BIOCHEMICAL, IMMUNOLOGICAL AND GENETIC CHARACTERISATION OF THE MAJOR OUTER MEMBRANE PROTEIN FROM AN OVINE ABORTION STRAIN OF *CHLAMYDIA PSITTACI*

by

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A thesis submitted for the degree of Doctor of Philosophy
University of Edinburgh
December 1989

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Biochemical, Immunological and Genetic Characterisation of the Major Outer Membrane Protein from an Ovine Abortion strain of Chlamydia psittaci.

by Tan Tin Wee, Moredun Research Institute, Edinburgh.

Abstract

Ovine enzootic abortion (OEA) is an economically important disease of ewes caused by a type of *Chlamydia psittaci*. Vaccines have been available to control this disease for more than 30 years. In the past decade, vaccine efficacy has been poor despite efforts to improve the inactivated whole organism vaccine currently in use.

The initial aim of this project was to characterise the antigenic structure of OEA *C.psittaci* and to identify potentially immunoprotective antigens. Retrospective analyses of sera taken from ewes of different immune status were carried out using immunoblotting. A single 39 to 40 kDa antigen, thought to be the major outer membrane protein (MOMP) of OEA *C.psittaci*, was implicated as an important immunogen.

A series of biochemical analyses including gel electrophoresis, immunoblotting, peptide mapping, surface radio-iodination, detergent fractionation, *in vitro* oligomerisation and protein microsequencing, as well as electron microscopy, showed conclusively that this antigen was indeed the MOMP of OEA *C.psittaci*, the analogue of well-characterised MOMPs of other chlamydial strains. OEA MOMP was found to be a major surface-exposed component of the outer membrane fraction of the chlamydial elementary body (EB), and appeared as fine ultrastructural particles (3 to 4 nm in diameter) densely packed on the outermost surface of chlamydial EBs. It possessed epitopes cross-reactive with other chlamydial MOMPs and is a site of heterogeneity between ovine *C.psittaci* types. Its solubility was enhanced in the presence of reducing agent and MOMP monomers formed disulphide cross-linked oligomers under non-reducing conditions *in vitro*. It was found to possess an N-terminus identical to *C.trachomatis* MOMP indicating that cleavage of the signal sequence occurs at the same site to produce a mature protein of 39.5kDa.

A method was adapted to isolate MOMP by detergent extraction. An outer membrane fraction, highly enriched in MOMP, was prepared in sufficient quantities for a vaccination-challenge experiment. The results showed that both purified whole elementary bodies and the outer membrane preparation, given in a single dose, could protect ewes from infection and abortion. To test the hypothesis that MOMP was a protective component in these vaccine preparations, sufficient quantities of purified MOMP were needed. A recombinant DNA approach was taken.

Firstly, the MOMP gene from a vaccine strain, S26/3, was completely sequenced and extensively analysed in order to develop good strategies of expressing recombinant MOMP (rMOMP). This monocistronic gene contained putative tandem promoter and rho-independent terminator sequences flanking a 1,167 base-pair open reading frame. Comparison with other MOMP gene sequences revealed four highly variable domains interdigitating five constant domains.

Using the polymerase chain reaction and specially designed primers, a specific sequence corresponding to the mature MOMP was amplified, cloned into M13 mptac18 viral expression vector and subcloned into three plasmid vectors, pUC8, pRIT5 and pRIT2T, for expression. Several rMOMPs representing the complete mature MOMP and a truncated MOMP were successfully expressed in *Escherichia coli*. Finally, further analyses showed that these rMOMPs retained antigenic properties of MOMP and that large quantities could be obtained in a highly purified form. Such rMOMPs can now be tested for the presence of epitopes which can protect pregnant ewes from ovine enzootic abortion.

DECLARATION

The work presented in this thesis was part of a larger project concerned with the investigation of chlamydial abortion in sheep. Except where indicated, a full role has been played in the initiation, design and execution of the experiments described herein and in the interpretation of the results.

TAN TIN WEE*
December 1989

* Note that in Chinese names (as in Korean and Japanese names), surnames (eg. Tan) precede given names. Given names commonly (but not always eg. Hua Su) comprise two Chinese characters (Tin Wee) and are sometimes hyphenated (eg. San-Ping Wang or Cho-Chou Kuo) or combined as one word (Hongsheng Huang). In the west, Tan Tin Wee would be rendered T.W.Tan or Tin Wee Tan to avoid confusion. The name, of course, is the romanised equivalent of the Chinese pronunciation, depending on the dialect or more commonly, using the standard Hanyu Pinyin pronunciation. Thus, Tan Tin Wee (Hokkien dialect) would be rendered Chen Dingwei or D.-W. Chen using the standard pronunciation commonly encountered in papers authored by mainland Chinese scientists. Where a "Christian" name has been adopted (eg. Peter Y. Chou or Emil Y. Chi or Jeffrey J. Ma), the Chinese given name is usually retained.

