of W11 as the hypothetical protein encoded by the UUR10_0137 gene.

The MBA is a major surface antigen that is recognised by the immune system during *Ureaplasma* infection and was proposed as one of the virulence factors of *Ureaplasma* spp. that play a role in antigenic variations and pathogenesis of *Ureaplasma* species (Watson *et al.*, 1990; Zheng *et al.*, 1995). Several previous studies have demonstrated both phase and size variations in the MBA *in vivo* as well as *in vitro* (Monecke *et al.*, 2003; Zheng *et al.*, 1995; Zimmerman *et al.*, 2011; Zimmerman *et al.*, 2009). It is also believed that these events of alteration in expression arise as a result of change in the number of the repeat units in the variable regions of the MBA, which might be caused by a slipped-strand mispairing mechanism (Teng *et al.*, 1994; Zheng *et al.*, 1994; Zheng *et al.*, 1995). In the study by Monecke and colleagues (2003) it was shown that subjecting representative serovars (SV3 and SV5) from both species of *Ureaplasma* to a selection pressure using polyclonal anti-MBA antibodies resulted in the emergence of escaping MBA-negative clones (Monecke *et al.*, 2003). In another study exposure of *Ureaplasma* to a similar selection pressure with antibodies against the MBA had also created escaping MBA-off variants. In these strains the MBA gene (UU375) was found to switch expression with an adjacent gene (UU376) through a DNA inversion event at short

inversion sequences, as the MBA 5' region was found to flip and drive expression of the adjacent, promoter-less UU376 gene (Zimmerman *et al.*, 2009). A later report by Zimmerman et al has suggested that the ORFs of both genes (UU375 and UU376) are part of gene family that comprise the locus of *mba* gene in *Ureaplasma* (Zimmerman *et al.*, 2011).

In this study, the MBA variants were identified by both MS identification using a proteomic approach and by immunoblot analysis using anti-MBA monoclonal antibodies. Antigenic alterations in the MBA expression, following serial challenges with NHuS, were observed in a number of representative serovars and clinical isolates, which is in agreement with many of the previous studies that indicated size variations in MBA. For example, in my study, size variations in MBA size were seen in two of the induced serum-resistant strains (SR-DFK-1 and SR-SV9) when compared to their parental serum-sensitive strains. Nevertheless, no change in MBA size was also observed in another equally induced serum-resistant strain (SR-HPA2). This later finding indicated that changes in MBA are not necessary for developing serum resistance in *Ureaplasma in vitro*.

Significantly, all observed changes in MBA size of *Ureaplasma* induced serum-resistant strains in this study were constantly associated with alterations and expression of other antigens, which seemed to be involved in developing serum resistance, as will be discussed later. Furthermore, similar observations (alterations in MBA size or no changes in MBA expression) were also demonstrated in other *Ureaplasma* strains in this study following immunological challenges with NHuS *in vitro*. Emergence of serum resistance among those strains was found to be with or without alterations in MBA expression. Further induced serum resistance without MBA alteration include the two different

isolates HPA56 and HPA57 which belong to the same serovar (SV3). Although both of them became resistant to serum killing following challenge with NHuS only the HPA57 isolate showed loss of one of the two isoforms of MBA following selection while the MBA of HPA56 remained unchanged. Besides, challenging an inherently serum-resistant strain with NHuS revealed that alteration in MBA expression cannot be induced when an isolate is already serum-resistant suggesting no direct relationship between MBA size variation and complement-mediated serum resistance is seen in *Ureaplasma*.

MBA size variation was also observed in isolates collected serially from premature babies. This experiment represented a real confrontation with the host immune defences (*in vivo* challenge) despite the fact that the immune system of premature babies is still underdeveloped. A number of last collected isolates from these new-born babies showed a significant alteration in their MBA size when compared to the first collected isolates (parental isolates). This provided additional evidence that *Ureaplasma* is able to change their MBA *in vivo* when faced with human immune defences. Similar observations were also shown in recent studies using animal models where alterations in MBA size *in vivo* were reported (Dando *et al.*, 2012; Knox *et al.*, 2010; Robinson *et al.*, 2013). Despite being able to observe these differences in the MBA size *in vivo*, no significant changes in complement susceptibility, which could be linked to such alteration in MBA size, among the serum-sensitive isolates over the time (both first and last strains remind the same) were observed. One possible explanation for this finding would be the fact that the immune system of the premature babies is still not completely mature to raise a significant immune response able to eliminate *Ureaplasma* colonisation.

Neonates and particularly premature babies have been shown to be deficient in several complement components, particularly the complement component C9, which is one of the

essential components required for forming the membrane attack complex that lyse bacterial pathogens (Lassiter *et al.*, 1992). In fact, such insufficiency could predispose these babies to several invasive bacterial infections. In this regard it is presumed that the serum-sensitive *Ureaplasma* isolates have maintained an external, non-invasive type of infection whereas if they had survived a strong complement attack that would enforce the emergence of serum resistant strains in those premature babies. However, the remarkable variations in MBA expression do suggest exposure to maternal or neonatal specific antibody as a driving force for altered MBA expression, which may be an effective mechanism (*in vivo*) for avoiding recognition. On the other hand, isolation of some serum-resistant isolates from premature babies may indicate colonisation with strain that was already resistant to serum killing, as serum-resistant serovars and clinical isolates were reported among *Ureaplasma* (as discussed in Chapter 3). Moreover, the serum used in the killing assay was from immune competent adults, where all arsenals of the immune system are fully developed. However, they were infected with other strains as the serum and isolate were not matched in these studies. To further validate such findings one would use a serum from the same premature babies and/or their mothers to test complement killing activity.

Antigenic variations in the MBA are believed to play an important role in avoiding recognition by the host immune system *in vivo*. In animal models, it has been suggested that the ability of *Ureaplasma* to colonise the amniotic fluid without any detectable histological inflammation of the chorioamnion was attributed to variations in this variable surface lipoprotein (Knox *et al.*, 2010). The authors of this study have also proposed that the severity of chorioamnionitis might depend on the number of MBA size variants in some serovars, as it was found that isolates with low number of MBA size variants are more virulent. In many mycoplasmas the high level of antigenic variations in surface

exposed lipoproteins is believed to help these microorganisms to evade the immune system of their hosts and establish chronic infections. MBA has been demonstrated as one of the pathogen-associated molecular patterns (PAMP) recognised by toll-like receptors 1, 2 and 6, and able of inducing the cytokine NF-κB and antibody production (Shimizu *et al.*, 2008; Triantafilou *et al.*, 2013). It has also been speculated that interaction of the MBA with TLR2, and not TLR4, might reflect the ability of *Ureaplasma* to cause chronic colonisation and low-level of inflammation in the amniotic epithelial cells that leads to premature rupture of membranes (PROM) and premature labour in pregnant women (Triantafilou *et al.*, 2013). Initiation of inflammatory responses by TLR2 engenders a passive inflammatory response (Hirschfeld *et al.*, 2001) that enables the microorganism to establish a chronic colonisation. Therefore, it can be assumed that *Ureaplasma* would be able to overcome such an immune response by changing the MBA via varying the number of the tandem repeats of their MBA gene in response to challenge with NHuS that contains *Ureaplasma*-specific antibodies and this probably happens by a slipped strand mispairing mechanism (Monecke *et al.*, 2003). Although, it has been previously reported that MBA-OFF variants of *Ureaplasma* strains could emerge following selection pressure *in vitro* (Monecke *et al.*, 2003; Zimmerman *et al.*, 2009), in this study only alterations in size and number of MBA variants were observed. No MBA negative clones were seen as MBA expressing species, sometimes with multiple separable isoforms, were constantly detected in all investigated *Ureaplasma* strains. It should be noted that all previous studies used rabbit anti-MBA polyclonal antibodies to induce selection pressure whereas in this study immunological pressure was achieved by using NHuS from health adult volunteers.

The second immunogenic protein that showed an interesting alteration coincident with serum resistance was the elongation factor Tu (EF-Tu). As an abundant protein, EF-Tu

was successfully identified using proteomics from several protein spots excised from stained 2-D gels. Following further investigation EF-Tu was revealed to be immunogenic and had a consistent change in its charge (seen as more acidic *pI* isoforms species) among all induced serum-resistant strains when compared to the parental serum-sensitive strains. This observation was not seen in a parallel challenged HPA5 isolate that remained serum-sensitive following several consecutive challenges with NHuS. EF-Tu is one of the most abundant proteins in bacteria and plays a main role in the translation process during protein synthesis (Weijland *et al.*, 1992). Besides its main function, EF-Tu has also been indicated to have other independent functions in bacterial cells. EF-Tu was suggested to be one of the cytoskeletal components of prokaryotic cells. Early studies have suggested EF-Tu as a structural protein since it was found to form filamentous structures *in vitro* (Beck, 1979; Beck *et al.*, 1978). Furthermore, in a number of bacterial species including *M. pneumonia, E. coli, N. gonorrhoeae, Bacillus subtilis, Listeria monocytogenes and Mycobacterium leprae* , EF-Tu was demonstrated under the cytoplasmic membrane or associated to the cell wall. These observations provided more evidence for the role of EF-Tu in the cytoskeleton of bacteria (Archambaud *et al.*, 2005; Dallo *et al.*, 2002; Defeu Soufo *et al.*, 2010; Jacobson and Rosenbusch, 1976; Judd and Porcella, 1993; Marques *et al.*, 1998).

EF-Tu was also suggested to play a role in bacterial virulence as it was found to mediate adhesion to host cells and establish colonization (Dallo *et al.*, 2002; Granato *et al.*, 2004). For example, Dallo *et al.*, (2002) indicated that EF-Tu in *Mycoplasma pneumoniae* is able to bind fibronectin and may also mediate adhesion to host cells. A similar function for EF-Tu as an adhesion protein was also reported in *Lactobacillus johnsonii* as it was shown that EF-Tu mediates adhesion to intestinal cells as well as working as a pro-inflammatory mediator. In addition, EF-Tu was found to be exposed on the surface of *P.*

aeruginosa mediating binding both FH and plasminogen, resulting in evading the activation of the complement system (Kunert *et al.*, 2007). In *Ureaplasma,* EF-Tu may also be associated to the surface membrane and play a role in pathogenesis as it was found to be immunogenic and had alternated charge that was coincided with serum resistance. Alterations in protein charges are believed to occur via a post-translational modification such as phosphorylation and the appearance of a protein as a chain of spots with same mass size in 2-D gels is suggestive of phosphorylation. EF-Tu was found to be phosphorylated in a number of microbes and proposed to be associated with survival and virulence (Archambaud *et al.*, 2005). In *M. genitalium* and *M. pneumoniae,* EF-Tu was also reported among the phosphorylated proteins (Su *et al.*, 2007). In this study, EF-Tu was observed as a series of spots with the same mass size and different *pI*. Therefore, this may indicate phosphorylation of EF-Tu that might play a role in mediating serum resistance among *Ureaplasma*. Further investigation on EF-Tu and its putative role in mechanism of serum-resistance will most likely give new insights into *Ureaplasma* pathogenesis.

The most interesting finding observed following emergence of serum resistance was an expression of a novel immunogenic protein with approximate mass size of 41 kDa. This antigen was putatively identified as UU820 protein (endo-1,4-beta-glucanase) using proteomic based method (1-DE based proteomics) in the genome of *U. parvum* serovar 1 (strain ATCC-27813). Switching on expression of a 41 kDa antigen was seen in all induced serum-resistant strains and one an inherently serum-resistant *U. urealyticum* isolate (W11; SV12). Interestingly, this immunogenic 41 kDa protein was only expressed in association with the development of serum resistance and challenged strains that had not become resistance after the serial selection pressure did not express it. This observation indicates that this protein is most likely to be involved in the mechanism of

serum resistance and requires more attention and investigation. Therefore, I put my efforts to try to define its role in serum resistance. Unfortunately, upon an extensive investigation, I was unable to establish a relationship between UU280 protein using specific created reagents and gene expression cassettes and the 41 kDa serum-resistance associated protein. Introducing an exogenous copy of the UU280 gene via transposon mutagenesis to known serum-sensitive isolates did not affect their susceptibility to complement activity and transmutants failed to express the recombinant UU280 protein as detected by immunoblot analysis. In addition, expression of an endogenous UU280 protein was detected in all serum- sensitive and resistant strains by anti-UU280 antisera. Moreover, the recombinant UU280 protein which was successfully expressed by recombinant *E. coli* cells was not recognized by the F7 human hyper-immune serum that binds to the 41 kDa protein. These observations suggest misidentification of the 41 kDa protein as UU280 using proteomics. Protein bands picked from 1-DE gels may contain more than one protein, and hence there is a possibility of contamination that could lead to a wrong identification by MS (Huang *et al.*, 2002).

Fortunately, transposon mutagenesis analysis (detailed in Chapter 5) has revealed an interesting phenotypic observation that might lead to resolve the mystery of the 41 kDa protein. A transmutant strain from the inherently serum-resistant *U. urealyticum* isolate W11 (SV12) showed an altered susceptibility to complement-mediated serum killing that was associated with a loss of the 41 kDa protein. The transmutant strain became susceptible to killing by seropositive sera when compared to its parental serum-resistant strain. Therefore, it was interesting to identify the disrupted gene in this particular strain as this may provide an accurate identification to the candidate 41kDa immunogenic protein. In fact, developing and applying of transposon mutagenesis in this study has provided another powerful tool to investigate genes involved in *Ureaplasma*

pathogenesis. Mapping the transposon insertion site of pMT85 plasmid in the genome of the W11 transmutant strain revealed the identity of the disrupted gene associated with the loss of that remarkable protein band. The disrupted gene was identified as a UUR10_0137 gene in the fully annotated genome of *U. urealyticum* serovar 10 (strain ATCC 33699) and the gene encodes for a hypothetical protein of unknown function. In spite of this significant progress in tracing and studying this 41 kDa protein, I was unable to proceed further and investigate the role of this newly identified candidate gene in serum resistance due to time constraints. Therefore, further studies in this regard would defiantly define the role that this protein and others play in mechanism of serum resistance and avoiding complement killing by *Ureaplasma*.

It is important to recognise that avoiding complement-mediated serum killing in *Ureaplasma* spp. could be mediated by several mechanisms as it was observed in this study. With the exception of the W11 isolate many inherently serum-resistant serovars and clinical isolates of *U. urealyticum* have been reported without seeing the expression of the new 41 kDa band protein (UUR10_0137) (as discussed in Chapter 3). Similar observation has also been reported among the other group of *Ureaplasma*, *U. parvum* (Beeton *et al.*, 2012). These findings may indicate that *Ureaplasma* can utilize multiple strategies to manipulate and subvert the complement killing and elude the immune system defence in order to survive and establish colonisation. These potential mechanisms would be areas very worthy of investigation. Using more than one strategy by a microbe to evade the complement system has been reported in several pathogenic microorganisms (Zipfel *et al.*, 2007). Of these mechanisms binding complement regulators to mask complement, producing proteases to inactivate complement components and expression of complement inhibitors are well-defined tactics in a number of pathogens. This project has highlighted and addressed some of these possible strategies employed by

Ureaplasma. Expression of new antigen (UUR10_0137) and phosphorylation of EF-Tu protein could be possible mechanisms used by *Ureaplasma* to evade host complement attack, as these molecules might act as complement regulators or inhibitors. A possible protein-protein interaction between the two proteins can be assumed as their altered expression following complement attack and the development of serum resistance was coincident. These findings need more investigation to reveal the exact roles that both proteins have as virulence factors in *Ureaplasma*.

Using and developing methodology to study virulence factors of these unusual bacteria require a huge amount of time for optimisation and adaptation. I have been able to address some of these difficulties and successfully developed and modified a powerful method (transformation of *Ureaplasma*) that would enable further investigation for these interesting observations. These tools are now available and doors are open for others in this field to benefit from these developments and observations to answer some important questions raised in this study.

Generally, induction of serum resistance among *Ureaplasma* strains in this study was not caused by loss of immune-epitopes (phase variation), since serum resistance was only seen when the new 41 kDa immunogenic protein was expressed in association with modifications in EF-Tu. Appearance of a new protein band (switching on gene) might be the expression of new protein (potential complement regulator or inhibitor) that could play a role in avoiding the bactericidal activity of NHuS. This study also indicates that, apart from the MBA, *Ureaplasma* are able to manipulate a number of other immunogenic proteins in order to evade complement activity. Proteomics and genetic based approaches such as transposon mutagenesis are currently the most efficient methods in the

microbiology field for studying microbial pathogenesis and biology. Therefore, they will be useful tools to investigated *Ureaplasma.*

Chapter 5

Random insertion and gene disruption via transposon mutagenesis of *Ureaplasma parvum* using a mini-transposon plasmid

5. Random insertion and gene disruption via transposon mutagenesis of *Ureaplasma parvum* using a mini-transposon plasmid

5.1 Introduction

While transposon mutagenesis has been widely and successfully used for many microorganisms including the closely related species, *Mycoplasma* spp., to disrupt and determine non-essential genes (Choi, 2009; Hutchison *et al.*, 1999), previous attempts with *Ureaplasma* spp. have been unsuccessful. There is evidence of transposon gene delivery into the *Ureaplasma* spp. genome that has occurred in nature. The fully sequenced genome for *U. urealyticum* serovar 9 (strain ATCC 33175), which exists as a whole genome shotgun sequence (NZ_AAYQ02000002.1) in the NCBI database, shows the presence of the tetracycline resistance *tetM* gene (UUR9_0151) adjacent to a conjugal transfer protein (UUR9_0147), transposase (UUR9_0146), and integrase (UUR9_0149). The *tetM* gene for this genome is accepted to be part of a Tn*916* a conjugative transposon also called integrative conjugative element (ICE). Tetracycline resistance for many *U. parvum* and *U. urealyticum* genomes have been reported to be due to transposon-associated *tetM* gene presence in the bacterial genome from isolates from distant countries, including: UK, France, South Africa, Russia, Tunisia, USA (Beeton *et al.*, 2009b; de Barbeyrac *et al.*, 1996; Govender *et al.*, 2012; Mardassi *et al.*, 2012; Roberts, 1990; Taraskina *et al.*, 2002) and donation from transposon-carrying *Enterococcus faecalis* to a close relative to *Ureaplasma* (*Mycoplasma hominis*) during co-culture was demonstrated through mating at a frequency of 10^{-6} to 10^{-7} (Roberts and Kenny, 1987).

Transposon-carrying plasmids were first successfully used to insert selectable markers in the genomes of *M. pulmonis, Mycoplasma hyorhinis* and *Acholeplasma laidlawii* in the late 1980's (Dybvig and Alderete, 1988; Dybvig and Cassell, 1987; Mahairas and Minion, 1989a, b), which showed random genome insertion allowing both investigation of disrupted genes as well as the delivery of exogenous genes. The methods and reagents used to study essential genes and the physiological effects of delivering exogenous genes has continued to expand and be refined (Lartigue *et al.*, 2009; Paralanov *et al.*, 2012).

Experimental infection in primates have definitively proven that intrauterine infection of *Ureaplasma*, as a sole pathogen, induces preterm birth and associated neonatal respiratory disease (Novy *et al.*, 2009); however, the ability to study the role of individual bacterial genes in pathogenesis experimentally has been hampered by a lack of tools to deliver or knock-out genes. To date, no report has been made to show successful experimental delivery of transposons or plasmids into *Ureaplasma* spp. Therefore, the aim of this chapter is to develop a genetic tool (transposon mutagenesis) that allows genetic manipulation of *Ureaplasma* spp.

5.2 Results

5.2.1 Transposon mutagenesis of *Ureaplasma*

Transformation with pMT85 was performed in parallel for nine representative strains of *U. parvum* and *U. urealyticum*. Between 1-5 successful transformants survived per 10^8 cells used in the transformation reaction for each experiment; however, all parallel transformations of ATCC strains of *U. urealyticum* failed with the exception of one clinical isolate of *U. urealyticum* (strain W11; SV12), which was successfully transformed. Using the broth culture method previously utilised to determine MIC for other antibiotics (Beeton *et al.*, 2009b), the gentamicin MIC_{90} for *U. parvum* was determined to be 44 mg/L and for *U. urealyticum* was 66 mg/L. Therefore, the gentamicin selection was performed at 128 mg/L (Figures 5.1). Following transformation, all transformed resistant clones were found to contain the *aac-aphD* resistance gene from pMT85, which was not found in untransformed controls (Figure 5.2). Classical insertion into the genome should only insert the genes bordered by the inverted repeats at position 1bp and 3437 bp in pMT85; therefore, PCR was used to investigate the presence of plasmid sequence beyond the second inverted repeat, including the transposase gene (Figure 5.2). Prototype serovar 3, strain HPA5, was successfully transformed in 16 separate experiments with pMT85 and only transfer of plasmid DNA bordered by the inverted repeats (i.e. no transposase gene sequence) was observed. Furthermore, 2 other different strains of serovar 3 (U6 and HPA56), 3 different strains from serovars 1 (O10, DFK1 and HPA78) and 2 strains of serovar 6 (HPA58 and SR-HPA2) of *U. parvum* were transformed, but not all of them behaved as the strain HPA5. Probing undigested genomic DNA from these isolates found that *aac-aphD* gene was located on the chromosome, and probing of HindIII-digested genomic DNA revealed that, with the exception of 3 strains, a single copy was inserted into the genome (Figure 5.3). Portions of the transposase (trp)

gene from pMT85 integrated into the genomes of two strains from serovar 1 (O10 and HPA78), one strain from serovar 3 (HPA56) and one strain from serovar 6 (HPA58) (Figure 5.2.B&C). Moreover, in 3 separate experiments with prototype serovar 1 strain (DFK-1), only two out of three transmutant strains followed classical integration (Figure 5.2.C). Sanger sequencing of purified genomic DNA from strains HPA56 and HPA58 confirmed that genomic integration included transposase sequence. Sequencing with primer 195R (designed to sequence across the IR at pMT85 position 1) confirmed genomic integration with interruption of the gene UU047 (predicted ATP/GTP binding protein) at position 390 bp in the coding region for HPA56 and interruption of the gene UU526 (hypothetical open reading frame) at position 543 bp in the predicted reading frame for HPA58, respectively. However, Sanger sequencing of genomic DNA with primer 3192F (designed to sequence across the IR at position 3437) showed no integration and the intact presence of the pMT85 transposase gene sequence. On the other hand, the genomic DNA sequencing of the transmutant strain HPA78 (Figure 5.2.C) with the same primers, did not confirm the presence of transposase gene as both IR were found to interrupt the gene UU440 for this strain.

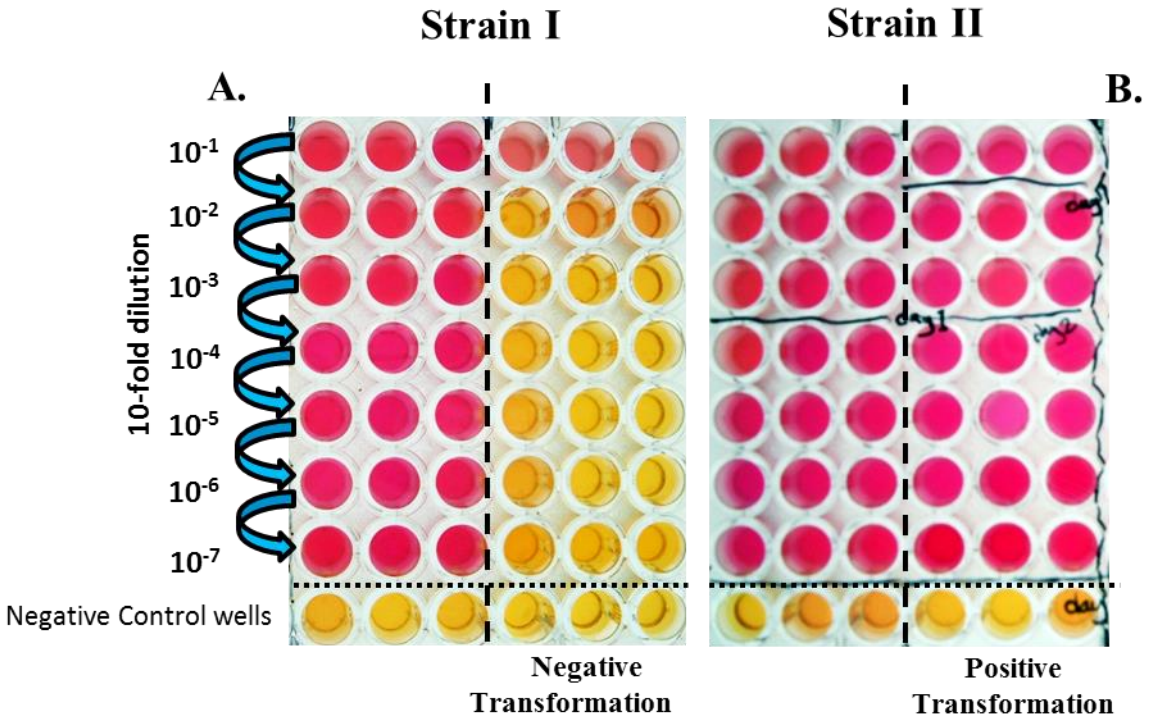


Figure 5.1 Schematic diagram illustrates *Ureaplasma* growth in USM and USM with gentamicin after transformation with pMT85 plasmid. Image **(A)** shows unsuccessful transformation, strain I (*U. urealyticum* SV2) grows only in USM without gentamicin; image **(B)** shows successful transformation, strain II (HPA5; *U. parvum* SV3) grows in both USM and USM with gentamicin.

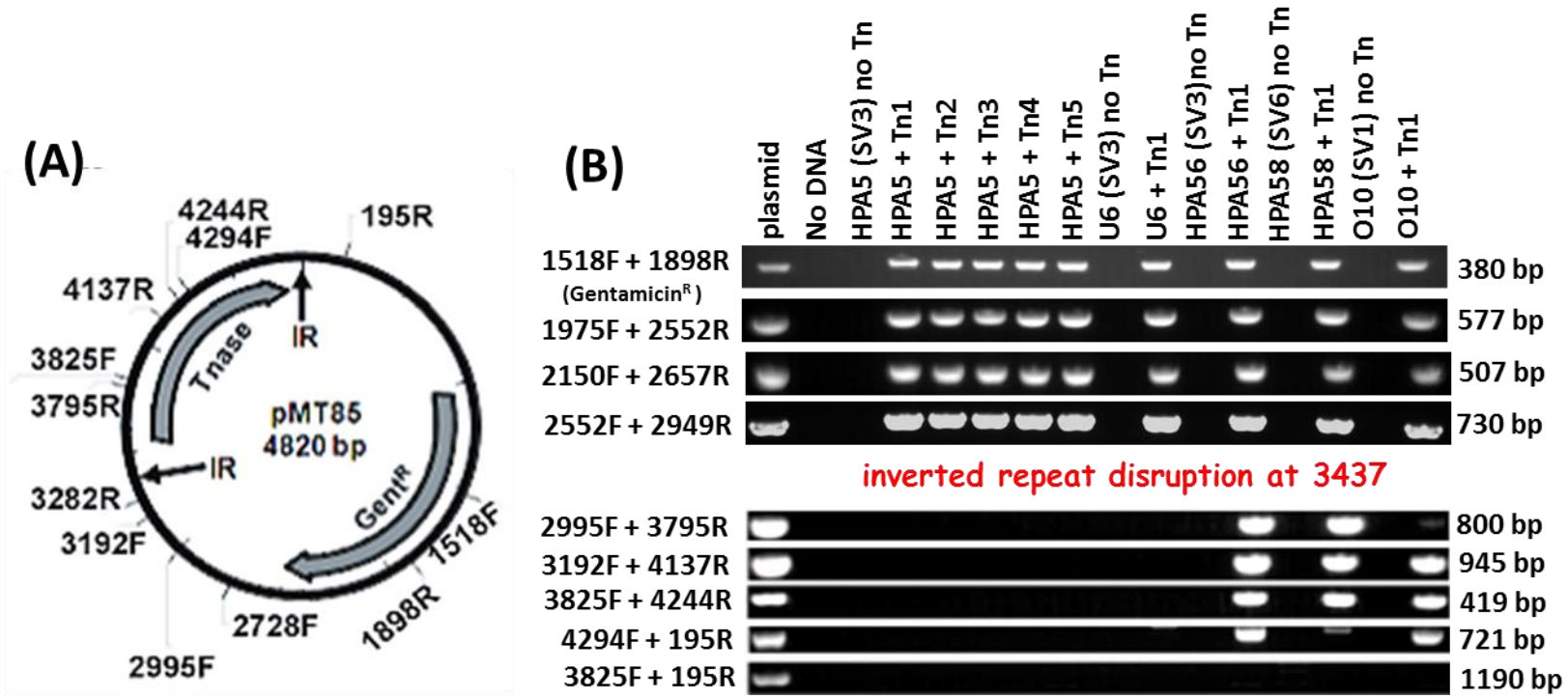


Figure 5.2 PCR mapping of regions of mini-transposon plasmid pMT85 **(A)** present in the genomes of gentamicin resistant clonal strains **(B&C)**. Presence of the gentamicin resistance gene (primers 1518F to 1898R) was only found in transposon mutated strains. PCR probing for different regions of the plasmid identified that strains such as HPA5, U6, SR-HPA2 and W11 only contain plasmid DNA from between the inverted repeat (IR) regions. Whereas, other transposon mutated strains contain mini-transposon plasmid DNA that include some of the transposase (Tnase) gene. Expected amplicon size is indicated to the right of the figure.

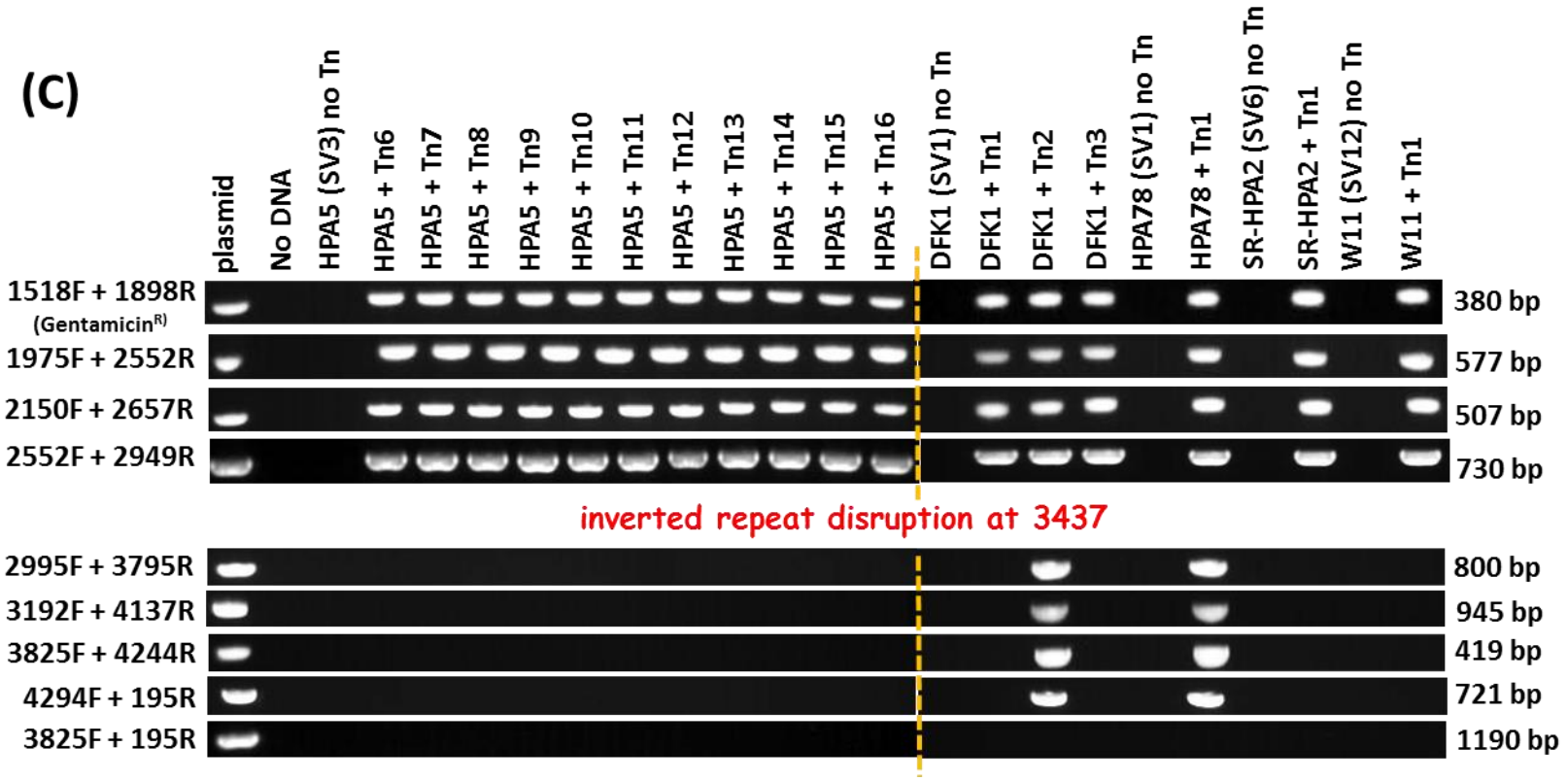


Figure 5.2 (continued) PCR mapping of regions of mini-transposon plasmid, pMT85, in the genomes of *Ureaplasma* transmutant strains.

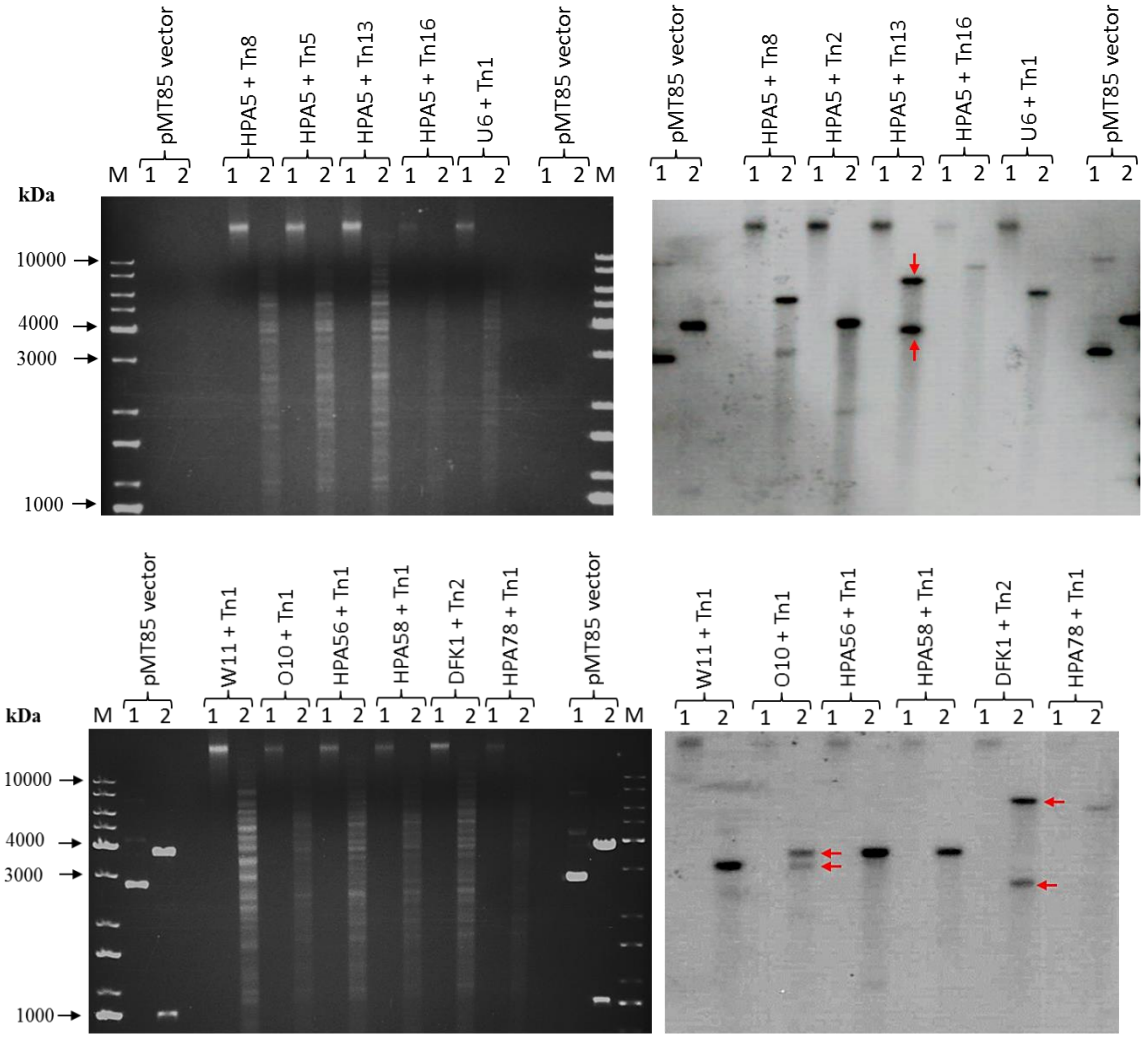


Figure 5.3 In gel DNA/DNA hybridization detection of gentamicin selection gene. DNA was extracted from a 100 ml culture of transposon mutated *Ureaplasma* and run on a 1% agarose gel comparing HindIII digested **(2)** and undigested **(1)** genomic DNA. Panels on the left show ethidium bromide visualisation of the DNA prior to probing with the gentamicin resistance probe (labelled with [^{32}P]) (visualised by autoradiography in the panels on the right). Strains in the upper panels: HPA5+Tn8 (UU390::mTn), HPA5+Tn5 (UU178/188), HPA5+Tn13 (UU520::mTn), HPA5+Tn16 (UU582::mTn) and U6+Tn1 (UU450::mTn); in the lower panels: W11+Tn1 (UUR10_137::mTn), O10+Tn1 (unknown), HPA56+Tn1 (UU047::mTn), HPA58+Tn1 (UU526::mTn), DFK1+Tn2 (unknown) and HPA78+Tn1 (UU440::mTn). Three strains (HPA5+Tn13, O10+Tn1and DFK1+Tn2) show 2 bands (red arrows) suggesting a mixed colony or 2 insertion sites. No undigested samples show any extra-chromosomal plasmid DNA. All the remaining examined isolates show a single insertion site into the genome. HindIII-digested and undigested pMT85 vector is shown along with the KAPA Universal DNA ladder for size comparison.

5.2.2 Interruption of *U. parvum* genes by random genomic insertion

Identification of plasmid insertion site and interrupted genes of *U. parvum* were performed by an unknown flanking DNA PCR followed by a basic PCR protocol to confirm results and screen other transformants (as described in chapter 2, Figure 2.3). Verification of gene interruption in transmutant strains was performed using primer pairs that were designed based on the genomic sequence for *U. parvum* serovar 3 (ATCC strain 700970) for genes UU390, UU450, UU520, UU582, and intergenic region between UU187 and UU188. These primers successfully amplified these genes by PCR in all parent strains of *Ureaplasma*, while single failure for each primer set to amplify these genes in mutated strains (Figure 5.4) was due to transposon integration and disruption of these genes. These disruptions were then confirmed by sequencing the integration interface (Figure 5.5) Sequencing of the junctions between transposon insertion site for these genes found 8 bp direct repeats that were unique for each clone, adjacent to the inverted repeat from pMT85 (Figure 5.5). The 8 bp direct repeat for the intergenic insert between UU187 and UU188 replicated the last two bases of the TAA stop codon from UU187 ensuring that UU187 was not disrupted. All disrupted genes identified in this study are listed in Table 5.1.

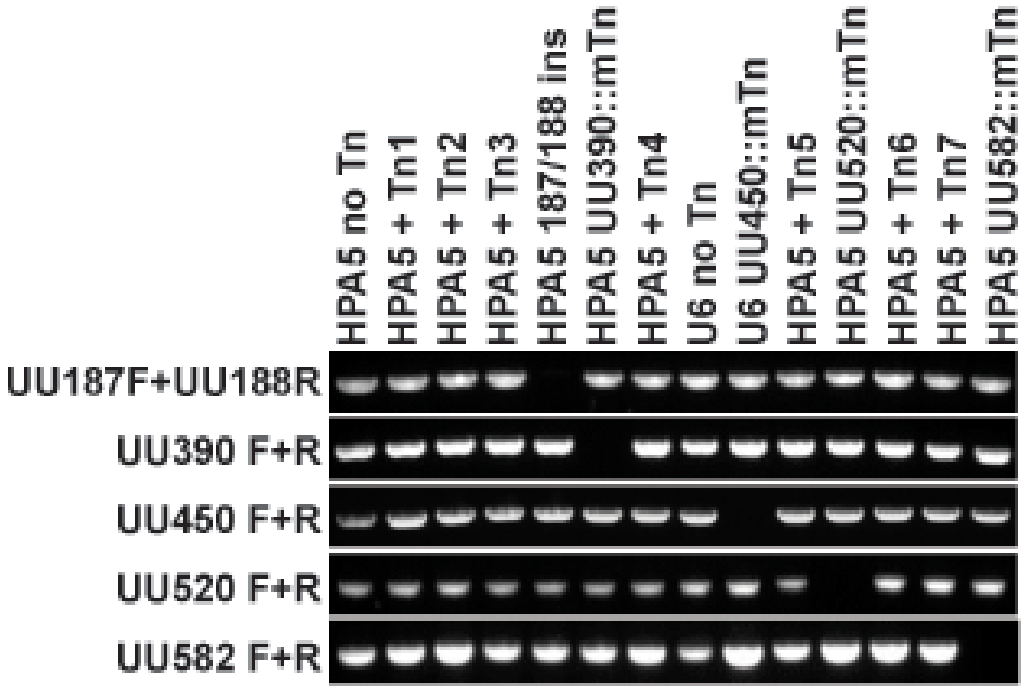


Figure 5.4 PCR amplification of *U. parvum* genes (as designated by ATCC strain 700970 nomenclature) UU390, UU450, UU520, and UU582 using primers designed within the coding region of these genes. An additional primer set amplifying the end of gene UU187 and the beginning of UU188 (including the 32 bp intergenic region) was also included. Non-mutagenised HPA5 and U6 serovar 3 *U. parvum* are included as controls. Single clones with disrupted genes are shown for each primer set as well as 7 additional clones successfully transformed with the mini-transposon to show specificity.

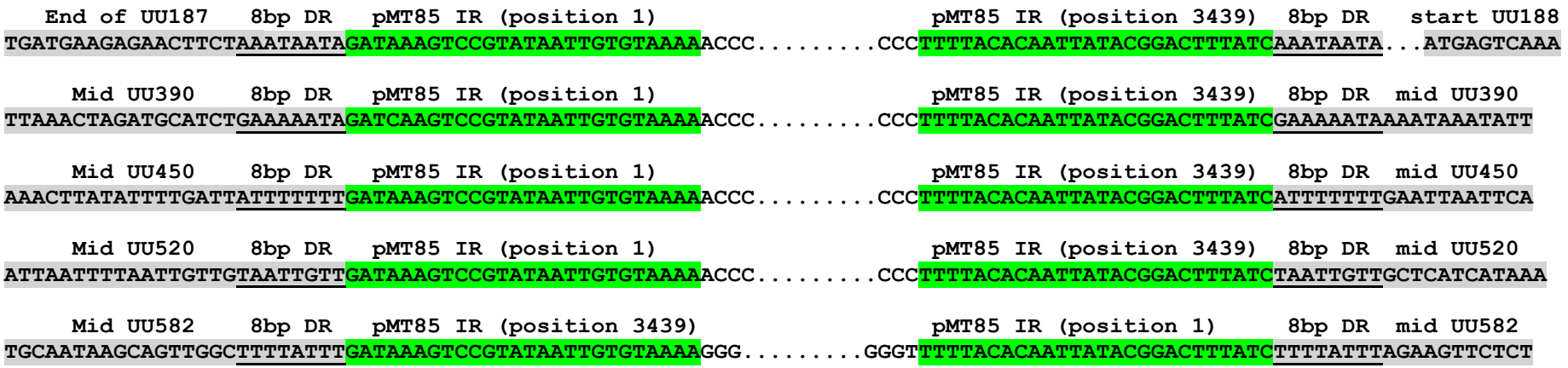


Figure 5.5 Sequence alignments of transposon insertion boundaries for HPA5 187/188 intergenic insertion, HPA5 UU390::mTn, U6 UU450::mTn, HPA5 UU520::mTn, and HPA5 UU582::mTn. Inverted repeats are highlighted in green, coding regions of genes are highlighted in grey. The Tn insertion sites are bordered by 8-basepair direct repeats, with 100% identity intrastrain, but unique when compared intrastrain (except being very AT-rich). The direct repeat for the 187/188 insertion shows that the direct repeat contains the stop codon for UU187 (TAA), therefore no disruption to either UU187 or the promoter for UU188 occurs.