My sincere thanks are due to the following:

The Animal Health Trust for providing me with the Wooldridge Farm Livestock Fellowship for the three and a quarter years of this project;

Dr Ian D. Aitken (Director, Moredun Research Institute), Dr David R. Snodgrass (Head, Microbiology Department) and Dr David Baird (Head, Biochemistry Department) for providing the research facilities;

Dr Ian Poxton for being my University supervisor and for reading the thesis;

Dr Maggie McClenaghan for the inheritance of chlamydial organisms for starting the work;

Dr Gareth Jones and Mr Ian Anderson for providing sheep serum samples, chlamydial strains culture facilities, and for collaborating in the vaccination-challenge work;

Mr Huang Hongsheng and Dr Dave Buxton for providing lymph and serum samples from their lymph node cannulation experiment;

Dr I. W. Smith for providing sera of zoonotically infected patients;

Mrs Susanna Dunbar for her expert assistance in molecular cloning techniques;

Mr Eddie W. Gray and Mrs Lesley Inglis for producing excellent electron micrographs;

Dr Richard Davies for hydroxyapatite column chromatography;

Ms Pat Dewar and Mr Andy Dawson for radio-iodinated antibodies;

Mr Douglas Thomson (University of Edinburgh - Veterinary Pathology) for the amphipathicity predictions;

Dr Andrew Lyall and Dr J.Collins (University of Edinburgh - Biocomputing Unit) for access to the programs CLUSTAL, PROSRCH and PATSRCH;

Dr Linda Fothergill-Gilmore for allowing me to have MOMP sequenced on her Applied Biosystems 470S protein sequencer as part of a Protein Microsequencing course;

Dr Mike Sharp, Dr Richard Davies, Dr Karen Stevenson, Dr Christina Wilson and Mr Neil Inglis for advice, comfort and cameraderie;

Mr Stuart Baxter for providing a Bluescribe clone carrying the MOMP gene;

Mr Frank Wright, Scottish Agricultural Statistics Service, for the CAI computation;

Ms Kay Hall and Mrs Kay Quinn for tissue culture support;

Mr Brian J. Easter and Mr Alan Inglis for superb photography;

Mr Mike McLauchlan and Mr John Spence for advice on using computers;

Staff of the Department of Clinical Studies for the welfare of experimental animals;

Service Room staff for the provision of sterile glassware and reagents;

Dr Julia Wong, Dr Roland Chong and Dr Joanna Oliver for commenting on chapters of the MS;

The Animal Diseases Research Association, the Animal Health Trust, the Society of General Microbiology, the Biochemical Society, the NATO Advanced Studies Institute, the organisers of the first Asia-Pacific Congress of Medical Virology and UnivEd Technologies Ltd for providing funds and rebates to attend courses and conferences; and

All the staff of the Moredun Research Institute for their advice and cheerful assistance.

I thank my family in Singapore for their concern and support, particularly my parents who have sacrificed much to fund my earlier education.

I am deeply indebted to Dr Alan J. Herring, my supervisor and mentor, for accepting me as a pre-doctoral student for this project, for guiding me from before the beginning of this project to the completion of this thesis, without whose advice, constant encouragement, stimulating and thought-provoking discussions, provision of facilities and material, this work could not be possible.

ABBREVIATIONS

AGE	agarose gel electrophoresis
ATP	adenosine triphosphate
BHK	baby hamster kidney
bp	base pairs
BSA	bovine serum albumin
CAPRA	cysteine and proline-rich anionic
CD	constant domain (protein sequence)
CELD	chick embryo lethal dose
CF	complement fixation/fixing
CLAC	cysteine, lysine and arginine-containing cationic
COMC	chlamydia outer membrane complex
cpm	counts per minute
CR	control region
CRP	cysteine-rich protein
CS	conserved sequence (nucleotide sequence)
d	day
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dpi	days post inoculation
dsDNA	double stranded DNA
DTH	delayed-type hypersensitivity
DTT	dithiothreitol
EB	elementary body
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
kb(p)	kilobase (pairs)
kDa	kilodalton
KDO	2-keto-3-deoxyoctonic acid
keV	kilo electron volt
GPIC	guinea pig inclusion conjunctivitis
HRP	horse-radish peroxidase
IPTG	isopropyl-β-D-thiogalactopyranoside
IDU	5-iodo-2'-deoxyuridine
Ig	immunoglobulin
IR	inverted repeat
LGV	lymphogranuloma venereum
LPS	lipopolysaccharide
mAb	monoclonal antibody
MCS	multiple cloning site
mCi	millicurie
2-ME	2-mercaptoethanol
mIF	microimmunofluorescence
Mn	meningopneumonitis
MOMP	major outer membrane protein
MoPn	mouse pneumonitis
M_r	relative molecular mass
MRI	Moredun Research Institute
mRNA	messenger ribonucleic acid
OD	optical density or absorbance
OEA	ovine enzootic abortion
OM	outer membrane

ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline with 0.5% Tween-20
PCR	polymerase chain reaction
pI	isoelectric point
RB	reticulate body
RE	restriction endonuclease
REA	restriction endonuclease analysis
rMOMP	recombinant MOMP
RNA	ribonucleic acid
SAPU	Scottish Antibody Production Unit
SDS	sodium dodecyl sulphate
SIG	signal sequence or signal peptide
SRP	signal recognition particle
SSC	standard sodium citrate
ssDNA	single stranded DNA
TCA	trichloroacetic acid
TCID	tissue culture infective dose
Tris	Tris(hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
TWAR	Taiwan acute respiratory
U	Units (of enzyme activity)
UWGCG	University of Wisconsin Genetics Computing Group
VD	variable domain (protein sequence)
VS	variable sequence (nucleotide sequence)
v/v	volume per volume
w/v	weight per volume
Xgal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

... he said unto him the third time, Lovest thou me?
And he said unto him, Lord thou knowest all things;
thou knowest that I love thee. Jesus saith unto him:
Feed my sheep...

John 21:17

But what things were gain to me those I count loss for Christ.
Yea doubtless, and I count all things but loss for the
excellency of the knowledge of Christ Jesus my Lord ...

Philippians 3:7,8

"He is no fool who gives what he cannot keep
to gain that which he cannot lose."

J.Elliot

CONTENTS

CHAPTER 1
GENERAL INTRODUCTION AND AIMS

ECONOMIC, VETERINARY AND MEDICAL IMPORTANCE OF CHLAMYDIAL ABORTION IN SHEEP

Ovine enzootic abortion (OEA), also known as enzootic abortion of ewes, is a major infectious cause of lamb loss in sheep farming (Aitken, 1983). The disease involves inflammation and necrosis of the placental tissues in pregnant ewes resulting in premature lambing, birth of weak lambs or abortion of the infected fetus. In 1950, Stamp and colleagues at the Moredun Research Institute (Edinburgh) identified the causative agent as *Chlamydia psittaci*, now known to be an obligate intracellular bacterium with a Gram-negative envelope. A series of studies testing various forms of experimental vaccines culminated in the development of an inactivated whole organism vaccine which has formed the basis of a commercial product (McEwen *et al*, 1951a, 1951b and 1955; Littlejohn *et al*, 1952; Hepple, 1952; McEwen and Foggie, 1954 and 1956; McEwen, 1954; Foggie, 1959). This vaccine consisted of a crude suspension of egg-cultured chlamydial organisms suitably inactivated and formulated as an emulsion in oil. Although not entirely eliminating the disease, the vaccine reduced the incidence of OEA to an economically acceptable level for many years up to the mid-1970s (Foggie, 1973). Vaccines against OEA represent the most successful and the only extensive application of immunoprophylaxis for any disease of chlamydial aetiology.

Resurgence of ovine enzootic abortion

In recent years, however, OEA has re-emerged to become a prevalent cause of infectious lamb loss. An epidemiological study carried out between 1975 and 1978 by Linklater and Dyson (1979) recorded an increase in outbreaks of OEA in south-east Scotland. Some outbreaks had even occurred in regularly vaccinated flocks. As no significant changes in sheep husbandry took place in the region at that time, the vaccine, which was virtually unchanged since the 1950s, was thought to be losing

its potency; or else, new strains of *C.psittaci* were emerging against which the vaccine offered little or no protection. Owing to the lack of suitable potency tests, the quality of the vaccine has been difficult to monitor. Heterologous challenge experiments in sheep (Aitken *et al*, 1981) and toxicity tests in mice (Russo *et al*, 1979; Johnson and Clarkson, 1986) have provided some indication that there might be antigenic variation in the field. In contrast, other studies on the virulence of several abortion isolates in mice (Anderson, 1986b) and comparisons of restriction endonuclease profiles of the chlamydial genome (McClenaghan *et al*, 1984) did not reveal any variations. Nevertheless, a second isolate (S26/3) obtained from a severe outbreak in 1979 was added to the vaccine which had contained only the original A22 vaccine strain.