Table 5.1 Genes disrupted in the genome of *Ureaplasma* transmutant strains

Gene name	Product	Gene length (bp)	Transmutant strain name
UU187 /UU188	DNA-directed RNA polymerase subunit beta/ DNA-directed RNA polymerase subunit beta	1434/1305	HPA5(SV3) + Tn5
UU390	Hypothetical membrane protein	816	HPA5(SV3) + Tn8
UU520	Hypothetical membrane protein	1863	HPA5(SV3) + Tn13
UU582	ATP-dependent RNA helicase	1332	HPA5(SV3) + Tn16
UU450	Conserved hypothetical membrane protein	1164	U6(SV3) +Tn1
UU047	ATP/GTP binding protein	2604	HPA56(SV3) +Tn1
UU526	Hypothetical protein	1512	HPA58(SV6) +Tn1
UU440	Membrane lipoprotein	1500	HPA78(SV1) +Tn1
UUR10_0137	Hypothetical protein	696	W11(SV12) +Tn1

5.2.3 Susceptibility of *Ureaplasma* transmutants to complement killing

Complement killing assay was performed to test the susceptibility of *Ureaplasma* transmutant strains to killing by human complement compared to their parents. All strains were exposed in parallel to their parents to seronegative serum or seropositive serum for 1 hr and then titrated out to determine the fold killing reduction of survival relative to the original parent strains after the challenge. The aim of this experiment was to screen any changes in complement killing that might have occurred as a result of gene disruption following transposon mutagenesis. In fact, this would be very helpful to understand the interaction between *Ureaplasma* and host immune system. My findings showed no variation in complement killing in all *U. parvum* transmutants, but the only transformed strain of *U. urealyticum* (W11 + Tn1) that did show a loss of resistance to serum killing. This particular transmutant strain became sensitive to seropositive serum (but remained resistant to seronegative serum) compared to the untransformed parent strain following transposon mutagenesis (Figure 5.6 and 5.7). The other 25 strains remained unchanged relative to their parent strains (i.e. the sensitive strains remained sensitive and the resistant strains remained resistant) (Figures 5.6 & 5.7).

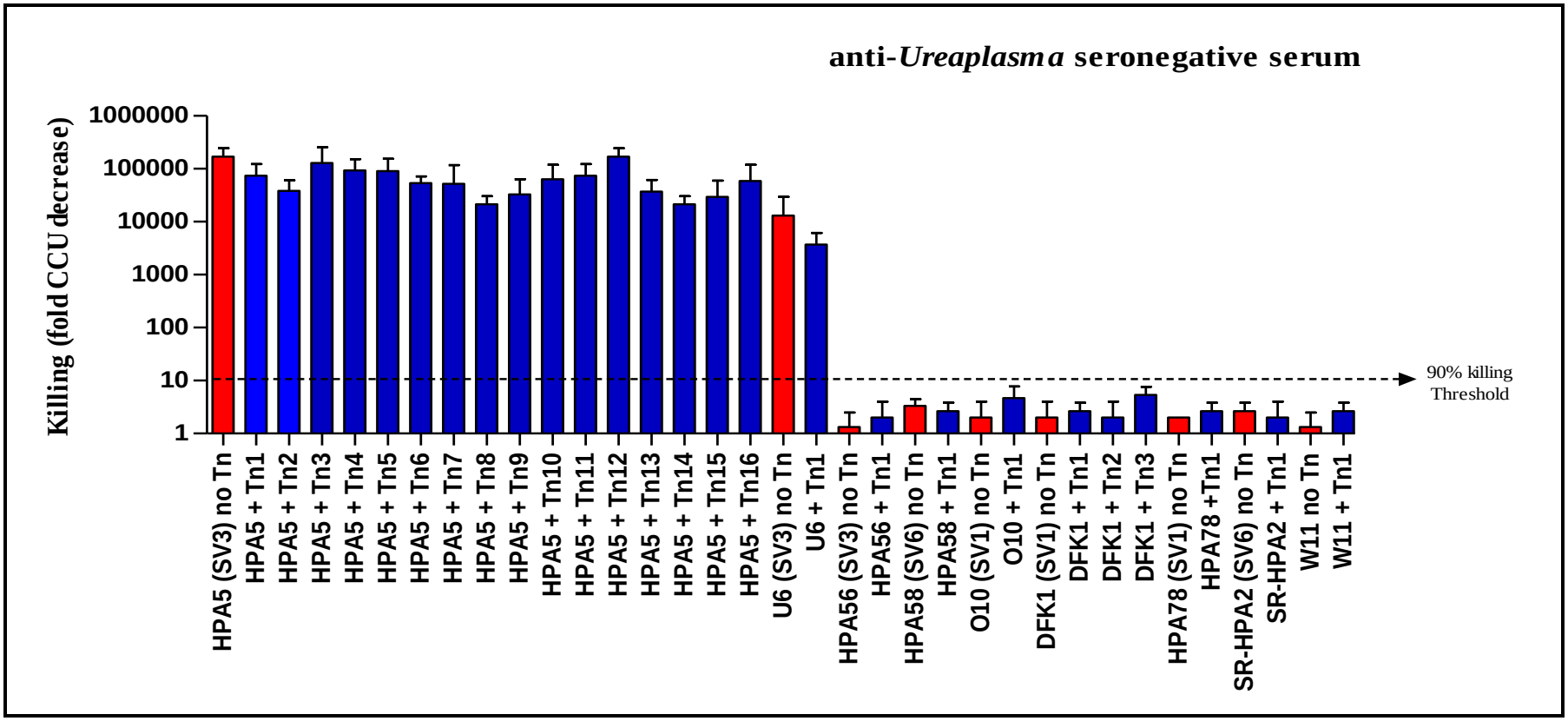


Figure 5.6 Complement activity against *Ureaplasma* transposon-mutated strains compared to their parents. Strains were challenged with seronegative serum (shows no reactivity by western blot); Red bars indicate parent strains and blue bars indicate transposon-mutated strains. The black dotted line indicates the 90% killing threshold. No changes observed between untransformed and transformed strains.

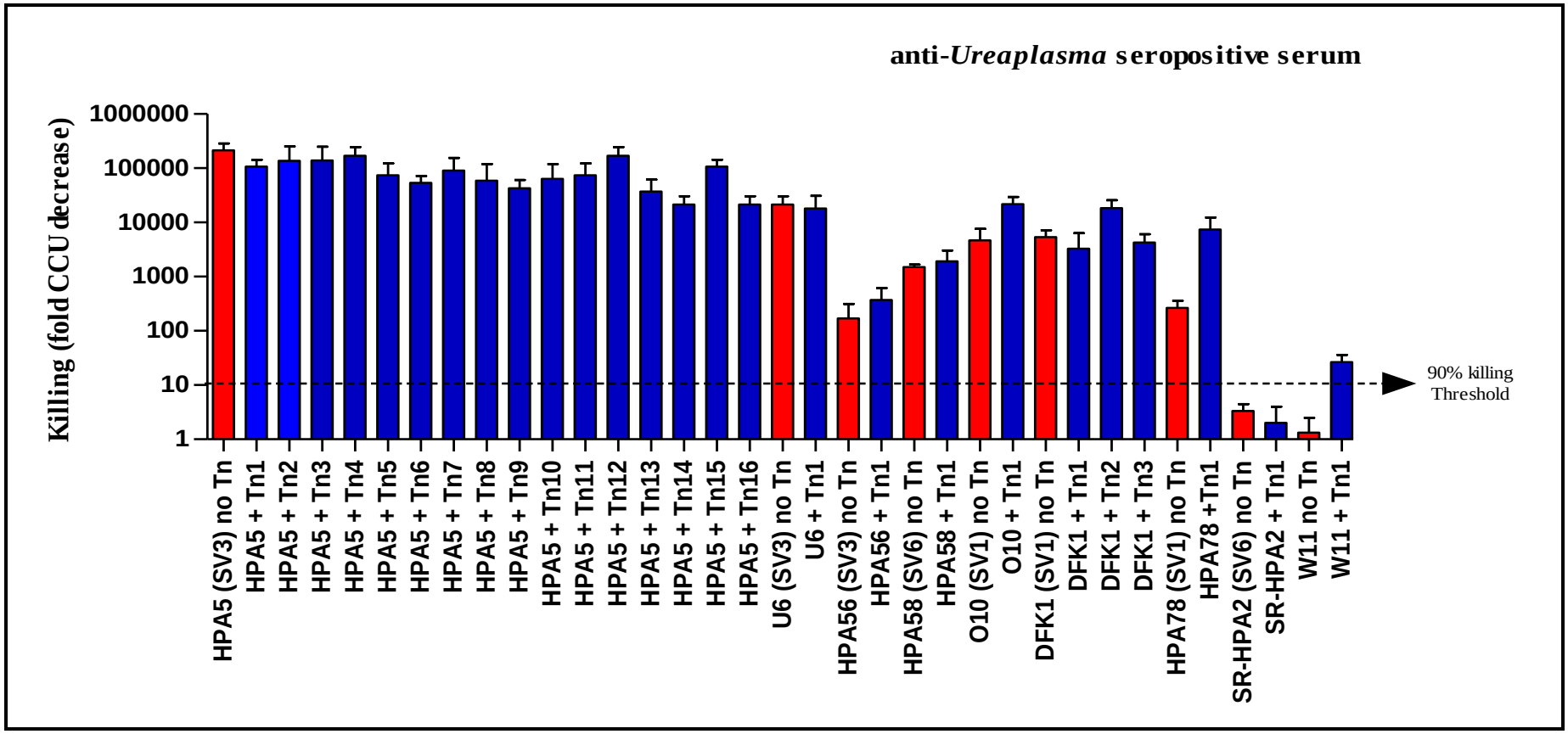


Figure 5.7 Complement activity against *Ureaplasma* transposon-mutated strains compared to their parents. Strains were challenged with seropositive serum; Red bars indicate parent strains and blue bars indicate transposon-mutated strains. The black dotted line indicates the 90% killing threshold. No changes observed between untransformed and transformed strains.

5.2.4 Screening *Ureaplasma* transmutants for altered expression of the major surface antigen

Immunoblot analysis using monoclonal anti-multiple banded antigen (MBA; UU375) antibody was carried out to examine if any of the transformed strains had altered size or expression of the major surface antigen, MBA, relative to the untransformed parent strains. In 26 transformed clones from four different *Ureaplasma* serovars, MBA-negative clones (phase variation) were never observed, and only three clones showed a small alteration to the MBA mass (Figure 5.8). The site of genomic integration for one strain (U6) is known to be at the gene UU450, which is not close to the gene encoding the MBA, therefore the observed MBA mass alteration for this strain is not due to direct interference with the coding gene. Of the three transposon-mutated DFK1, one altered MBA size to a lower size and the other expressed two MBA size variants while the third remained unchanged compared to the original strain. HPA78 transposon-mutated strain had 2 MBA bands and following transmutation was reduced to a single MBA band; this likely represents selection of a single strain from a mixed parent population, as clonal strains, based on my experience, only exhibit a single MBA species (Figure 5.8).

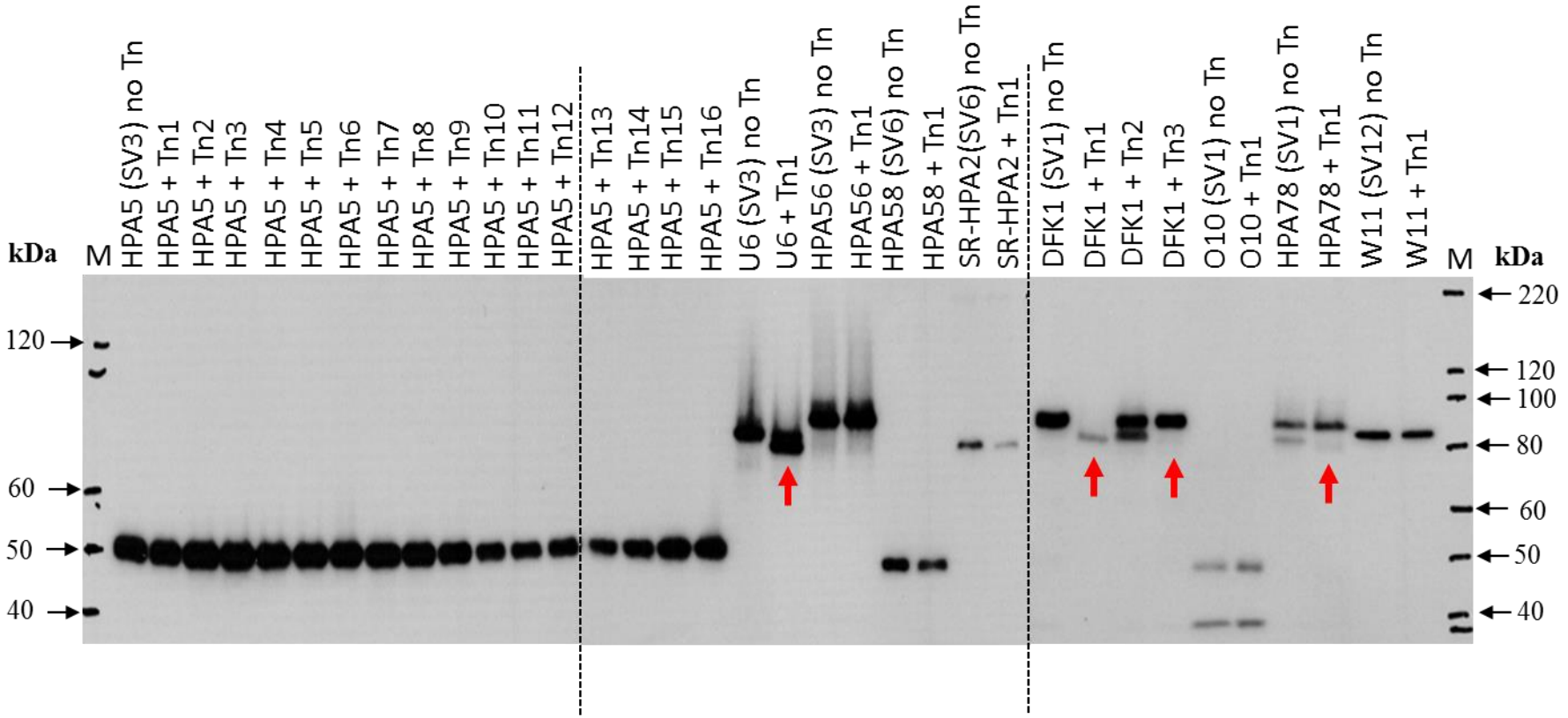


Figure 5.8 Immunoblot analysis of SDS solubilised total cell bacterial protein probed with monoclonal anti-multiple banded antigen antibody. Of 26 individual transformation experiments only three strains (U6, DFK1 and HPA78) showed altered mobility of the MBA following transposon mutagenesis (+ Tn). Red arrows indicate changes in MBA.

5.2.5 Screening *Ureaplasma* transmutants for altered expression in immunogenic proteins recognised by seropositive serum

To investigate any changes in expression of immunogenic proteins, other than the MBA, following the transposon mutagenesis, all *Ureaplasma* transmutants and original strains were screened with immunoblot analysis using the high titer seropositive serum. As presented in chapter 3 of my thesis, *Ureaplasma* spp. were capable of altering their immunogenic proteins after immunological challenge with human serum *in vitro*. These changes in immunogenicity were observed in the multiple banded antigens, MBA, as well as other immunogenic proteins. The results of this experiment showed that of 26 transmutant strains, only one had a change in immunogenic proteins. The *U. urealyticum* strain W11+Tn1 lost a 41 kDa band compared to the parent strain (Figure 5.9 and 5.10). Apart from the alterations seen in MBA mobility that were presented previously , all *U. parvum* transmutant strains did not show any other variation in immunoproteins detected by human serum.

The immunogenic change (loss of 41 kDa protein) in W11 transmutant strain relative to the serum-resistant parent strain was an interesting finding in this study, as it was associated with alteration in complement susceptibility. Following transposon mutagenesis, the transmutant strain of W11 became significantly more susceptible to complement-mediated killing (Figure 5.10). While remained resistant to seronegative serum, the transmutant strain was readily killed by sera containing anti-*Ureaplasma* antibodies (seropositive sera). Genomic DNA analysis identified a single genomic insertion site by in gel hybridization for the *aac-aphD* gene (Figure 5.4). Sanger sequencing of purified genomic DNA sequencing from the transmutant strain of W11found that the gene UUR10_0137 (ATTCC strain 33699 serovar 10 gene annotation numbering) was disrupted at amino acid 126 of 231.

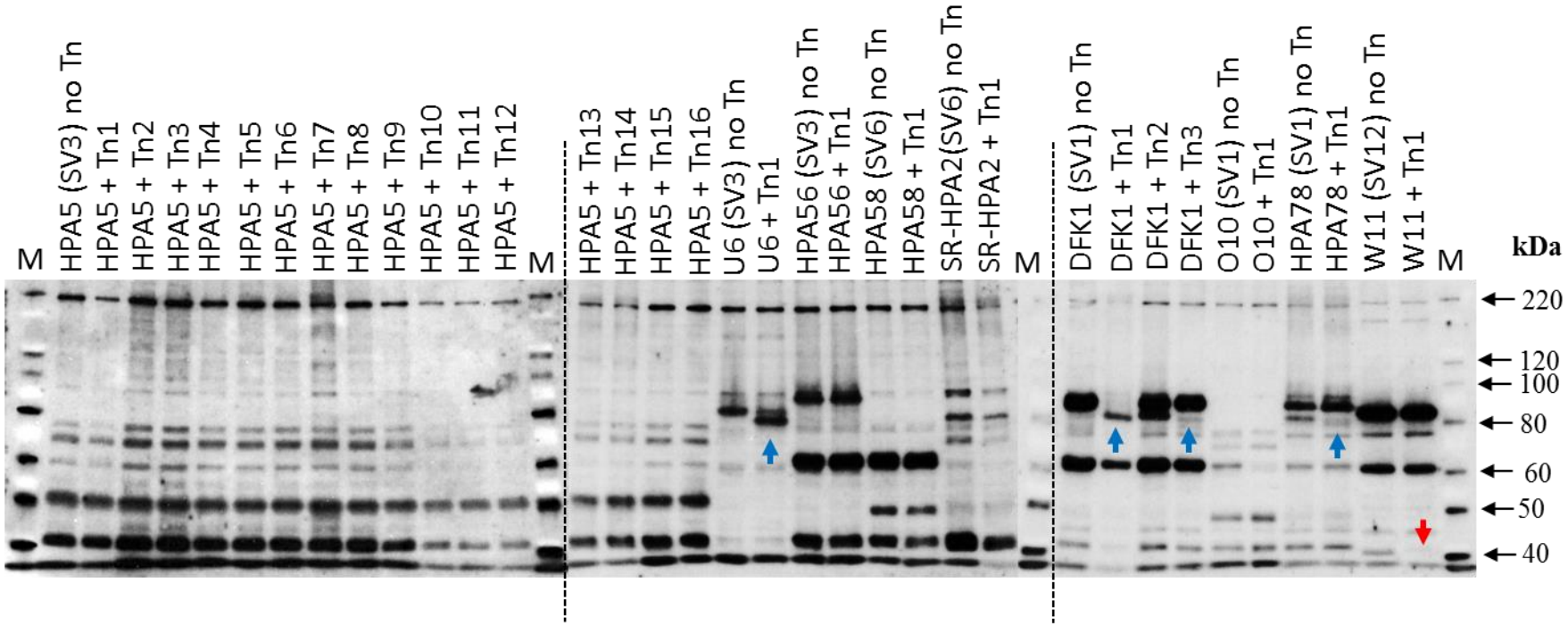


Figure 5.9 Immunoblot analysis of SDS solubilised total cell bacterial protein probed with seropositive serum. The transposon-mutated strain of *U. urealyticum* W11 lost a 41 kDa band (red arrow) compared to the parent strain following transposon mutagenesis (+ Tn) when detected by human high seropositive. And apart from the changes in MBA (blue arrows), none of 25 *U. parvum* transposon-mutated clones (+Tn) showed alterations in expression of other immunoproteins detected by humane serum. M= molecular marker.

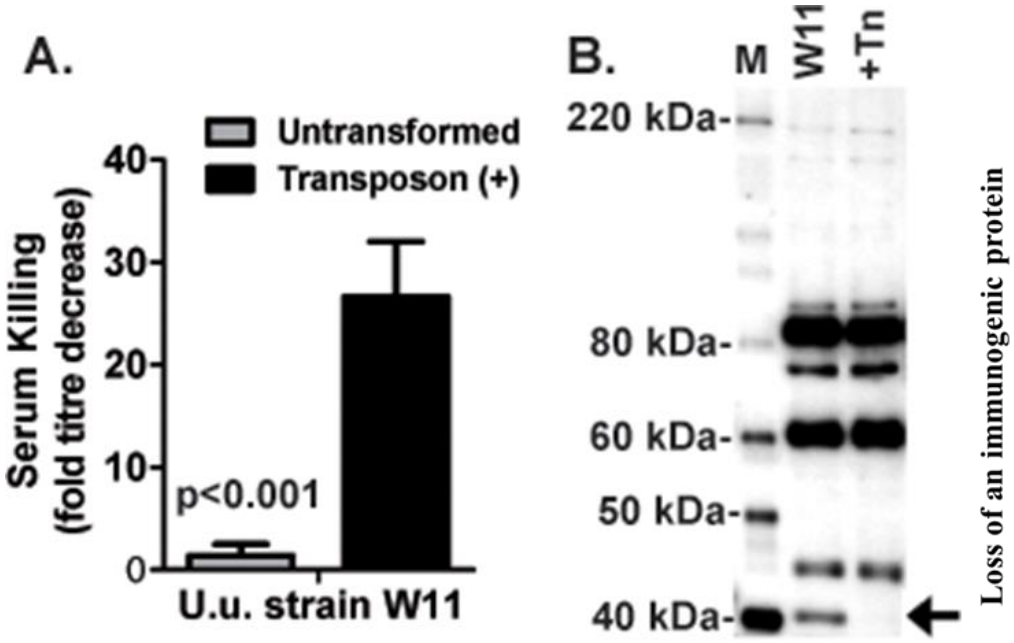


Figure 5.10 Serum killing **(A)** and immunoblot analysis using human high titre seropositive serum **(B)** for parental *U. urealyticum* strain W11 (SV12) and following successful transposon mutagenesis (+Tn). Serum killing was increased significantly following 1 hr challenge with human serum containing antibodies; and analysis of this serum shows the serum-sensitive transposon mutated strain has lost a 41 kDa band that was immunoreactive with the challenging serum. Bar graph shows mean +/- SEM of experiments performed in triplicate. Representative immunoblot from three repeat experiments shown.

5.2.6 Disruption of RNA helicase (UU582) gene altered *Ureaplasma* growth kinetics

Random transposon insertion was found to disrupt the gene UU582 as shown in Figures 5.4 and 5.5. UU582 encodes the only copy of an ATP-dependent DeaD-box bacterial RNA-helicase in the *U. parvum* genome and disruption of this gene was found to alter final titre and growth kinetics for the bacteria at a range of incubation temperatures. Of all the transposon mutated *Ureaplasma* strains, only the disruption of gene 582 (UU582:: mTn) had this effect. Maximum bacterial titre was obtained for parent and all other transposon mutated strains by 36 hr at 37°C (Figure 5.11), while UU582::mTn did not reach maximum titre until 60 hr and had a 3 to 4 log reduction in final bacterial titre. As DEADbox RNA-helicase mutants are reported to be unable to replicate at lower temperatures (Owttrim, 2013), I also investigated growth kinetics at 33°C and 25°C (an example of growth for the parent strain at these temperatures is shown in figure 5.12). Under these conditions the maximum titre for other *Ureaplasma* strains took longer to attain and UU582::mTn titres were 10^8-fold lower at 25°C.

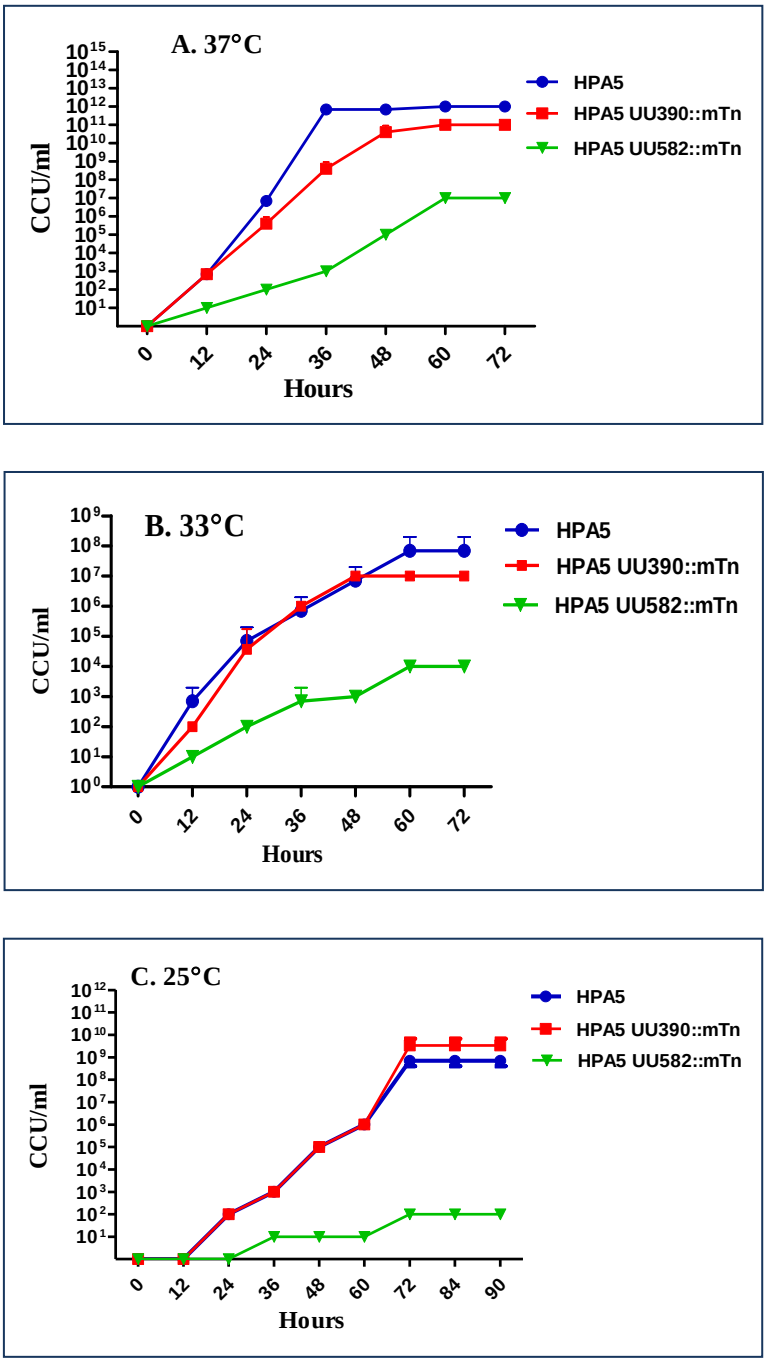


Figure 5.11 Growth kinetics for *U. parvum* parent strain (HPA5) compared to membrane protein disruption (UU390::mTn) or DEAD-box RNA-helicase gene disruption (UU582::mTn) strains when incubated at 37 °C (A), 33 °C (B) or 25 °C (C). Strains were titrated out in a 10-fold dilution series and growth measure at time points indicated by urease conversion of urea to ammonium ions. *Ureaplasma* growth is shown as colour (pH indicator) changing units per ml. Mean and standard deviation of dilutions performed in triplicate. Results were consistent through three repeated experiments.

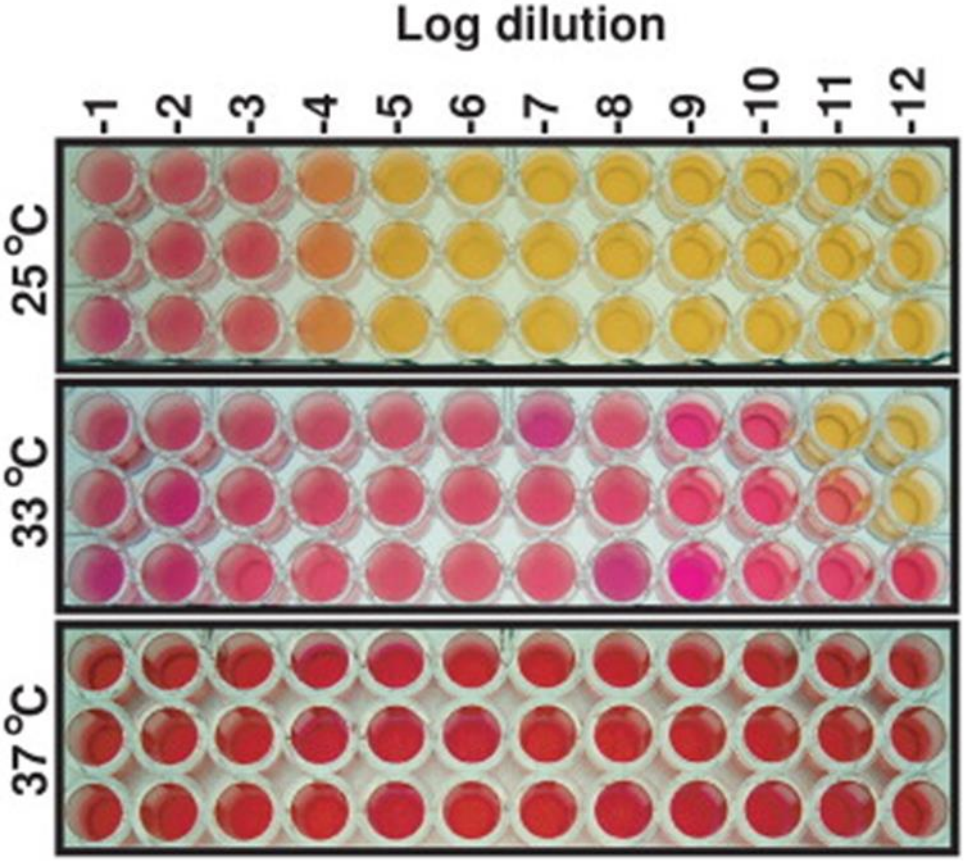


Figure 5.12 Growth of *U. parvum* HPA5 in triplicate at 25, 33, and 37 ºC as measured by colour change of USM. 180 µl were placed in each well and 20 µl of prototype laboratory *U. parvum* strain HPA5 was inoculated into the first well (in triplicate). Filter tips were changed between each dilution (transfer of 20 µl) across the plate. The plates were then incubated in separate incubators at the listed temperatures and the growth documented at various time points. These images were taken at the 48 hr time point for one of the repeats of the experiment.

5.3 Discussion

Large scale analysis of random genome integration has been used for *Mycoplasma* spp. to determine the essential genes of a minimum genome, the underlying presumption being that essential genes cannot be disrupted. *M. genitalium* has the smallest genome and saturating Tn mutagenesis on this organism led to the proposal of a between 265-350 genes as being the minimum required to sustain self-replicating independent life (Hutchison *et al.*, 1999). Further refinement of this method identified all 43 RNA-coding genes to be essential and 382 of 482 *M. genitalium* protein-coding genes to be essential for culture growth of *M. genitalium* (Glass *et al.*, 2006). These studies were hugely influential in the construction of the minimum synthetically assembled genome based on *M. genitalium* in 2008 (Gibson *et al.*, 2008). However, comparison of the smallest *Mycoplasma* and *Ureaplasma spp.*, which vary in their use of glycolysis, arginine-metabolism and urea-metabolism for survival, showed *U. parvum, M. hominis* and *M. genitalium* only have an overlap of 247 coding sequences (Pereyre *et al.*, 2009) suggesting much is yet to be learned from minimum genome analysis of the other mollicutes.

Comparison of 19 sequenced genomes for human *Ureaplasma* spp. has identified an average of 608 predicted genes for each *U. parvum* genome and 664 for *U. urealyticum* (Paralanov *et al.*, 2012). A total of 1020 possible predicted protein coding genes, including singletons, were identified with a core conserved genome of 515 genes. Now that transposon mutagenesis is routinely successful with *U. parvum*, determination of non-essential genes in a microbe that does not use the glycolysis pathway will add empirical verification to the composition of a hypothetical minimum gene set. However, I acknowledge that my studies have not examined whether transposon insertion into the genome had altered gene expression. Two of the transposon-mutated strains showed

phenotypic alteration, and I would predict that all of the other identified open-reading frame disruptions that I identified should result in a failure to express fully functional proteins, as the predicted open reading frames were disrupted between 150-704 bp into the expected coding region. However, as they are hypothetical open reading frames of unknown function, they may not be expressed in parent strains. The one exception may be the integration between UU187(rpoB)) and UU188 (rpoC), both of which are predicted to be homologues of DNA-directed RNA polymerase subunit beta; as the stop codon of UU187 and the intergenic region between the genes was conserved, expression of both of these genes may be conserved and no phenotypic alteration was observed for this strain. Most of the disrupted genes were of unknown function and only listed as predicted open-reading frames (Table 5.1). Genes UU390, UU450, and UU520 may encode membrane proteins, UU047 is predicted to encode a conserved hypothetical ATP/GTP-binding protein, UU526 is predicted to encode an MBA paralogue that should be expressed as a surface-associated membrane lipoprotein, as is UU440 which is also predicted to be a hypothetical membrane lipoprotein, while UUR10_137 is a predicted protein of unknown function.

I have also identified an intermediate level of gene class here, where disruption of the only annotated RNA helicase in the *U. parvum* genome (UU582) resulted in a significant physiological growth alteration that would likely affect the ability of the resultant strain to survive *in vivo*. RNA helicases largely belong to superfamily two of the six families of nucleic acid helicases, and the *U. parvum* RNA helicase in particular belongs to the DEAD-box family based on the signature sequence, Asp-Glu-Ala-Asp (Fairman-Williams *et al.*, 2010). While many bacteria encode a few RNA helicases, a substantial number of sequenced bacterial genomes only contain a single DEAD-box helicase (Zolldann *et al.*, 2005). Although *E. coli* encodes 5 RNA helicases, assessment following individual

disruption of each found that the Δ*deaD* mutation was primarily responsible for observed growth defects at 37°C including increased doubling time and Δ*deaD* (as well as the Δ*srmB*) mutation exhibited a cold sensitive phenotype (Jagessar and Jain, 2010), very similar to my observations for *U. parvum.*

I was unable to successfully deliver the mini-transposon to a range of *U. urealyticum* strains despite being performed in parallel with the same conditions and reagents used to successfully mutagenise *U. parvum.* The only exception was a single experiment where a clinical strain (W11) was successfully transformed resulting in delivery of the gentamicin resistance gene. This strain exhibited my second observed phenotypic change: altered survival following serum challenge. Previously Beeton *et al.* (2012) had characterised the complement sensitivity of *U. parvum* strains and found that some strains (such as HPA5) were very sensitive and killed by seronegative serum, some strains were readily killed only by serum containing anti-*Ureaplasma* antibodies (such as HPA2 and DFK-1), and others were inherently resistant. However, I did not observe any alteration of serum sensitivity or resistance of the mutagenised *U. parvum* strains compared to the parent strains in this study (Figures 5.8 and 5.9). As presented in chapter 3 of my thesis, characterisation of the W11 strain found it to be completely resistant to all previously characterised seropositive sera containing anti-*Ureaplasma* antibodies. However, following transposon integration into the W11 genome, the resultant strain was sensitive to killing by seropositive sera. The altered phenotype was co-incident with loss of a 41 kDa protein detected by the human high titre seropositive serum used to challenge the *Ureaplasma* strains and the single transposon insertion site was found to disrupt the hypothetical gene UUR10_137 (ATCC 33699 serovar 10 gene annotation numbering). The predicted mass of this open reading frame is 27 kDa, but the size may be increased by post-translational modification. It is also possible that the disruption of this gene may not have any direct bearing on the

loss of the expression of the 41 kDa protein or alteration of serum resistance. Further experiments are required to determine if isolated expression of this gene is capable of solely mediating serum resistance.

The major surface antigen MBA (gene UU375 in *U. parvum* serovar 3 ATCC strain 700970) has also previously been shown to be susceptible to phase variation following bacterial stress (Zimmerman *et al.*, 2011; Zimmerman *et al.*, 2009) or alteration of size (Robinson *et al.*, 2013). I only observed three strains with alteration in MBA size and no strains with loss of MBA expression. Therefore, the temperature-shock and selection in gentamicin associated with transformation do not appear to trigger phase variation and I would expect that loss of MBA expression would require gene disruption.

In general, I have succeeded in developing a methodology that is capable of delivering a mini-transposon to the *U. parvum* genome, which results in random gene disruption. This methodology can now be utilised to determine the minimal genome contingent for a bacterial class that do not utilise glycolysis to survive and shed further light on core essential genes. Characterisation of disrupted gene strains with pathogenesis studies in experimental *in utero* model infections will also be a key to identifying pathogenic markers within the *U. parvum* genome, as it has been shown to initiate preterm labour and chronic lung disease in preterm neonates experimentally as a sole pathogen (Novy *et al.*, 2009). Transposon mutagenesis will also be valuable in enabling delivery and expression of exogenous genes to *U. parvum* for *in vivo* tracking and possibly as a mucosal vaccine delivery tool of the future.

Chapter 6

Expression of foreign genes in *Ureaplasma*

6. Expression of foreign genes in *Ureaplasma*

6.1 Introduction

Expression of foreign genes, in most Mollicutes, has been challenging due to the unusual codon usage that this group of bacteria have. During their evolution, Mollicutes have gone under a remarkable genome reduction co-incident with a shift to very low G + C content. As a result of this, an alteration in usage of the UGA (opal) stop codon to tryptophan codon has occurred, with the only exception of the species *A. laidlawii*, which have maintained a normal tryptophan codon usage (the UGG codon) (Razin *et al.*, 1998). In fact, the unusual codon usage makes it difficult to express *Ureaplasma* genes in other bacteria and vice versa as this would lead to truncated immature gene products. Previous attempts to solve this issue have not been fully successful (Jarhede *et al.*, 1995).

As it has become possible to deliver and disrupt genes in *Ureaplasma* spp. by means of transposon mutagenesis (as discussed in Chapter 5), the aim of this final results chapter was therefore to investigate the ability of these microbes to express exogenous genes subsequent to successful delivery. The *mCherry* that encodes for a monomeric red fluorescent protein (RFP) mCherry was delivered to and investigated for expression in *Ureaplasma*. Furthermore, expression of this foreign gene was examined under several parameters that influence gene expression efficiency, such as the strength of the transcriptional promoter, the codon-usage, and the number of copies of the gene to evaluate the effect of these factors on expression of exogenous genes in *Ureaplasma*. In other prokaryotes such as *E. coli* ("the work-horse for gene expression"), it has been

found that many factors including the above mentioned parameters influence expression of foreign genes (Glick and Whitney, 1987). Fluorescent proteins have been widely used in many different applications, for instance as probes that enable tracing and localizing microbes *in vivo.* Optimisation of *RFP* gene expression as a probe for *Ureaplasma* would be a useful technique that opens doors for studying the biology and pathogenesis of these tiny pathogens. Generation of fluorescent *Ureaplasma* would also be a useful method that allows for tracing and studying *Ureaplasma* infection in animal models.

6.2 Results

6.2.1 Generation of pMT85 expression vector with modified *mCherry* inserts

In this study, all *mCherry* inserts under investigation were constructed and cloned into pMT85 Tn*4001* mini-transposon plasmid as an expression vector (Figure 6.1). As previously described in chapter 5, this plasmid was successfully delivered to several *Ureaplasma* isolates through PEG-mediated transposon mutagenesis. Construction of the plasmid with inserts of interest was as described in chapter 2. The *mCherry* inserts were modified and synthesized in different ways to investigate some fundamental factors that control and affect gene expression (see Table 2.10). Vector pMT85 plasmids carrying various inserts with different copy numbers, selected transcriptional promoters and/or optimized codon-usage were generated and cloned into *E. coli* where plasmid vector constructs were amplified and validated before being introduced into *Ureaplasma* strains, where efficacy of expression was evaluated. Nucleotide sequence and detail of the pMT85 vector are shown in Appendix I.

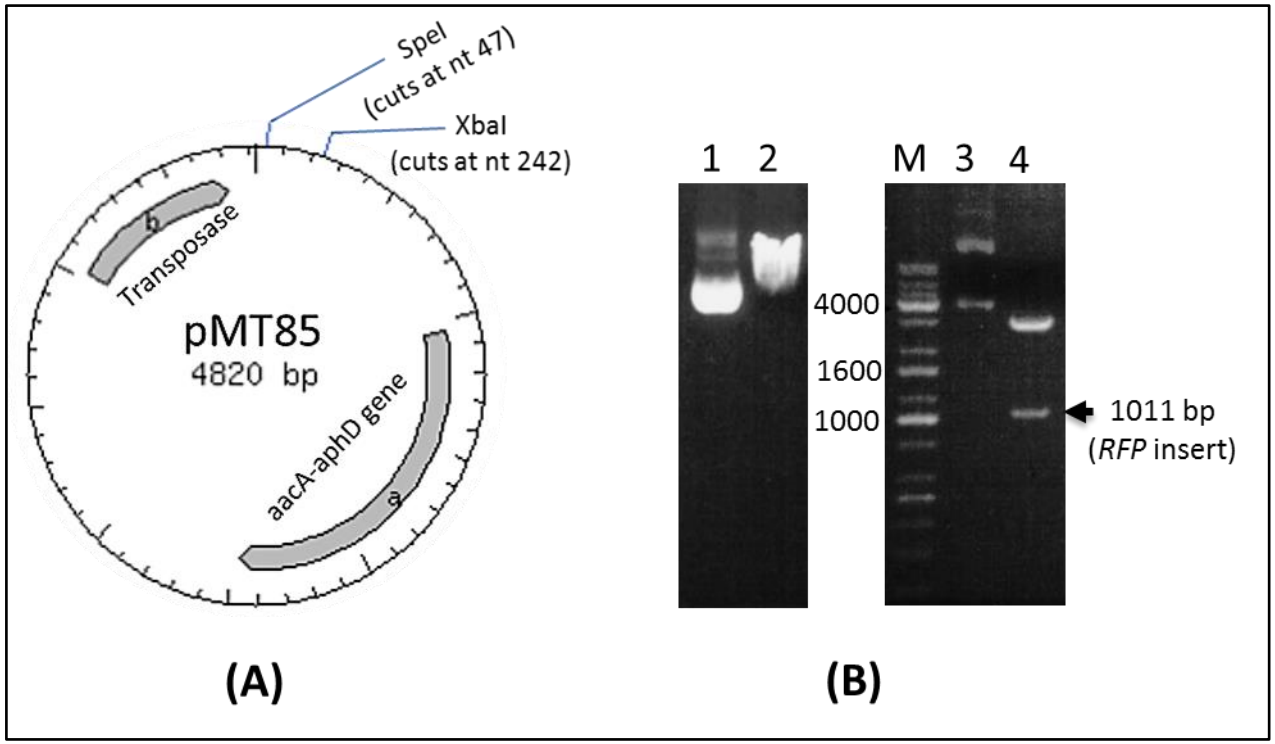


Figure 6.1 A map of pMT85 showing the cloning site of *mCherry* inserts (A) and restriction digestion profile of pMT85 vector with an insert (B). Lane 1: Undigested pMT85 plasmid; lane 2: pMT85 plasmid digested by XbaI; lane M: KAPA universal ladder; lane 3: Undigested pMT85 plasmid with *mCherry* insert; lane 4: pMT85 plasmid with single RFP (*mCherry*) insert digested and released by XbaI.