A continuing problem

Despite the introduction of this bivalent vaccine, Veterinary Investigation Centres throughout England, Scotland and Wales have recorded a steady increase of ovine abortion due to chlamydial infection in recent years (Wilsmore and Dawson, 1986). Furthermore, OEA has also arisen in parts of the country previously free from the disease. Currently, about 20% of all fetopathies per year are attributed to chlamydial infection. In 1988, this represents 46% of all diagnosed fetopathies (Veterinary Investigation Data Analysis - VIDA). Such losses in sheep production due to OEA amount to as much as £10 million per year (I.D.Aitken, personal communication).

Within the European community, chlamydial infection in ruminants has been identified as a disease of emerging importance especially within member countries possessing large sheep and goat populations, including Britain, France, Germany, Greece and Italy (Aitken, 1986). Chlamydial abortion in sheep is also a serious problem in North America (Storz, 1971; Shewen, 1980). There have been reports of this disease in Australia but the situation is not as serious presumably because of differences in animal husbandry and environmental factors (Seaman, 1985).

Danger to pregnant women

The prevalence of *C.psittaci* in sheep also serves as a reservoir for zoonotic infection of humans. Such an infection is more acute compared to OEA and has life-threatening sequelae (see next section). In recent years, there has been an increasing awareness of the infectious potential of ovine abortion *C.psittaci* for pregnant women (Buxton, 1986; Helm *et al*, 1989). Although a serological survey did not find the risk of infection by *C.psittaci* in the farming populations of north-west England to be high (Hobson and Morgan-Capner, 1988), the lack of specificity for *C.psittaci* in the test reagents and the limited scope of the survey are causes for speculation as to the extent of infection among humans. Hence *C.psittaci* of ovine origin should not be perceived solely as an animal pathogen. Owing to its veterinary, economic and medical importance, this type of *C.psittaci* and the diseases it causes warrant continued surveillance and sustained research. In particular, modern techniques of molecular biology should be brought to bear on the problem in a concerted and systematic way.

THE NATURE OF THE DISEASE

OEA is a seasonal disease of ewes and is widespread throughout flocks of all breeds in the UK. It occurs predominantly in lowland flocks which are intensively managed at lambing time (Aitken, 1983). A high level of environmental contamination by infected ewes in lambing fields or pens is thought to be an important link in the infectious chain. Susceptible lambs and ewes acquire latent infections which emerge in the following lambing season (McEwen *et al*, 1951a; Foggie, 1954). Early chlamydial abortions within a flock can also be a source of infection for other pregnant ewes which become infected and abort in the same lambing season (Blewett *et al*, 1982). Excreted organisms and infected abortices contaminate the soil and are thought to be sources of infection through ingestion and inhalation.

Clinical symptoms and diagnosis

There are rarely any signs of impending abortion. Behavioural changes and occasional vulval discharge may be present shortly before abortion but these signs are seldom noticed under farming conditions. Ewes usually abort during late gestation but cases as early as 70 - 100 days gestation have been reported. The usual indicator is the discovery of dead lambs 2-3 weeks before lambing. Initial diagnosis is made on the appearance of the fetal membranes. Necrotic placental cotyledons, oedematous thickening of the inter-cotyledonary regions of the chorion and well-formed aborted fetuses are typical signs of chlamydial abortion. Confirmation of diagnosis is either by direct isolation of the organism in eggs or cell culture, or more conveniently, by the demonstration of chlamydial EBs in smears made from affected tissues (modified Ziehl-Neelsen stain - Stamp *et al*, 1950). Retrospective diagnosis by serology (complement fixation test - Stamp *et al*, 1952) can also be performed since post-abortion ewes tend to possess high antibody titres.

Treatment and prevention

C.psittaci is sensitive to antibiotics such as tylosin, erythromycin, tetracycline and oxytetracycline. Blanket treatment can only moderate the severity of the incidence and in any case is dependent on rapid and definitive diagnosis. Therapy may reduce the number of organisms shed but does not eliminate infection or reverse the damage already done to the placenta.