The first construct insert generated and used in this study consisted of a tandem *mCherry* insert (GRT), i.e. two separate copies of *mCherry*, under the control of the expression promoter of elongation factor Tu (EF-Tu) gene of *U. parvum* serovar 14 (ATCC strain 33697 as provided by GeneBank id: emb|Z34275.1); each independent copy of the *mCherry* had its own promoter, and the codon- usage of the gene was optimized for *Ureaplasma* spp. expression by converting the amino acid sequence of synthetic monomeric red fluorescent protein (GeneBank id: gb AAV52164.1) into a synthetic gene by using the most common *U. parvum* codons utilised (Figure 6.3), as provided by the website:

http://www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=134821&aa=14&style=N

The tandem *mCherry* insert (GRT) and all other inserts in this study (see Table 2.10) were cloned into the XbaI site of the pMT85 vector (Figure 6.1). Constructed pMT85 vector with forward (GRT^1-F) and reverse (GRT^2-R) orientations of tandem *mCherry* insert were obtained and confirmed by sequencing before being delivered into *Ureaplasma* cells. The placement of the tandem *mCherry* insert altered by cloning the tandem expression construct into the SpeI site of the pMT85 vector (Figure 6.1) to make another different construct (GR-1Tn) (Figure 6.2) that was equivalent to GRT inserts. However, the GR-1Tn plasmid could then receive a second tandem expression construct into the XbaI site resulting in a total of 4 copies of *mCherry* (GR-2Tn) (Figure 6.2). Furthermore, plasmid constructs with one copy and two copies of single *mCherry* (GR-½Tn and GR-½Tn(2)) were separately constructed using a ½ of the tandem *mCherry* insert described above under the control of the same promoter (EF-Tu) used with tandem *mCherry* (Figure 6.2). The codon usage of the ½ tandem *mCherry* insert was identical to that of tandem *mCherry* insert; the only difference was that the three tryptophan UGA codons (*Ureaplasma* tryptophan codon) in the coding region of the synthetic gene were

replaced by the standard UGG tryptophan codon (Figure 6.3). These constructs with different copy number of *mCherry* were mainly optimized to be expressed exclusively in *Ureaplasma* and generated to investigate the effect of copy number on expression of exogenous genes; however, the constructs differing only by the use of tryptophan codon were designed to determine the influence of that single codon. I also examined the expression of a previously reported *mCherry* construct that was expressed in *M. pneumoniae* by Zimmerman and Herrmann (2005), an insert I have designated GRZ, which was a recombinant fusion protein of the *M. pneumoniae* high expression protein Pmp200 and mCherry. This construct was originally designed to assess expression of a small, cysteine-rich, peptide that is only 29 amino acid long (PmP200) using the monomeric red fluorescent protein (mRFP1) in *M. pneumoniae* as a reporter (Zimmerman and Herrmann, 2005). Expression of the GRZ construct was driven by EF-Tu promoter from *M. pneumoniae* and modified by adding a 7x His-tag to the C-terminus. The codon-usage of this particular insert was not modified and only one copy was present in the pMT85 vector.

OPT-*mCherry* insert (GR-m) was an additional construct generated that was codon-optimized based on the reported tRNA genes identified by genomic analysis of 19 serovars by (Paralanov *et al.*, 2012); however, the UGG codon was used for this construct so that it was also expressed in *E. coli* (Figures 2 and 3). The promoter used in this insert was EF-Tu promoter of *U. parvum* SV14, and the insert was also modified by adding BamHI site downstream the promoter to allow flexibility to change prompters (XbaI/SpeI/promoter/BamHI/RFPgeneSacI/XbaI). Following cloning into pMT85,, three different pMT85 constructs with one (GR-1m), two (GR-2m) and three copies of RFP insert (GR-3m) were generated by chance and identified by characterization of several

colonies (Figure 6.4) and were introduced into both *E. coli* and *Ureaplasma* to investigate expression level mediated by copy number.

The last insert constructed and used in this study was an *Ureaplasma*-optimized *mCherry* insert (uRFP-his) that was synthesized as an identical codon usage to the original tandem *mCherry* construct, utilizing the UGA codon for tryptophan (Figure 6.3). However, this insert, has an additional 6-histidine tag added in frame at the end of the gene, and the promoter controlling its expression was the multiple banded antigen (MBA) promoter of *U. parvum* serovar 1. For direct comparison to the tandem *mCherry* construct, two copies of uRFP were cloned into pMT85 (one into the SpeI site, one into the XbaI site) prior to delivery into *Ureaplasma* to examine expression (Figure 6.2.)

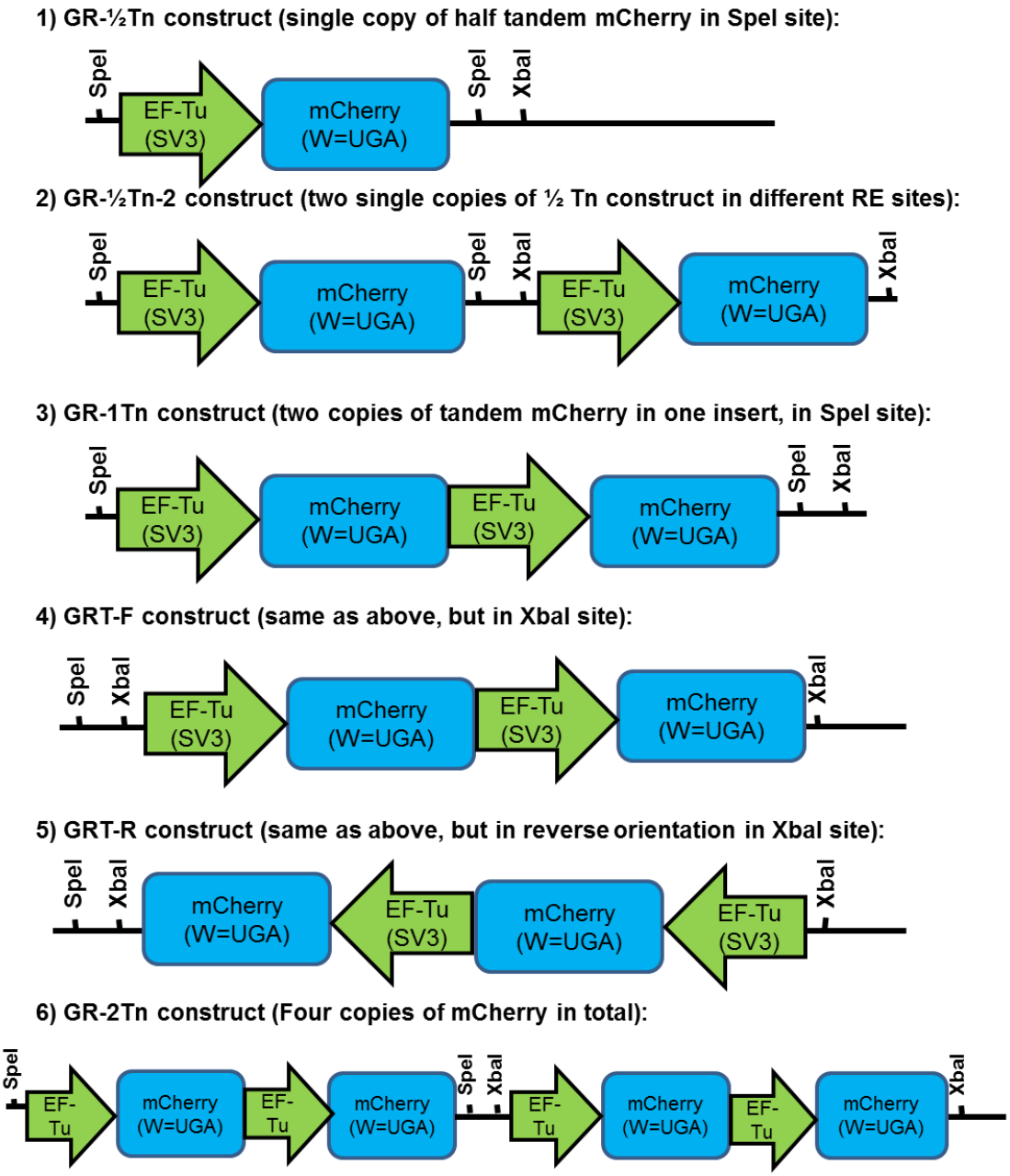
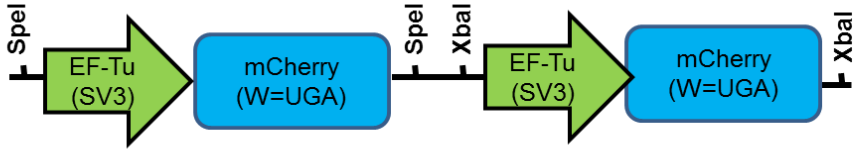


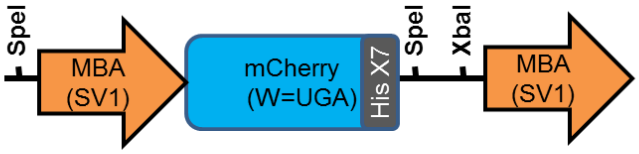
Figure 6.2 Diagrammatic representation of *mCherry* expression constructs delivered into the *Ureaplasma* strains by mini-transposon plasmid, pMT85. Two separate *mCherry* constructs (identified by blue and violet rectangles) were created that differed by the codons utilised to encode the synthetic gene. Of critical importance were those constructs where tryptophan was determined by the UGA codon (*Ureaplasma* expression only) relative to the UGG codon (all prokaryotes). A grey box identifies constructs where 7 consecutive histidine residues (His-tag) have been added in frame prior to the stop codon. The relationship of the different constructs utilising the EF-Tu promoter and *mCherry* are shown by the insertion of single promoter-gene or double tandem promoter-gene expression constructs into the available XbaI and SpeI restriction sites (RE) to determine copy number on each plasmid. Green arrows indicate EF-promoter and orange ones indicate MBA-promoter.

Continued

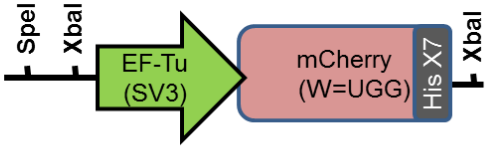
7) GR-½ Tn(2) construct (two single copies, one in XbaI site, one in SpeI site):



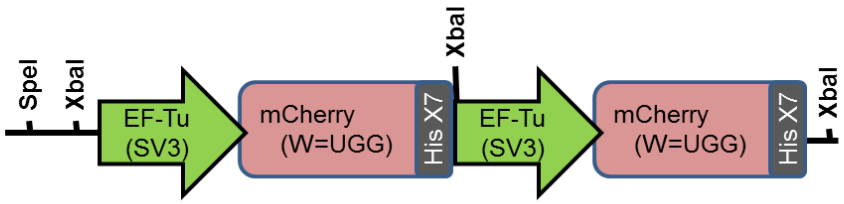
8) uRFP construct (same as above, but different promoter and fusion His-tag added):



9) GR-1m construct (same promoter as original construct, but different codons):



10) GR-2m construct (two single copies of above construct inserted in the XbaI site):



11) GR-3m construct (three single copies inserted in the XbaI site):

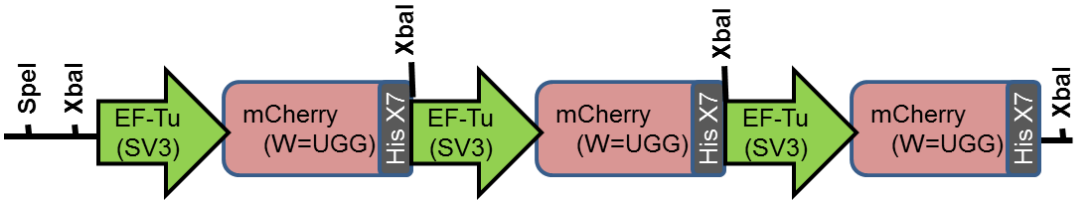


Figure 6.2 (continued) Diagrammatic representation of *mCherry* expression constructs delivered into the *Ureaplasma* strains by the mini-transposon plasmid, pMT85. Two separate *mCherry* constructs (identified by blue and violet rectangles) were created that differed by the codons utilised to encode the synthetic gene. Of critical importance were those constructs where tryptophan was determined by the UGA codon (*Ureaplasma* expression only) relative to the UGG codon (all prokaryotes). A grey box identifies constructs where 7 consecutive histidine residues (His-tag) have been added in frame prior to the stop codon. The relationship of the different constructs utilising the EF-Tu promoter and *mCherry* are shown by the insertion of single promoter-gene or double tandem promoter-gene expression constructs into the available XbaI and SpeI restriction sites (RE) to determine copy number on each plasmid. Green arrows indicate EF-promoter and orange ones indicate MBA-promoter.

Opt mCherry UGG

```
GUA V 0.36 AGC S 0.08  AAG K 0.10 GGC G 0.15  GAC D 0.11  AAC N 0.19 GCA A 0.37 AUC I 0.15 UUC F 0.11
CAC H 0.25  UAC Y 0.24 UGG W 0.25  UUG L 0.05

atggtaagcaagggcgaagaagacaacatggcaatcatcaaggaattcatgcgtttcaag
 M  V  S  K  G  E  E  D  N  M  A  I  I  K  E  F  M  R  F  K
gtacacatggaaggcagcgtaaacggccacgaattcgaaatcgaaggcgaaggcgaaggc
 V  H  M  E  G  S  V  N  G  H  E  F  E  I  E  G  E  G  E  G
cgaccatacgaaggcacacaaacagcaaagttgaaggtaacaaagggcggcccactacca
 R  P  Y  E  G  T  Q  T  A  K  L  K  V  T  K  G  G  P  L  P
ttcgcatgggacatcttgagcccacaattcatgtacggcagcaaggcatacgtaaagcac
 F  A  W  D  I  L  S  P  Q  F  M  Y  G  S  K  A  Y  V  K  H
ccagcagacatcccagactacctaaagttgagcttcccagaaggcttcaagtgggaacgt
 P  A  D  I  P  D  Y  L  K  L  S  F  P  E  G  F  K  W  E  R
gtaatgaacttcgaagacggcggcgtagtaacagtaacacaagacagcagcctacaagac
 V  M  N  F  E  D  G  G  V  V  T  V  T  Q  D  S  S  L  Q  D
ggcgaattcatctacaaggtaaagttgcgaggcacaaacttcccaagcgacggcccagta
 G  E  F  I  Y  K  V  K  L  R  G  T  N  F  P  S  D  G  P  V
atgcaaaagaagacaatgggctgggaagcaagcagcgaacgtatgtacccagaagacggc
 M  Q  K  K  T  M  G  W  E  A  S  S  E  R  M  Y  P  E  D  G
gcactaaagggcgaaatcaagcaacgattgaagctaaaggacggcggccactacgacgca
 A  L  K  G  E  I  K  Q  R  L  K  L  K  D  G  G  H  Y  D  A
gaagtaaagacaacatacaaggcaaagaagccagtacaattgccaggcgcatacaacgta
 E  V  K  T  T  Y  K  A  K  K  P  V  Q  L  P  G  A  Y  N  V
aacatcaagctagacatcacaagccacaacgaagactacacaatcgtagaacaatacgaa
 N  I  K  L  D  I  T  S  H  N  E  D  Y  T  I  V  E  Q  Y  E
cgtgcagaaggccgacacagcacaggcggcatggacgaattgtacaagcaccaccaccac
 R  A  E  G  R  H  S  T  G  G  M  D  E  L  Y  K  H  H  H  H
caccaccactaa
 H  H  H  -
```

Half-tandem: UGA

```
atggtttcaaaaggtgaagaagataatatggctattattaaagaatttatgcgtttaaa
 M  V  S  K  G  E  E  D  N  M  A  I  I  K  E  F  M  R  F  K
gttcatatggaaggttcagttaatggtcatgaatttgaaattgaaggtgaaggtgaaggt
 V  H  M  E  G  S  V  N  G  H  E  F  E  I  E  G  E  G  E  G
cgtccatatgaaggtacacaaacagctaaattaaaagttacaaaaggtggtccattacca
 R  P  Y  E  G  T  Q  T  A  K  L  K  V  T  K  G  G  P  L  P
tttgcttgagatattttatcaccacaatttatgtatggttcaaaagcttatgttaaacat
 F  A  W  D  I  L  S  P  Q  F  M  Y  G  S  K  A  Y  V  K  H
ccagctgatattccagattatttaaaattatcatttccagaaggttttaaatgagaacgt
 P  A  D  I  P  D  Y  L  K  L  S  F  P  E  G  F  K  W  E  R
gttatgaattttgaagatggtggtgttgttacagttacacaagattcatcattacaagat
 V  M  N  F  E  D  G  G  V  V  T  V  T  Q  D  S  S  L  Q  D
ggtgaatttatttataaagttaaattacgtggtacaaattttccatcagatggtccagtt
 G  E  F  I  Y  K  V  K  L  R  G  T  N  F  P  S  D  G  P  V
atgcaaaaaaaaacaatgggttgagaagcttcatcagaacgtatgtatccagaagatggt
 M  Q  K  K  T  M  G  W  E  A  S  S  E  R  M  Y  P  E  D  G
gctttaaaaggtgaaattaaacaacgtttaaaattaaaagatggtggtcattatgatgct
 A  L  K  G  E  I  K  Q  R  L  K  L  K  D  G  G  H  Y  D  A
gaagttaaaacaacatataaagctaaaaaaccagttcaattaccaggtgcttataatgtt
 E  V  K  T  T  Y  K  A  K  K  P  V  Q  L  P  G  A  Y  N  V
aatattaaattagatattacatcacataatgaagattatacaattgttgaacaatatgaa
 N  I  K  L  D  I  T  S  H  N  E  D  Y  T  I  V  E  Q  Y  E
cgtgctgaaggtcgtcattcaacaggtggtatggatgaattatataaataa
 R  A  E  G  R  H  S  T  G  G  M  D  E  L  Y  K  -
```

Figure 6.3 Codon usage for key constructs (OPT-*mCherry* UGG, Half-tandem UGA and uRFP). Where best *U. parvum* codon is used in sequence, the codon is highlighted at first use.

Continued

uRFP

AGU S 0.38 actually higher than tandem S UCA S 0.24

```
atggttagtaaaggtgaagaagataatatggctattattaaagaatttatgcgtttaaa
 M  V  S  K  G  E  E  D  N  M  A  I  I  K  E  F  M  R  F  K
gttcatatggaaggtagtgttaatggtcatgaatttgaaattgaaggtgaaggtgaaggt
 V  H  M  E  G  S  V  N  G  H  E  F  E  I  E  G  E  G  E  G
cgtccatatgaaggtacacaaacagctaaattaaaagttacaaaaggtggtccattacca
 R  P  Y  E  G  T  Q  T  A  K  L  K  V  T  K  G  G  P  L  P
tttgcttgagatattttaagtccacaatttatgtatggtagtaaagcttatgttaaacat
 F  A  W  D  I  L  S  P  Q  F  M  Y  G  S  K  A  Y  V  K  H
ccagctgatattccagattatttaaaattaagttttccagaaggttttaaatgagaacgt
 P  A  D  I  P  D  Y  L  K  L  S  F  P  E  G  F  K  W  E  R
gttatgaattttgaagatggtggtgttgttacagttacacaagatagtagtttacaagat
 V  M  N  F  E  D  G  G  V  V  T  V  T  Q  D  S  S  L  Q  D
ggtgaatttatttataaagttaaattacgtggtacaaattttccaagtgatggtccagtt
 G  E  F  I  Y  K  V  K  L  R  G  T  N  F  P  S  D  G  P  V
atgcaaaaaaaaacaatgggttgagaagctagtagtgaacgtatgtatccagaagatggt
 M  Q  K  K  T  M  G  W  E  A  S  S  E  R  M  Y  P  E  D  G
gctttaaaaggtgaaattaaacaacgtttaaaattaaaagatggtggtcattatgatgct
 A  L  K  G  E  I  K  Q  R  L  K  L  K  D  G  G  H  Y  D  A
gaagttaaaacaacatataaagctaaaaaaccagttcaattaccaggtgcttataatgtt
 E  V  K  T  T  Y  K  A  K  K  P  V  Q  L  P  G  A  Y  N  V
aatattaaattagatattacaagtcataatgaagattatacaattgttgaacaatatgaa
 N  I  K  L  D  I  T  S  H  N  E  D  Y  T  I  V  E  Q  Y  E
cgtgctgaaggtcgtcatagtacaggtggtatggatgaattatataaacatcatcatcat
 R  A  E  G  R  H  S  T  G  G  M  D  E  L  Y  K  H  H  H  H
catcatcattaa
 H  H  H  -
```

Figure 6.3 (continued) Codon usage for key constructs (OPT-*mCherry* UGG, half-tandem *mCherry* UGA and uRFP). Where best up codon is used in sequence the codon is highlighted at first use.

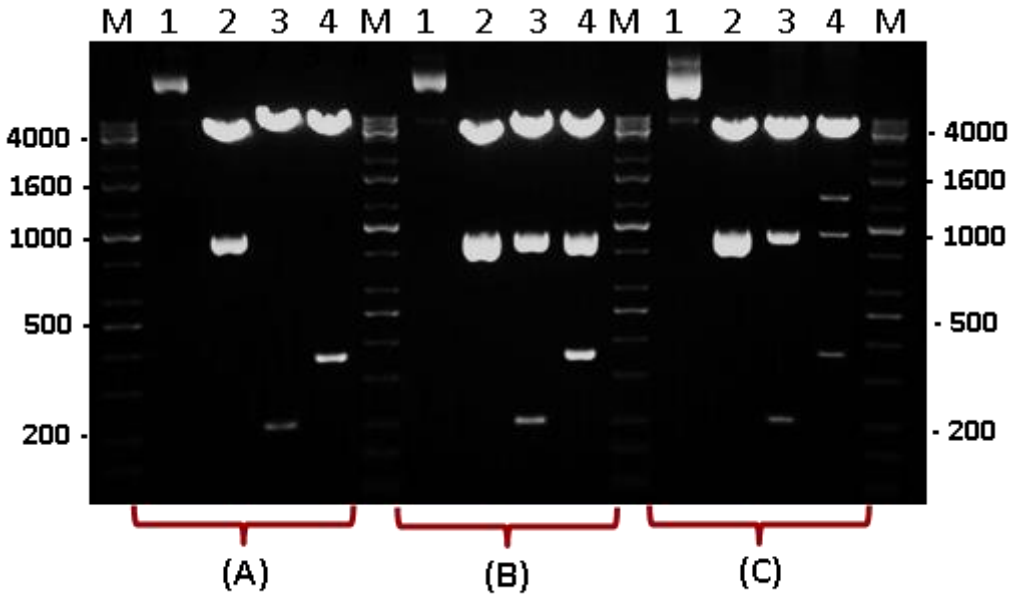


Figure 6.4 Mini-transposon plasmids (pMT85) carrying different copy-number of OPT *mCherry* insert. Purified plasmids with one (GR-1m) **(A)**, two (GR-2m) **(B)** and three (GR-3m) **(C)** copies of *mCherry* insert were digested with three different restriction enzymes XbaI, SpeI (3) and BamHI (4) to verify the copy number of the *mCherry* insert. Lanes: (1) undigested pMT85 plasmid and M; molecular weight marker (the KAPA universal ladder). The copy number was determined based on the differential bands produced by BamHI digest, which is not encoded within the insert (4).

6.2.2 Expression of tandem *mCherry* insert in *Ureaplasma*

The tandem *mCherry* insert was the first *RFP* insert to be delivered to and observed for expression in *Ureaplasma*. The pMT85 plasmid vector carrying a tandem *mCherry* insert was successfully delivered to several *Ureaplasma* strains as confirmed by the presence of the gentamicin resistance gene by PCR using gentamicin primers as previously detailed in chapter 5 of this thesis. Using immunoblot analysis, the findings showed that all *Ureaplasma* strains transformed with this RFP insert were able to express the fluorescent protein mCherry compared with untransformed parental negative controls and clones with empty pMT85 vector (Figure 6.5). The RFP was positively detected by anti-mCherry antibody and appeared as a single band with approximately 23 kDa combined with a double band with higher molecular mass size (about 30 kDa), which is probably a glycosylated isoform of RFP. The mCherry protein was completely absent in parental strains and strains cloned with an empty pMT58 vector. Expression of emitted red fluorescence (mCherry) was also observed in *Ureaplasma* colonies growing on USM agar medium using a fluorescent microscopy. Although RFP was clearly detected by Western blotting, the emission of red fluorescence from colonies viewed under a fluorescent microscope was of a low degree (Figures 6.6). An attempt to measure the red fluorescent protein from bacterial cells growing in broth media (USM) using a fluorometer failed. In contrast, no mCherry expression in *E. coli* cells transformed with pMT85- tandem *mCherry* (GRT) was seen (Figure 6.8), which is expected as the UGA-tryptophan tRNA is only present in *Ureaplasma* and other mollicutes and this protein would be truncated at 62 amino acids (first occurrence of tryptophan in the coding region out of 256 amino acids for the full-length protein).

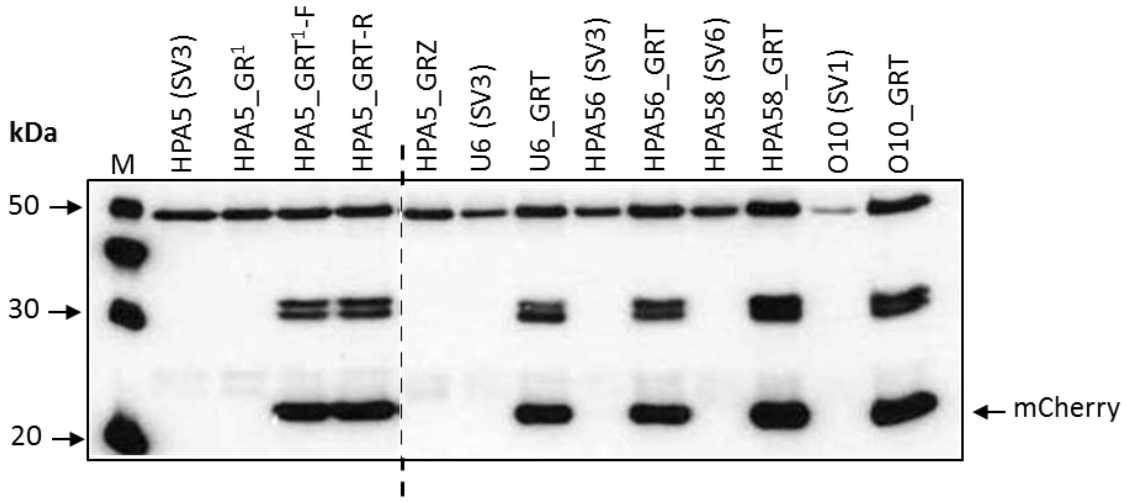
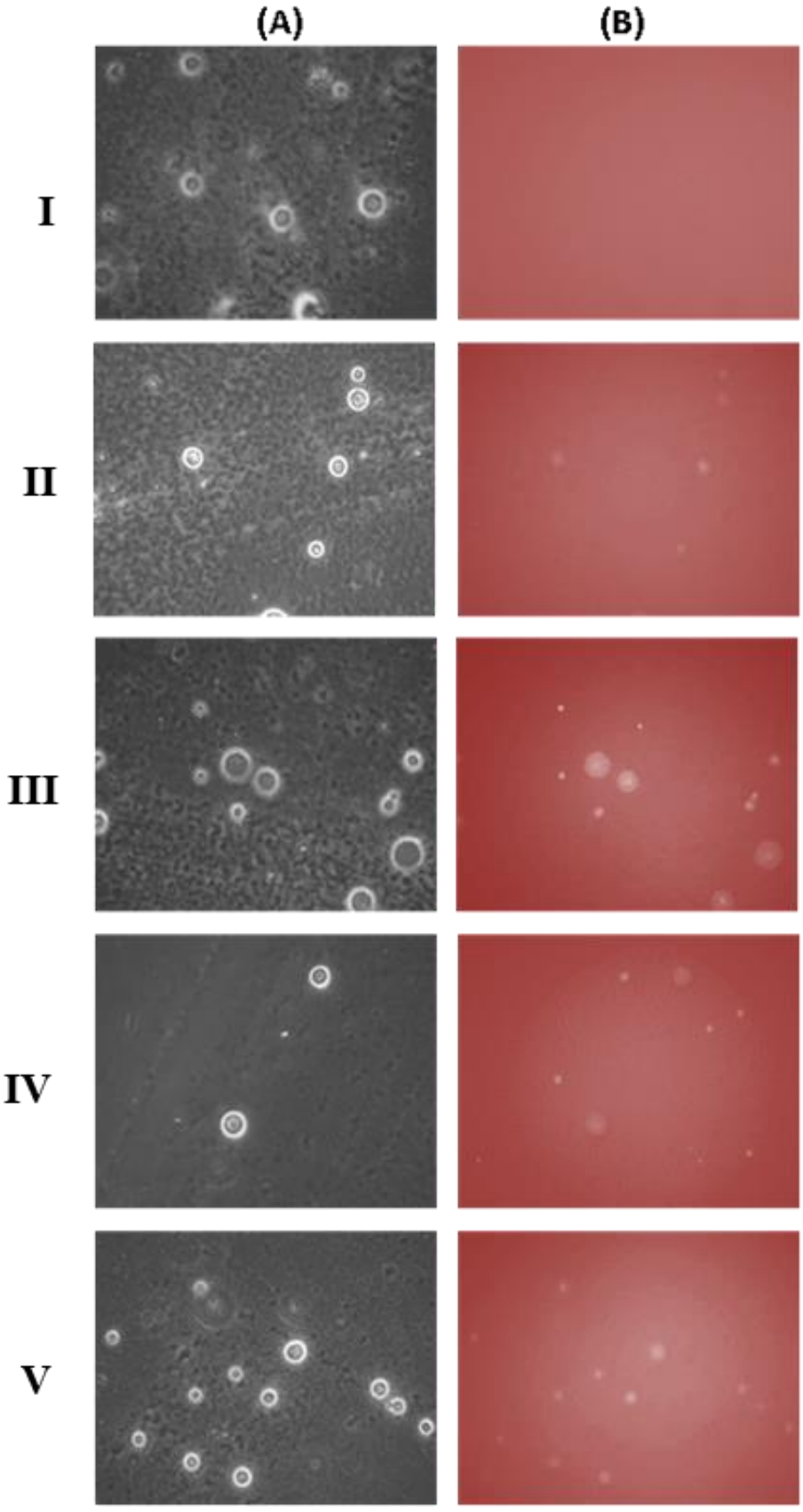


Figure 6.5 Expression of RFP in a number of representative transformed strains of *Ureaplasma.* Total protein extract was separated by SDS-PAGE (12.5%) and blotted onto nitrocellulose membrane. anti-mCherry polyclonal antibody was used to detect the RFP expression in transmutants with tandem *mCherry* insert (GRT) compared to untransformed parents and one HPA5 transmutant strain with empty vector. Another HPA5 transmutant clone carrying different *RFP* insert, Zimmerman *mRFP1* construct (GRZ) was also run alongside. With exception of HPA5_GRZ clone, all transformed clones were found to express the delivered exogenous *RFP* gene (tandem *mCherry* insert) with molecular mass of about 23 kDa associated with a larger double band of about 30 kDa size (probably glycosylated RFP as it was seen in all clones expressed RFP). Lane M= molecular mass marker.

Figure 6.6 Detection of RFP expression by *Ureaplasma* colonies. Colonies from representative transformed strains carrying different constructs of *mCherry* were screened and observed using a fluorescence microscope. Colonies from the same microscopic filed are shown in both visible light (A) and fluorescence (B). Strains from up to down: Parental strain HPA5 (SV3) **(I)**, transformed HPA5 strains: with forward tandem *mCherry* (HPA5_GRT-F) , with reveres tandem *mCherry* (HPA5_GRT-R) **(III)**, with tandem *mCherry* in SpeI cloning site (HPA5_GR-1Tn) **(IV)**, and two copies tandem *mCherry* (HPA5_GR-2Tn) **(V)**. All transmutants expressed a low degree of RFP and no fluorescence was observed in the parental HPA5 strain.


(A)
(B)
I
II
III
IV
V

6.2.3 Optimization of *RFP* gene expression in *Ureaplasma*

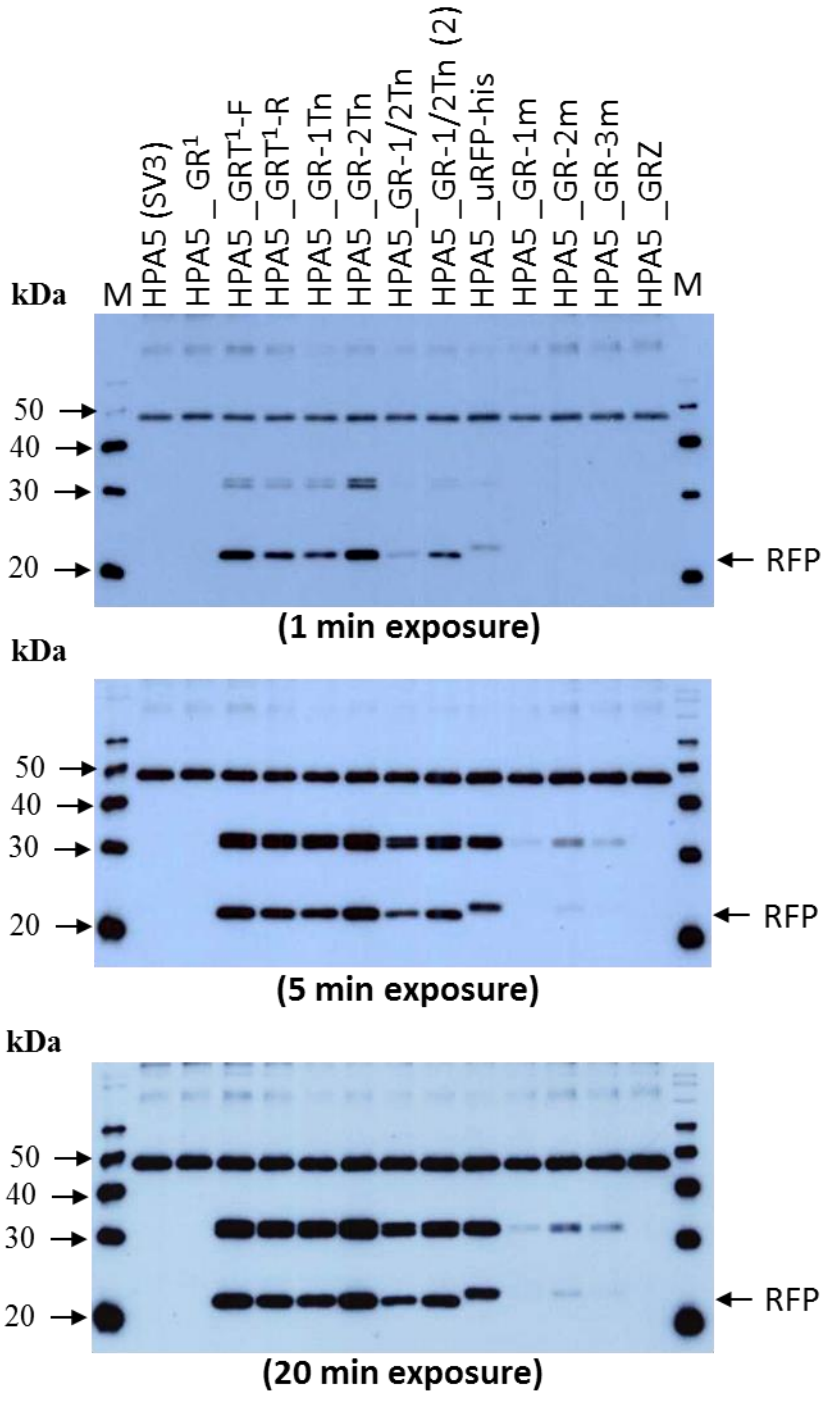
To investigate some of the factors that affect the expression level of foreign genes in *Ureaplasma*, a number of constructed *mCherry* inserts were created to examine codon-usage, copy number of *mCherry* and/or use of different promoters as stated above. The pMT85 vectors with all these different inserts with varying parameters were transferred into the *U. parvum* serovar 3, HPA5 isolate. Following successful transformation, expression levels of RFP were investigated by immunoblot analysis. Whole cell lysates were extracted, and 10 µg protein loads from all transmutant strains under investigation were run alongside each other and separated using 1-DE (12.5 % SDS-PAGE). Expression level of RFP was investigated by comparing intensity of mCherry protein bands obtained from all transmutant clones. As it can be seen in figure 6.7, RFP was found to be successfully expressed, albeit with different levels of expression by most of the transmutant strains (HPA5_GRT[1]-F, HPA5_GRT[1]-R, HPA5_GR-1Tn, HPA5_GR-2Tn, HPA5_GR-½Tn and HPA5_GR-½Tn-2, HPA5_uRFP-his, HPA5_GR-1m, HPA5_GR-2m, and HPA5_GR-3m). The only exception was the transmutant strain HPA5_GRZ that showed no detectable RFP by western blot analysis. This transmutant strain contained the *RFP* insert that was constructed as fused protein with EF-Tu promoter from *M. pneumoniae*, and was not codon-usage optimized for *Ureaplasma.* Variations in expression level observed among the transmutants were basically attributed to different modified parameters tested. By analyzing the results, it was found that all *mCherry* inserts derived by EF-Tu promoter of *U. parvum* SV14 were expressed at a higher level compared with the insert under the control of the MBA promoter of SV1. This observation was especially obvious in comparison to the transmutant strain HPA5_GR1½Tn, which only contained one copy of ureaplasma-optimized *mCherry*

insert with EF-Tu promoter. By comparison HPA5_uRFP-his had two copies of the same *Ureaplasma*-optimized *mCherry* insert, but controlled by the MBA promoter of SV1. Despite being having different copy numbers of *mCherry*, the two transmutants showed similar levels of RFP expression seen as immunoreactive bands with comparable intensity. By comparison, *Ureaplasma* containing two copies of *mCherry* (as for uRFP-his) but under the EF-Tu promoter (GRT-F/GRT-R) showed enhanced levels of expression compared to the transmutant strain HPA5_uRFP-his (Figure 6.7).

The majority of transmutant strains carried *mCherry* inserts were codon-usage-optimized for *Ureaplasma*, however, there were three transmutants (HPA5_GR-1m, HPA5_GR-2m and HPA5_GR-3m) which carried increasing copy numbers of *mCherry* that were codon-usage optimized based on identified tRNA species in the analysed 19 *Ureaplasma* genomes and using the UGG tryptophan codon so that it could be expressed in *E. coli* as well (Figure 6.3). The expression of this construct was under the control of EF-Tu promoter from SV14. Plasmid vectors carrying 1, 2 and 3 identical copies of this construct were created and characterized (Figure 6.2). Following, delivery into HPA5, the immunoblot results revealed that expression of these constructs was almost negligible relative to the other successfully delivered constructs (Figure 6.7). However, due to the use of the UGG codon for tryptophan, these constructs were expressed in *E. coli* and at much higher levels compared with expression in *Ureaplasma* (Figure 6.8). The effect of the copy number of *mCherry* on RFP expression in *E.coli* could be measured using a fluorometer for HPA5_GR-1m, HPA5_GR-2m and HPA5_GR-3m; transmutants with more than one copy numbers of the gene showed an enhanced expression compared with that of one copy insert (Figures 6.9).

The effect of gene copy number on level of expression was also investigated in constructs that were codon-usage optimized for *Ureaplasma.* As previously mentioned in section 6.2.1 of this chapter, constructs with different copy number of tandem *mCherry* and ½ tandem *mCherry* inserts were generated and transmutants with one copy (HPA5_GR½Tn), two copies (HPA5_GRT[1]-F, HPA5_GRT[1]-R, HPA5_GR-1Tn and HPA5_GR½Tn(2)), and four copies (HPA5_GR-2Tn) were created and investigated. While the right promoter and codon-optimization were crucial factors for expression of foreign genes in *Ureaplasma,* the results also showed that increasing gene copy number for *mCherry* had an increasing effect on expression of RFP in *Ureaplasma* as well. Of all transmutants studied, those with 2 and 4 copies of *mCherry* showed the highest expression, with 4 copies being the best detected by immunoblot analysis (Figure 6.7). Attempts to measure fluorescence of mCherry produced by *Ureaplasma* cells growing in USM using a fluorometer were unsuccessful as both untransformed and transformed *Ureaplasma* cells gave similar reading. In addition, the negative control USM broth (uninoculated) also gave a comparable fluorescence reading to that of USM with growing bacteria. It is believed that there are several factors might cause such problems, which mainly relate the ingredient of media. The presence of phenol red and 10% porcine serum in the growth medium are thought to confound the ability to detect additional fluorescence over the autofluorescence and/or quenching (Straight, 2007).

Figure 6.7 Comparison of RFP expression under different parameters by *U. parvum* SV3 (isolate HPA5). Total cellular protein was extracted and 10 µg protein of each sample was loaded and separated by SDS-PAGE (12.5%). Separated proteins were then blotted to a nitrocellulose membrane. RFP was detected with anti-mCherry polyclonal antibody as a band of approximately 23 kDa combined with detectable higher molecular mass double band. Lanes: parental strain (HPA5; SV3); HPA5 transmutant clone with empty pMT85 vector (HPA5_GR[1]); HPA5 transmutant strain with forward orientation tandem *mCherry* insert (HPA5_GRT[1]-F); HPA5 transformed clone with revers orientation tandem *mCherry* insert (HPA5_GRT[1]-R); HPA5 transmutant clone with one copy tandem *mCherry* insert cloned into the SpeI site of the vector (HPA5_GR-1Tn); HPA5 transmutant clone with two copies tandem *mCherry* insert (HPA5_GR-2Tn); HPA5 transmutant clone with half tandem *mCherry* insert (one copy *mCherry* insert) (HPA5_GR- ½ Tn); HPA5 clone with two copies half tandem inserts (HPA5_GR- ½ Tn-2); HPA5 transmutant clone with two copies of *RFP* with His-tag and SV1 MBA promoter (HPA5_U7); HPA5 transmutant clone with one copy optimized *mCherry*3 (HPA5_GR-1m), HPA5 transmutant clone with two copies optimized *mCherry*3 (HPA5_GR-2m), HPA5 transmutant clone with three copies optimized *mCherry*3 (HPA5_GR-3m); HPA5 transmutant clone with one copy modified *mRFP1* (Zimmerman fused protein) (HPA5_GRZ). M: molecular mass marker.


kDa
M
HPA5 (SV3)
HPA5 _GR1
HPA5 _GRT1-F
HPA5 _GRT1-R
HPA5 _GR-1Tn
HPA5 _GR-2Tn
HPA5_GR-1/2Tn
HPA5 _GR-1/2Tn (2)
HPA5 _uRFP-his
HPA5 _GR-1m
HPA5 _GR-2m
HPA5 _GR-3m
HPA5 _GRZ
M
50
40
30
20
RFP
(1 min exposure)
kDa
50
40
30
20
RFP
(5 min exposure)
kDa
50
40
30
20
RFP
(20 min exposure)

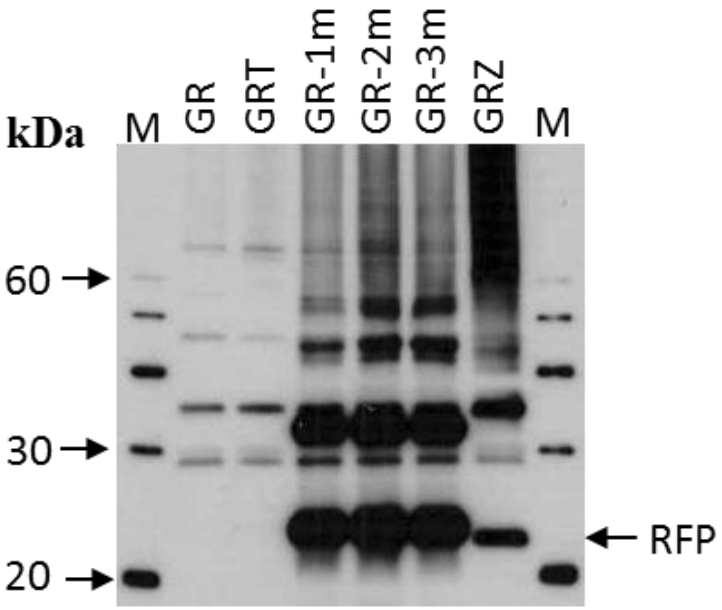


Figure 6.8 Immunoblot of *E. coli* transmutant strains harbouring different constructs of *mCherry*. Total cell protein extract (10 µg) was separated by SDS-PAGE (12.5%) and blotted onto nitrocellulose. RFP was detected with anti-mCherry polyclonal antibody. Transmutant strains: GR (with empty pMT85), GRT (with tandem *mCherry* insert), GR-1m (with one copy Opt-*mCherry* insert), GR-2m (with two copies Opt-*mCherry* insert) GR-3m (with three copies Opt-*mCherry* insert) and GRZ (with Zimmerman insert). RFP was expressed by all transmutant strains (approximately 25 kDa) compared to negative control (GR) and transmutant strain harbouring *mCherry* insert optimised for *Ureaplasma* (GRT). Lane M: molecular weight marker.