Vigilance is necessary to detect an outbreak early. Proper disposal of infected material, segregation of aborting ewes, and hygiene will often prevent spread of the infection to other susceptible animals. Care can be taken to select replacement stock from sources with no known history of OEA but this does not guarantee infection-free animals. Although previous exposure to chlamydiae can be deduced serologically, there are no tests that can distinguish infected animals from animals

which are vaccinated and protected. Post-abortion ewes also possess chlamydia-specific antibodies and are protected against abortion in subsequent pregnancies (Stamp *et al*, 1950). They may, however, continue to excrete infectious organism in vaginal discharges or in the faeces, thereby contaminating the environment.

The difficulty in identifying the immunological status of sheep - whether naive and susceptible, vaccinated and protected, infected and about to abort, or convalescent - justifies the use of extensive vaccination as a long-term policy for disease control; this had been practised from the 1950s to the 1970s with success. In the past decade, however, reduction in vaccine efficacy has been cause for concern. Attempts have been and are being made to improve the quality of the current vaccine. Complicating factors include the possible emergence of vaccine-resistant or more virulent OEA strains, the lack of serotyping tests to monitor the epidemiological aspects of the disease, and a limited understanding of the pathogenetic events and the basis of vaccine-induced immunity.

Pathology and pathogenesis

The anatomy of the ovine placenta with particular reference to ovine abortion has been described in detail by Studdert (1968). Chlamydiae colonise the chorionic epithelium at a particular stage of gestation; from approximately the 70th day of pregnancy, they can be detected in the placenta within trophoblastic cells. The primary lesions are localised at the chorionic epithelium, lining the sites of haematomas that are normally found in the ovine placenta from 60 days gestation. Presumably, infectious elementary bodies (EBs) of chlamydiae are released from site(s) of "latency", migrate via a haematogenous route, congregate at the haematomas and infect the apposing trophoblastic cells (Figure 1.1ABC).

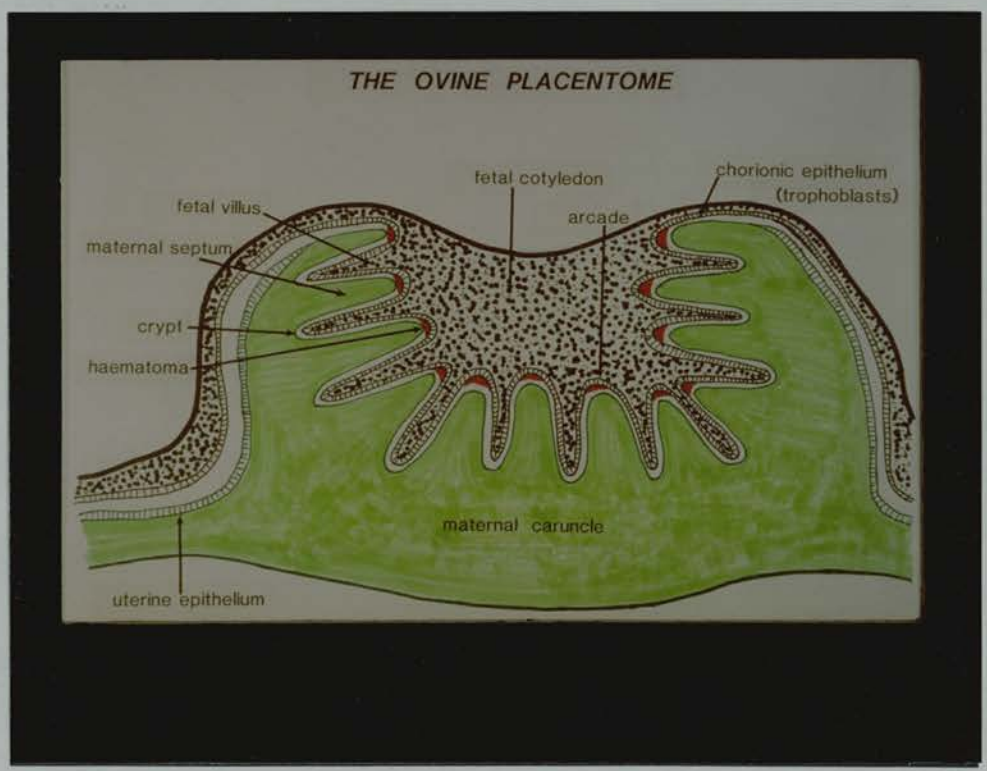


Figure 1.1A. Schematic section of an ovine placentome.(Courtesy of Dr Gareth E. Jones).