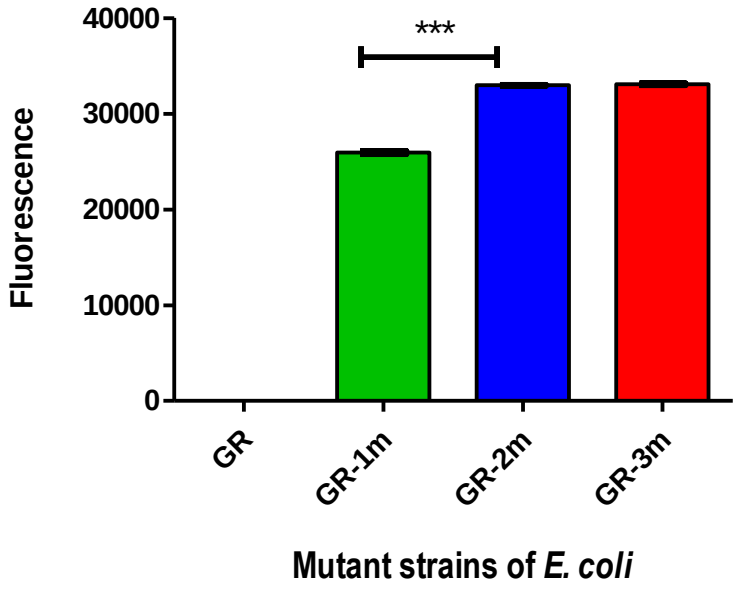


Figure 6.9 A graph showing the effect of the number of copies of the *mCherry* on expression by recombinant *E. coli*. RFP from three transmutant *E. coli* strains containing different copy number of *mCherry*: one (GR-1m), two (GR-2m) and three (GR-3m) was measured by fluorometer and compared to a transmutant strain carrying an empty pMT85 vector (GR) as a negative control. All transformants were grown overnight in L.B broth with gentamicin (15µg/ml) and the O.D at 600nm was adjusted to 1 before measurement of RFP. Transmutants with more than one copy number of the gene show a higher level of expression. Note that there was a significant increase in expression in transmutant strains carrying two or three copies when compared to transmutant strain carrying only one copy *** = $p<0.0001$. Data are presented as a mean cellular fluorescence ± SEM of triplicate samples of each transmutant strain under investigation.

6.3 Discussion

Ureaplasma spp. are among the smallest and simplest self-replicating and free-living bacteria known to date. The evolution of these tiny microbes has occurred through genomic reduction and reduction of G+C content from their ancestors that are believed to be Gram-positive bacteria (Dybvig, 1990). As a result of evolutionary process, many non-essential genes were lost resulting in several genomic and biological changes such as an absolute reliance on their host for nutrient supply, unusual codon usage for tryptophan, and lack of classical cell wall. These features have made it difficult to investigate *Ureaplasma* using some of the powerful molecular genetic tools that are available for studying the biology and pathogenesis of many well-known microorganisms. The first report of foreign DNA delivery in Mollicutes was when DNA extracted from a *Mycoplasma* "virus" (L2) was introduced into *A. laidlawii* using a PEG-mediated transfection (Sladek and Maniloff, 1983). Taking the advantage of the first successful transformation in *Ureaplasma,* the pMT85 Tn*4001*-minitransposon vector, in this study, was used to introduce the red fluorescent gene, *mCherry,* as an exogenous gene into *Ureaplasma* cells and investigate its expression under the control of several factors that affect the level of expression. Fluorescent proteins are widely used as labeling tools to investigate protein localization, protein-protein interactions, cell division, gene expression in many microorganisms (Chudakov *et al.*, 2010). The first documented expression of recombinant fluorescent proteins in Mollicutes was achieved in *M. pneumoniae,* when a GFP gene (*EGFP*) was fused with the gene encoding for the cytadherence associated protein HMW2 to determine its subcellular localization (Balish *et al.*, 2003) . In this work, the fusion protein construct was cloned into a modified pMT85 vector that was then used to transform the bacterial cells, and the results showed

that HMW2 protein is a part of the attachment organelle of *M. pneumoniae*. Afterwards, the subcellular localization of a number of other *M. pneumoniae* proteins including P65, HMW2, P41 and P24 were also identified by a similar labeling approach (Kenri *et al.*, 2004). In my study, expression of RFP was achieved by *Ureaplasma* in most cases, and the level of expression was varied and shown to be responsive to a number of parameters that are known to have a major impact on expression of foreign genes in other bacteria (Fakruddin *et al.*, 2013; Glick and Whitney, 1987).

The effect of transcriptional promoter on expression was one of the key parameters to be investigated in this study. I have selected and used three different promoters: 1) EF-Tu gene promoter (from SV3) that drives the expression of one of the most abundant bacterial proteins, EF-Tu protein, 2) the MBA gene promoter (from SV1) that drives the expression of a major surface lipoprotein of *Ureaplasma* spp., the MBA and 3) the EF-Tu gene promoter from the closely-related species *M. pneumoniae*. Among these promoters, EF-Tu promoter of SV14 was found to be the strongest as all *mCherry* inserts under the control of this promoter were effectively expressed, and the MBA promoter was also a good promoter, but not as good as the EF-Tu promoter. On the other hand, the expression of the RFP fusion protein driven by the *M. pneumoniae* EF-Tu promoter failed, whether this failure was resulted from the promoter or other factors related to the gene construct is an issue that has not been defined in this study as the main target of this work is to get positive expression of RFP that can be examined. However, this construct was reported to efficiently express in *M. pneumoniae* by Zimmerman and Herrmann. The results of this work indicated that choosing a strong promoter is a key aspect for driving expression of *mCherry* in *Ureaplasma*. In other mollicutes, a variety of chosen promoters have been utilized. Of these, the *S. citri* spiralin gene promoter that drives the most abundant protein

of *S. citri* has been the most widely used promoter for expression of foreign genes. This promoter has been effectively used for driving expression of several exogenous genes, for example, antibiotic selection genes (*tetM, cat, aacA-aphD*) and reporter genes (*lacZ* and *GFP*) and was indicated to be a strong promoter (Duret *et al.*, 2005; Duret *et al.*, 2003). Many other strong promoters have also been used for controlling expression of foreign genes and proved to be useful such as the promoter of the P40 gene of *M. agalactiae* and the promoters of P65 adhesin and *tuf* genes of *M. pneumoniae* (Balish *et al.*, 2003; Baranowski *et al.*, 2010; Kenri *et al.*, 2004).

Another important factor that was shown to have a great effect on expression of foreign genes in *Ureaplasma* was the codon-usage of the gene. In fact, there are 61 different trinucleotide codons that encode for the 20 known amino acids, and a number of these codons can encode for the same amino acid. Variations in codon-usage preference have been reported among many prokaryotes, and the frequency of codon-usage by a particular microorganism is believed to be greatly influenced by the cognate tRNAs pool (Glick and Whitney, 1987). Furthermore, and as indicated earlier, these species have a unique codon-usage for the amino acid tryptophan: The UGA codon in all other bacteria encodes for the open-reading frame termination stop codon Opal, therefore it was important to take this in consideration when optimizing the codons used to encode the red fluorescent protein mCherry to get maximal gene product. All *RFP* inserts that were codon-usage optimized for *Ureaplasma* were positively expressed regardless the level of expression as detected by western blot analysis, whereas one *RFP* insert (OPT-*mCherry*) that was codon-usage manipulated to function in both *Ureaplasma* and *E. coli* showed low expression in *Ureaplasma*, but high expression in *E. coli*. This may indicate that changing the codon-usage away from those preferred by *Ureaplasma* may have adverse

effect on expression in *E. coli*. Furthermore, another insert that was successfully expressed in *M. pneumoniae* was not expressed in *Ureaplasma*. This insert contained two fusion genes (mRFP1 and MP200RNA) but neither of these genes was optimized for *Ureaplasma* and was used as constructed by the authors (Zimmerman and Herrmann, 2005). This may explain why it did not express in *Ureaplasma* due to the importance of codon-optimization.

The copy number of the exogenous gene, *mCherry*, was also evaluated in my study and found to improve the efficiency of RFP expression in *Ureaplasma*. Inserts with more copies of *mCherry* (e.g. tandem *mCherry* and 2 copies tandem *mCherry*) were showed to enhance the expression degree, with four copies (two tandem *mCherry*) being the best. Using more than one copy number of a gene on the same vector has been indicated to improve expression by stabilizing the cloned protein (Glick and Whitney, 1987). However, it should be noted that gene copy number is probably less important than use of a strong transcriptional promoter and codon-usage optimization.

In summary, I have shown that introducing foreign genes into *Ureaplasma* using transposon-based approach is possible. This novel accomplishment will undoubtedly open doors for other researchers in this field to explore and understand *Ureaplasma* biology and pathogenicity in more detail. During this work, I also have tried to address and examine some of the key factors that play an important role in expression of exogenous gene in bacteria: the promoter, the codon-usage, and the gene copy number. It should be noted that the level of expression achieved in this study is still low and did not achieve a level sufficient for use in intracellular or *in vivo* tracking during infection. Therefore, further investigations of expression optimization are needed to get the

maximal possible expression in these unusual microorganisms and create recombinant fluorescent *Ureaplasma*, which will indeed make it possible to visualizes and study these tiny microbes both *in vivo* and *in vitro*. Some of the possible aspects that require more investigation are the stability of the foreign gene, the metabolic state of *Ureaplasma* cells, fusing RFP to *Ureaplasma* genes and/or using different vectors as well as other factors that could enhance foreign gene expression in bacteria.

Chapter 7
General Discussion

7. General discussion

7.1 Discussion

In this study, I applied a range of powerful techniques and sought to enhance our knowledge about the strategies and mechanisms utilised by *Ureaplasma* to overcome the complement-mediated serum killing. The development of a new genetic tool (transposon mutagenesis) within this study was a novel and potentially important advance that has provided a powerful tool and opened doors for future work to investigate the biology and pathogenicity of *Ureaplasma*. Our understanding of *Ureaplasma* pathogenesis and its role in human diseases has been hampered by the lack of genetic tools that would identify and determine virulence factors responsible for differential pathogenicity among *Ureaplasma* (Waites *et al.*, 2005). Now as this genetic method has become available, genes involved in virulence of *Ureaplasma* can be identified and studied. In other microbial pathogens, many powerful tools are available and have made it possible to reveal and study many virulence factors. In fact, this has largely enhanced our understanding of the biology and pathogenesis of microorganisms as well as immunology. Of these techniques, genomic and proteomic approaches are amongst the most widely and most successfully used tools (Choi, 2009; Schmidt and Volker, 2011).

The need for a genetic method that would allow investigation of *Ureaplasma* virulence genes, especially those that might be involved in mechanisms of serum resistance has driven me to seek a way to develop a transposon mutagenesis method that could be successfully applied to identify the mechanisms underlying serum resistance of these unique bacteria. In this project, serum resistance was highlighted and addressed and was found to be a common phenomenon among *Ureaplasma* serovars and clinical isolates that

could contribute to their role in human diseases. Therefore, it would be of great importance to define the underlying mechanisms by which *Ureaplasma* overcome this aspect of the host innate immunity. I developed PEG-mediated transformation protocol that was successfully applied to deliver a gentamicin resistance marker (6'-aminoglycoside N-acetyltransferase) to a number of representative serovars of *U. parvum* as well as one clinical isolate of *U. urealyticum* (W11; SV12). Mapping transposon insertion and investigating the disrupted genes in some transmutant strains was also achieved. The findings showed that the insertion of transposon in the *Ureaplasma* genome occurred randomly with only one insertion of transposon in most cases resulting in disruption of non-essential genes. I also used this method successfully to deliver exogenous genes to *U. parvum*; the red fluorescent protein gene (*mCherry*) was introduced, as a measurable exogenous gene, into the genome of *Ureaplasma.* This allowed me to examine the ability of *Ureaplasma* to express foreign genes and determine the optimal promoter, codon usage and copy number to facilitate future expression and testing of any identified serum resistance genes. Most importantly this method was also used for the first time in an attempt to investigate expression and physiological effect of a candidate gene (UU280) (the foremost candidate to encode the gene for the 41 kDa-serum resistance associated protein identified by proteomics). However, a second candidate gene for the 41 kDa protein was identified through transposon gene disruption of the gene UUR10_0137 coincident with loss of serum resistance in *U. urealyticum* strain W11 (discussed in details in Chapters 4 and 5).

As was the case for developing any new method, limitations exist and require further modification and optimization, especially when applied to challenging microorganisms such as *Ureaplasma*. In other mycoplasmas, the cultivation and growth conditions of these fastidious microbes have been proven to limit the transformation and genetic

manipulation (Dybvig and Voelker, 1996; Razin, 1985). In my study, this was one of the major obstacles I faced during the early failed attempts of *Ureaplasma* transformation. *Ureaplasma* and *Mycoplasma* are different in their growth requirements: *Ureaplasma* needs urea as a main source of energy, whereas *Mycoplasma* requires arginine (Fiacco *et al.*, 1984). These important supplements are added to the growth medium; however, in the case of *Ureaplasma,* hydrolysis of urea by the urease also produces ammonia, which is self-toxic for *Ureaplasma* (Ford and MacDonald, 1967). This dramatically affected the possibility of getting and selecting transformed cells as their numbers are usually very low and fail to thrive. I circumvented this barrier by repeated serial sub-culturing post-transformation in the USM containing gentamicin to rescue the growing transmutant cells. Other factors that have generally made it difficult to develop genetic tools for manipulating mycoplasmas include the lack of natural plasmids for most mycoplasmas, the lack of suitable growth media with improved ingredients, the paucity of antibiotic resistance selection markers, and the unusual codon usage of UGA as tryptophan codon (Dybvig and Voelker, 1996).

Although this method successfully transformed several representative serovars of *U. parvum* in this study, all attempts to introduce transposon Tn*4001* into the ATCC prototype strains of *U. urealyticum* serovars were unsuccessful. This may indicate species variations in genetic manipulation among *Ureaplasma*. Despite the large homology between the two species, there is a difference in the genome size of the two species; *U. urealyticum* (0.83 - 0.94 Mb) have a larger genome size than *U. parvum* (0.75 - 0.77 Mb). Further protocol refinement to achieve success both species of *Ureaplasma* is required. Similar challenges in introducing transposon Tn*4001* into the genomes of some species of the genus *Mycoplasma* have been reported. For example, attempts to introduce the Tn*4001* transposon to *M. arthritidis* and *M. pulmonis* have failed. The reason for

unsuccessful genetic manipulation was assumed to be functional failure of the antibiotic selection marker (the gentamicin resistance gene), as changing the gentamicin resistance gene by inserting the chloramphenicol acetyltransferase gene or the *tetM* tetracycline resistance gene into Tn*4001* have resolved the problem in both species (Dybvig *et al.*, 2000). Another limitation of the method was also found to be the low degree of transformation efficiency, but this is a common transformation problem encountered with most mycoplasmas. The modified protocol in this study was based on a PEG mediated transformation protocol and the other methods of transformation using electroporation have not been attempted for *Ureaplasma* in my study. Therefore, the next set of experiments should utilise electroporation based methods to see if this overcomes transposon mutagenesis in *U. urealyticum*. Currently, electroporation protocols are widely used with the closely related species of *Mycoplasma* (Baranowski *et al.*, 2014; Maglennon *et al.*, 2013; Sharma *et al.*, 2014; Shimizu *et al.*, 2014). Other factors that affect the transformation and delivery of exogenous genes have been reported to be the presence of restriction and modification systems, which are commonly found in Mollicutes and restrict gene delivery (King and Dybvig, 1994; Voelker and Dybvig, 1996). Such systems have also been hypothesised based on ORF homology in *Ureaplasma* spp., with *U. urealyticum* having more potential restriction modification enzyme genes than *U. parvum* (Dybvig and Voelker, 1996; Paralanov *et al.*, 2012). Whether these systems are functional and restricted the transformation of *U. urealyticum* have not been addressed in this thesis. However, one clinical isolate of *U. urealyticum*, as mentioned above, was successfully transformed suggesting that these barriers are unlikely causes of transformation failure of the other serovars of *U. urealyticum*. In the study by Lartigue and colleges (2009) for genome transplantation of *M. mycoides subsp. capri* from yeast to *M. capricolum*, such barriers of genetic manipulation were overcome by

inactivation of the restriction enzyme in the recipient cells and protecting the genome of the donor cell via methylation using extracts of *M. capricolum* (Lartigue *et al.*, 2009).

Regardless of all these limitations, genetic tools and protocols for studying these groups of bacteria have significantly been refined and improved. As a genetic tool, transposon mutagenesis has been improved dramatically and was successfully used to insert selectable markers in *M. pulmonis*, *M. hyorhinis*, and *A. laidlawii* (Dybvig and Alderete, 1988; Dybvig and Cassell, 1987; Mahairas and Minion, 1989a, b). In addition, genetic approaches to study essential genes and the physiological effects of delivering exogenous genes continue to expand and be improved (Algire *et al.*, 2009; Paralanov *et al.*, 2012). As *U. parvum* are now amenable to genetic manipulation using the modified protocol of transposon mutagenesis work can begin on extensive definition of essential genes for this organism.

Serum resistance of *Ureaplasma* was the main target of investigation in this study as it is known to be one of the determinants of pathogenicity in bacteria (Fierer *et al.*, 1972). Susceptibility of *U. parvum* to complement-mediated serum killing has been recently investigated. In that study, it was found that sensitivity to complement killing by normal human sera from normal volunteers varied amongst serovars and even amongst strains from the same serovar (Beeton *et al.*, 2012). These findings suggested differential pathogenicity factors (virulence factors) among serovars and clinical isolates that could allow them to evade the complement activity. In this study, I investigated the effect of complement on the other species of human *Ureaplasma* (*U. urealyticum*). My study demonstrated similar findings, as *U. urealyticum* also showed different susceptibility to serum killing ranging from high sensitivity to complement-mediated serum killing to absolute serum resistance, even in the presence of specific antibodies. As prevalence of

serum resistance was observed among *Ureaplasma* spp., it was important to try to define the underlying mechanisms that contributed to resistance. Achieving this goal would greatly increase our knowledge of *Ureaplasma* pathogenicity. In order to address this issue, I successfully created a number of serum-resistant strains from originally serum-sensitive strains through repeated sublytic challenge with NHuS. These induced serum-resistant strains were subjected to further investigation in an attempt to identify proteins that were induced, lost or had been otherwise altered associated with serum resistance. Identification of the deferentially expressed proteins and studying the role of some of these proteins in serum resistance were attempted using proteomic methods and the newly developed genetic method in this project.

Upon investigation, I found that three immunogenic proteins were differentially expressed or altered following the repeated sublytic challenge and developing serum resistance: MBA, EF-tu and a novel protein (approximately 41kDa size) suspected to be UU280 (by proteomics) but later re-identified as the UUR10_0137 gene that encodes a hypothetical protein. Altered expression of the MBA was not a new finding in my study, as many previous studies have reported both phase and size variations in this major surface antigen of *Ureaplasma* both *in vivo* and *in vitro*. And as a variable surface antigen, MBA has been proposed to be one of the virulence factors of *Ureaplasma* (Dando *et al.*, 2012; Knox *et al.*, 2010; Monecke *et al.*, 2003; Robinson *et al.*, 2013; Teng *et al.*, 1994; Zheng *et al.*, 1995; Zimmerman *et al.*, 2009). In *M. pulmonis*, a closely related species, the size of the variable surface protein (VsaA) of this bacterium was found to contribute to serum resistance. Strains expressing long tandem repeats of VsaA (40 repeats) were shown to be more resistant to complement killing than those expressing short VsaA (5 or less tandem repeats) (Simmons *et al.*, 2004; Simmons and Dybvig, 2003). I also reported variations in the MBA size (in *vitro* and *in vivo*); therefore, I sought

to reveal the potential role of such antigenic variations of MBA in serum resistance. I examined the association between serum resistance and MBA type and size in *Ureaplasma* and found that such correlation did not exist among the species of *Ureaplasma*. As both serum-resistant and serum-sensitive *Ureaplasma* strains were shown to express a variety of MBA species that varied in size (small or large) and serovars (one or more variants) this does not appear to contribute to serum resistance. Furthermore, an induced serum-resistant strain (SR-HPA2) became resistant without any alterations in the MBA, while one isolate (SA-HPA5), which was subjected to repeated sublytic challenge with NHuS, had slight alteration in the MBA but did not develop serum resistant (as discussed in chapter 4). Although the results showed no relationship between the MBA and serum resistance in *Ureaplasma,* these findings do not exclude a role for MBA in *Ureaplasma* pathogenicity, which remains to be further investigated.

In contrast, I found that the other two proteins (EF-Tu and the 41 kDa protein) were more likely to be involved in mechanisms of serum resistance as both of them were consistently altered in expression or isoform in all induced serum resistant strains. In my work, EF-Tu was shown to be immunogenic and had altered charge (seen as more acidic *pI* isoforms species), which is likely due to phosphorylation given the retention of molecular mass with the altered *pI*. EF-Tu is a multifunctional protein and has been proposed to play a role in bacterial pathogenesis (Dallo *et al.*, 2002; Granato *et al.*, 2004). EF-Tu in *P. aeruginosa* has been shown to play a role in complement evasion by mediating binding to FH and plasminogen (Kunert *et al.*, 2007). Therefore, the possible involvement of EF-Tu in serum resistance of *Ureaplasma*, based on observations in my study, can be anticipated and needs further investigation to be confirmed.

The 41 kDa immunogenic protein was a very interesting finding in this thesis. Using proteomic post-decay analysis of proteins separated by 1 dimensional SDS-PAGE, this

protein was identified as UU280 protein (endo-1,4-beta- glucanase). However, the role of this protein in serum resistance was ruled out after examination in serum resistant and sensitive strains as well as failure to alter phenotype after delivery by transposon mutagenesis (discussed in Chapter 4). Loss of this 41 kDa protein in one of the gene-disrupted transmutant strains, W11 (SV12), coincided with loss of complement resistance. The only disrupted gene in this transmutant strain was identified as UUR_0137 gene; therefore, this gene is more likely to be the gene encoding for the candidate 41 kDa protein. The role of this gene as a virulence gene mediating serum resistance would be highly recommended for further investigation to elucidate its function and contribution to serum resistance. An example of such experiments would be by delivering this gene to a serum-sensitive strain and investigate its expression and ability to mediated serum resistance. Another alternative way is trying to knock out the gene responsible for the 41 protein expression in known induced serum-resistant strains (created in this study) via transposon mutagenesis and see if this can alter their complement susceptibility. The additional experiment attempting to re-induce the expression of 41 kDa protein with recurrent complement challenge with examination of maintenance of gene disruption should also be performed.

The acquisition of complement regulators to the surface of microbial pathogens is a common mechanism of evading complement killing (Blom *et al.*, 2009; Kraiczy and Wurzner, 2006). This kind of mechanism has not been addressed in my study and is a potential future work. Both EF-Tu and UUR_0137 gene product (the 41 kDa protein) could be surfaced expressed and either recruit complement regulators or directly regulate complement themselves as a mechanism of complement evasion in *Ureaplasma*. Therefore, induced serum-resistant strains, as well as inherently resistant strains, of *Ureaplasma* need to be investigated for their ability to bind regulators of the complement

activation such as FH, C4bP or other regulators. This will provide a new insight in possible tactics utilized by *Ureaplasma* to manipulate the host immune defences.

It should be admitted that proteomic approaches used in this study to identify the candidate proteins were also another challenge. Large scale culture of *Ureaplasma* growth was required to produce sufficient amounts of total cell protein lysate for analysis by proteomics. In spite of that effort, the 2-DE-based proteomic method (2-DE in combination with MS) failed to identify the key proteins that were under investigated in this study, with exception of the EF-Tu. 2-DE is a powerful method for protein separation; however, it has some drawbacks such as inability to resolve hydrophobic, very basic or high molecular weight proteins. In addition, low-abundance proteins (e.g. regulatory proteins and receptors) cannot usually be visualised by conventional staining methods. They are often masked by other high-abundance proteins when analysing whole cell protein lysate. Moreover, membrane proteins are among those proteins that are difficult to visualise and analyse using 2-DE analysis, as reviewed in (Beranova-Giorgianni, 2003). These limitations may have caused the failure of identification of some interesting proteins in my study. For example, although the MBA is a major surface antigen of *Ureaplasma* (membrane protein), it has not been identified in any of the protein spots analysed. In addition, I could not see any clear difference in protein expression at molecular mass sizes around 40 kDa, where the 41 kDa protein should be located on 2 DE gel, suggesting its low abundance among other cell proteins of *Ureaplasma*. Therefore, I chose an alternative method, which is 1-DE in combination with MS. This approach allowed a single band to be visualised and excised for identification by MS. Despite being able to identify the potential proteins (UU280 protein and MBA) using this approach, it seemed later that the 41 kDa protein was misidentified with another more prevalent protein present in the same excised band (UU280). In fact,

1- D bands are believed to contain more than one protein at the same time (Huang *et al.*, 2002). As the 41 kDa protein is probably a low abundance protein, contamination with other high abundance proteins may cause failure in identification.

7.2 Summary and conclusion:

The work carried in this thesis was aimed to investigate mechanisms underlying serum resistance of *Ureaplasma* spp. by creating serum-resistant models of laboratory *Ureaplasma* strains and developing and using some powerful tools to study the role of potential factors. My original contribution to the knowledge in this work was the development of transposon mutagenesis method that can now be used to study virulence genes of *Ureaplasma.* The overall hypothesis of this study was that induced serum resistance of *Ureaplasma* sensitive strains would result in identifiable phenotypic and genotypic alterations that could be correlated to pathogenicity of *Ureaplasma* in future studies. Monitoring and investigating induced serum-resistant strains using proteomics and genetic tools revealed significant changes in two candidate proteins coincident with serum resistance. The first was the EF-tu protein that had altered *pI* isoforms, which is most likely to be as a result of phosphorylation even. The observed change in this protein was consistent in all serum-resistant strains, which suggests a possible contribution in mechanism of serum resistance, possibly as a mediator for binding complement regulators at the cell surface (Figure 7.1). The second candidate protein was a novel 41 kDa protein that was uniquely expressed in all induced serum-resistant strains. Expression of this new protein in all resistant strains strongly indicated its involvement in mechanism(s) of serum resistance of *Ureaplasma*. The possible gene that encodes for this protein has putatively been identified as UUR10_0137 in the genome of *U. urealyticum* serovar 10 (ATCC 33699 strain); based on induced serum sensitivity coincident with

disruption in one transmutant strain using the new developed genetic method (transposon mutagenesis). Although the gene product of UUR10_0137 gene is not known (hypothetical protein), this protein is now identified and proposed to have a role in serum resistance of *Ureaplasma* (Figure 7.1). Whether this 41 kDa protein functions directly or indirectly as a regulator or inhibitor of complement activation needs to be determined. Confirmation of a gene that mediates complement resistance would dramatically increase our understanding of *Ureaplasma* pathogenicity and provide a target for future human studies with preterm birth and *Ureaplasma* infection.

7.3 Future work and recommendations

The findings presented in this study have answered some questions and provided an amount of new information that has added to our knowledge about mechanisms of serum resistance of *Ureaplasma*; however, many other questions are still unanswered and require further experiments. With regards to mechanisms underlying serum resistance of *Ureaplasma*, this study has paved the way towards identifying and understanding factors that could contribute to serum resistance.

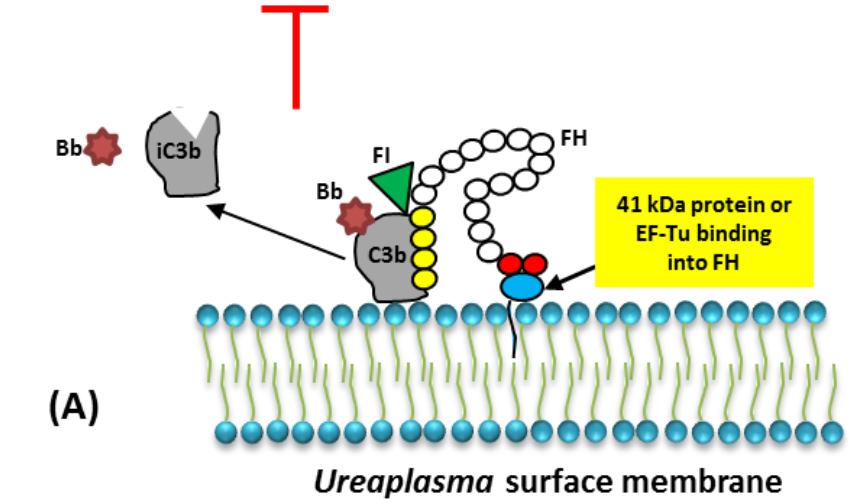
The next obvious investigations should determine if disruption of UUR_0137 blocks the ability to induce the 41 kDa protein and to deliver UUR_0137 under exogenous high-level expression promoter control to a serum sensitive strain to determine if serum sensitivity phenotype is changed. There is a general trend towards strains with low mass MBA to be very sensitive to non-immune human sera, therefore transposon mutagenesis delivery of a high mass MBA gene (with confirmation of expression by immunoblot) should be performed to confirm that this a single alteration determines overt serum sensitivity. The ability of EF-Tu (total and more acidic *pI* isoforms) to be expressed on the bacterial cell surface needs to be investigated; however, the small (<200 nm on

average) size of the bacteria presents a significant difficultly in investigating this by any method other than immuno-electron microscopy or other technically challenging methods. It is also possible to delivery phospho-mimetic EF-tu mutants by transposon mutagenesis if the pI is determined to be important to resistance or surface expression. The role of EF-tu surface expression on recruiting FH and C4bP can then be determined. Once the mechanisms have been clarified specifically characterised resistant and sensitive strains could be used to infect animal models to determine the importance of these alterations to pathogenicity of *Ureaplasma*.

Figure 7.1 Schematic illustration represents proposed mechanisms of complement evasion by *Ureaplasma.* Both *Ureaplasma* surface exposed immunogenic proteins, the EF-Tu and the novel 41 kDa protein (a possible product of the UUR10_0137 gene), may mediate recruitment and binding of the complement regulators FH **(A)** and/or C4BP **(B)** to *Ureaplasma* surface membrane. FH bound to *Ureaplasma* surface membrane functions as a cofactor for factor I mediating degradation of the C3 convertase (C3bBP), thus inhibiting the activation of complement via the alternative pathway **(A)**. Whereas, binding C4BP to the surface membrane mediates the cleavage of C4b and degradation of the C3 convertase (C4b2a) leading to inhibition of complement activation by both the classical and lectin pathways **(B)**. These proposed mechanisms might be ways used by *Ureaplasma* to avoid the complement-mediated serum killing.

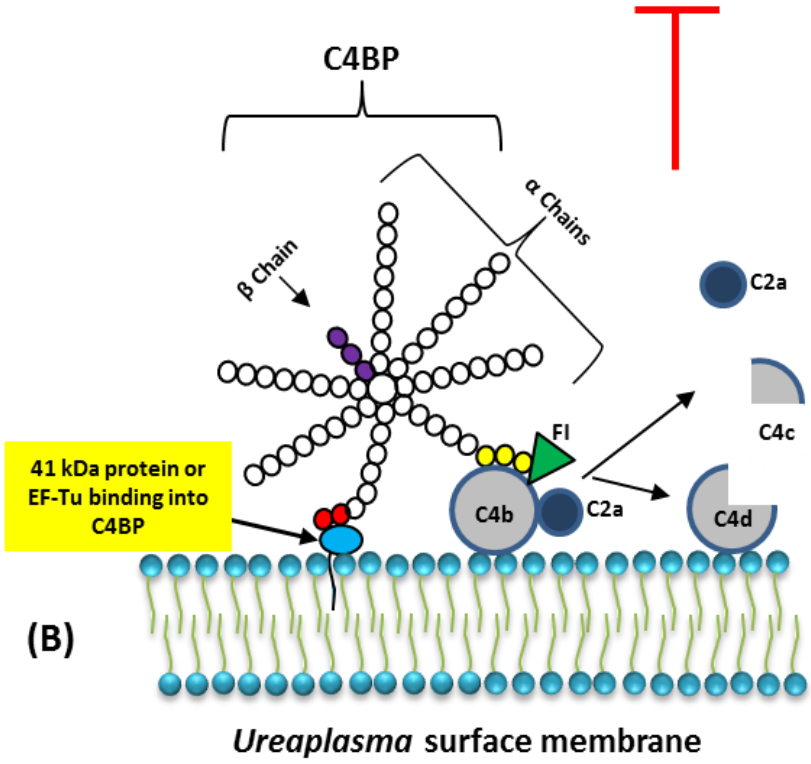
FH inhibits the activation of the alternative pathway by:

- Acting as a main cofactor for FI mediating the cleavage of C3b into inactive form, iC3b
- Acting as decay accelerating factor for C3bBP complex
- Prevents the assembly of the C3 convertase (C3bBP complex) by binding C3b



C4BP inhibits the activation of the classical and lectin pathways by:

- Binding C4b and preventing the assembly the of C3 convertase ,C4b2a complex.
- accelerating the decay of the C4b2a complex by separating C2a from the complex and preventing re-assembly of the C4b2a complex
- Acting as a cofactor for FI in cleaving C4b into two inactive forms (C4c and C4d)



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Appendixes

Appendixes

Appendix I: Sequence and detail of the pMT85 vector

Legend:
Inverted repeat 1 : 1-26
Inverted repeat 2: 3412-3438
Gentamicin resistance: 1036 - 2475 479 a.a.
bifunctional AAC/APH (AAC(6'): 6'-aminoglycoside N-acetyltransferase and APH(2'): 2''-aminoglycoside phosphotransferase [synthetic Mycoplasma genitalium JCVI-1.0]
Sequence ID: gi|166079093|gb|ABY79711.1|Length: 495 (100% match)
transposase 3538-4710 390 a.a.
IS256, transposase [Enterococcus faecalis V583]
Sequence ID: gi|29374776|ref|NP_813928.1|Length: 390 (100% match)
>pMT85gen

GATAAAGTCCGTATAATTGTGTAAAAACCCATAGCTTTGGACACACACTAGTACGGA
TCCACCCGCAATTACTGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTT
TATACTTCCGGCTCGTATATTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGG
AAACAGCTATGACCTTGATTACGGAATTCACGGCCGGGGGGGCCACCCCACCAATTG
ACGCGGCCGCAACTCTAGAGGATTCATCGGCCGTCGTTGCCTGGTTTCCGGCACCAG
AAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAGGCCGATACTGTCGTCG
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ATCCCATTACGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCGACGGGTTGTTACT
GCGCTCACATTTATATGCTGACTGAAAGCTGGCTACAGGAAGGCCAGACGCGAATTA
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ATTTTGATAAGTAGAAATGGTAAAAACATTGTATAGCATTTTACACAGGAGTCTGGA
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ATAATAAAATTATAGGATTTGCATATTGCTATACACTTTTAAGACCTGATGGAAAAA
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Appendix II: Additional Figures

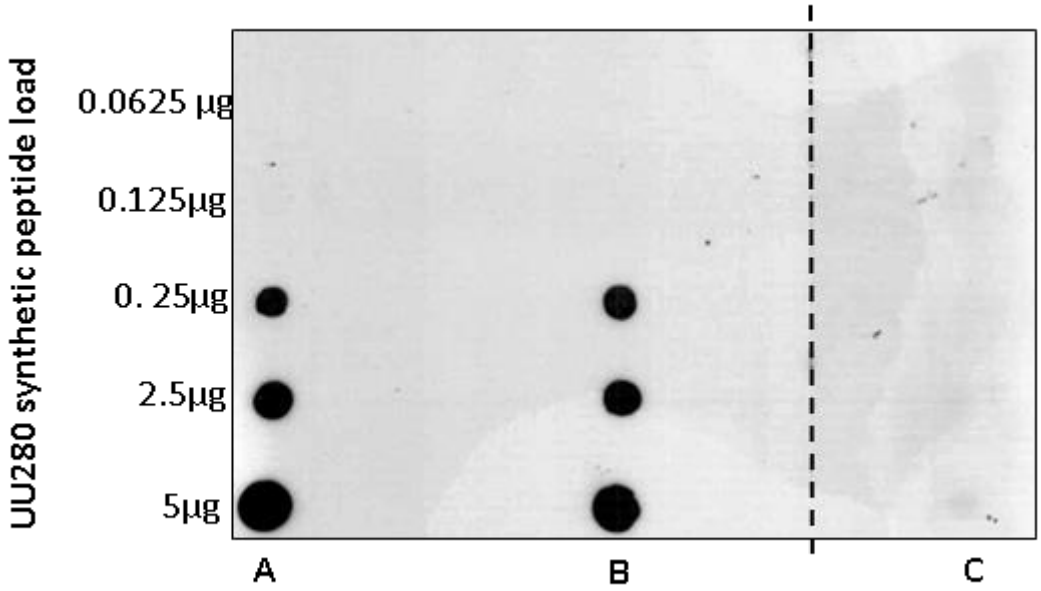

Figure II-1 Validation of anti-UU280 polyclonal antibodies for Western blot. Dot blot analysis shows reactivity of UU280 synthetic peptide (23 aa -322-343) with two anti-UU280 antisera (1:50) and seropositive serum. (A) and (B): anti-UU280 antisera detected strongly the UU280 peptide (in dilutions higher than 0. 25 µg). No reactivity was seen with seropositive serum (C) after a longer exposure.

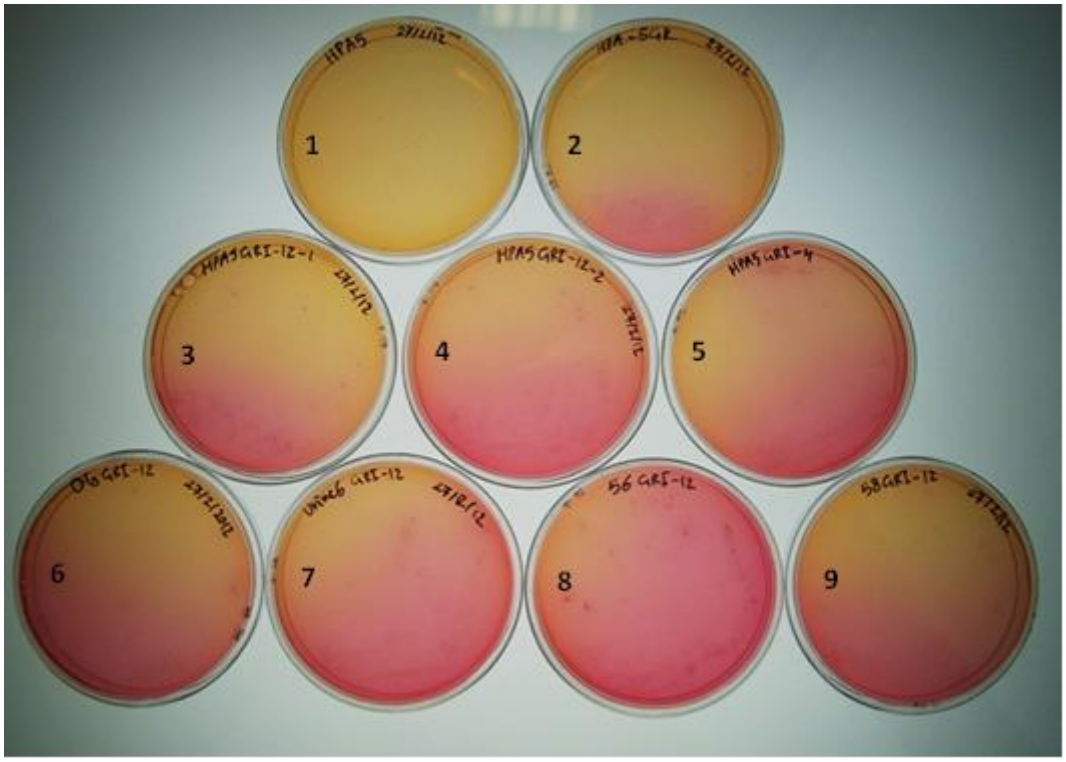

Figure II-2 Growth of *U. parvum* transformed strains on USM agar plates with 128 mg/L gentamicin. *Ureaplasma* growth is shown as change in colour of the medium from yellow to red around the tiny colonies. Plate 1 shows no growth of untransformed strain, HPA5 (negative control); plates 2-9 show growth of transmutant strains carrying the gentamicin resistant gene after successful transformation, HPA5 (SV3) (3-5) and others (O10(SV1), Urine6 (SV3), HPA56 (SV3), HPA58 (SV6)).

Appendix III: Talk and Poster Presentations from this work

Aboklaish, A.F., and **Spiller O. B.** Induced serum resistance of *U. parvum* occurs without loss of immunodominant epitopes. *Talk presentation*, **20th Congress of the international organization for Mycoplasmology,** June 2014, Blumenau, Brazil.

Aboklaish, A. F., Dordet-Frisoni, E., Citti C., Glass J. I., **Spiller O. B.** Random insertion and gene disruption via transposon mutagenesis of *Ureaplasma parvum* using a mini-transposon plasmid, *Poster presentation***, Society of General Microbiology Annual Conference,** April 2014. Liverpool, UK.

Aboklaish, A. F., Dordet-Frisoni, E., Citti C., Glass J. I., **Spiller O. B.** Transposon-mediated gene-delivery to *Ureaplasma parvum*: development of a powerful tool for tracking infection and for functional genomic studies. *Talk presentation,* **19th Congress of the International Organization for Mycoplasmology (IOM)**, July 2012, Toulouse, France.

Aboklaish, A.F., Brewis, I. A., and Spiller, O.B. Delivering genes to *Ureaplasma parvum*: a powerful genetic tool to study the molecular bases of pathogenicity, *Poster presentation,* **South West** & **South Wales Microbiology Forum,** Sep 2012. Swansea, UK

Aboklaish, A.F., Brewis, I. A., and Spiller, O.B. Immunodominant Epitopes for *Ureaplasma parvum*: identification and alteration following induced resistance under immunological pressure, *Talk presentation,* **I3 IRG Meeting**, Sep 2011, Cardiff, UK

Aboklaish, A.F., Kotecha, S., and Brad Spiller, O.B. The bactericidal activity of normal human serum against *Ureaplasma urealyticum, Poster and 5-min talk presentation*, **South West** & **South Wales Microbiology Forum,** Sep 2010. Cardiff University, UK

Appendix IV: Publications

Aboklaish, A. F., Dordet-Frisoni, E., Citti, C., Toleman, M. A., Glass, J. I. and Spiller, O. B. (2014). Random insertion and gene disruption via transposon mutagenesis of *Ureaplasma parvum* using a mini-transposon plasmid. *International Journal of Medical Microbiology* **304:**1218-1225.

Triantafilou, M., De Glanville, B., **Aboklaish, A. F.,** Spiller, O. B., Kotecha, S. and Triantafilou, K. (2013). Synergic activation of toll-like receptor (TLR) 2/6 and 9 in response to *Ureaplasma parvum* & *urealyticum* in human amniotic epithelial cells. *PLoS One* **8:**e61199.

The End