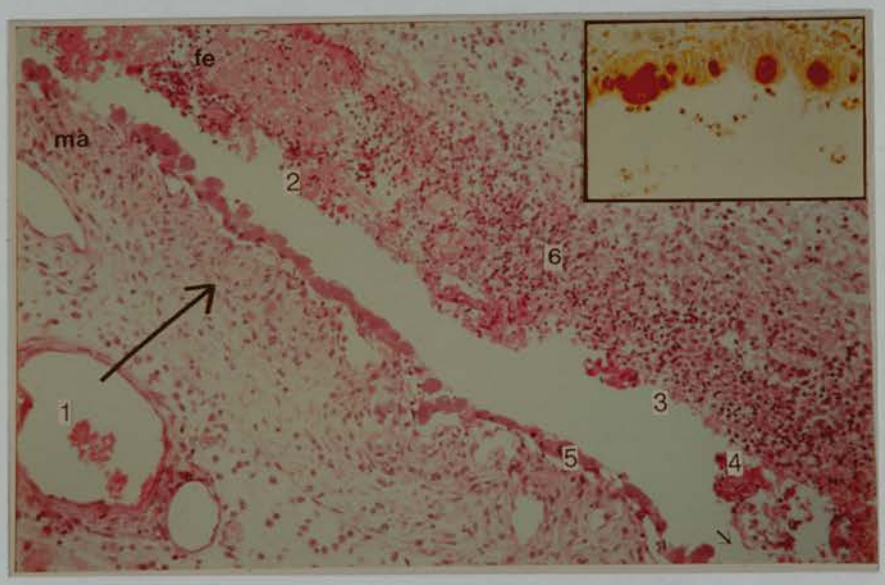


Figure 1.1B. Section of an infected ovine placentome (haematoxylin and eosin stain; x180). 1. Infectious elementary bodies (EBs) arrive at the placentome via the maternal circulation possibly from an initial tonsillar infection. 2. EBs migrate (long arrow) across the placental tissues to infect the trophoblastic epithelial cells. 3. Loss of infected cells and progressive necrosis. 4. Release of cellular debris and more infectious EBs. 5. Infection of apposing uterine epithelium and lateral spread to the inter-cotyledonary regions (short arrow). 6. Local infiltration by fetal and maternal macrophages, neutrophils and other lymphocytes. Maternal side (ma). Fetal side (fe). Inset. Infected trophoblastic epithelium immunostained with anti-chlamydial antibodies (Finlayson *et al*, 1985) showing large inclusion bodies full of chlamydial organisms (x250); counterstained with haematoxylin. (Courtesy of Dr David Buxton.)

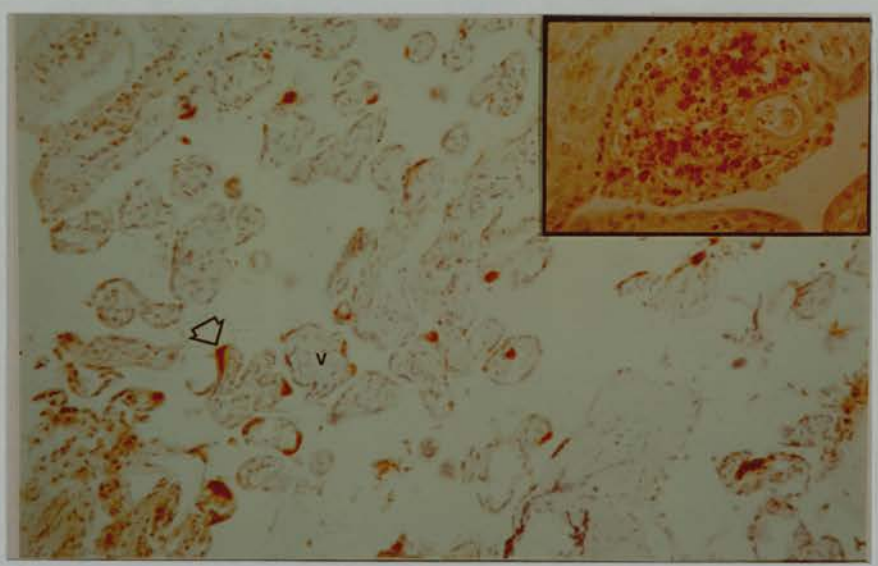
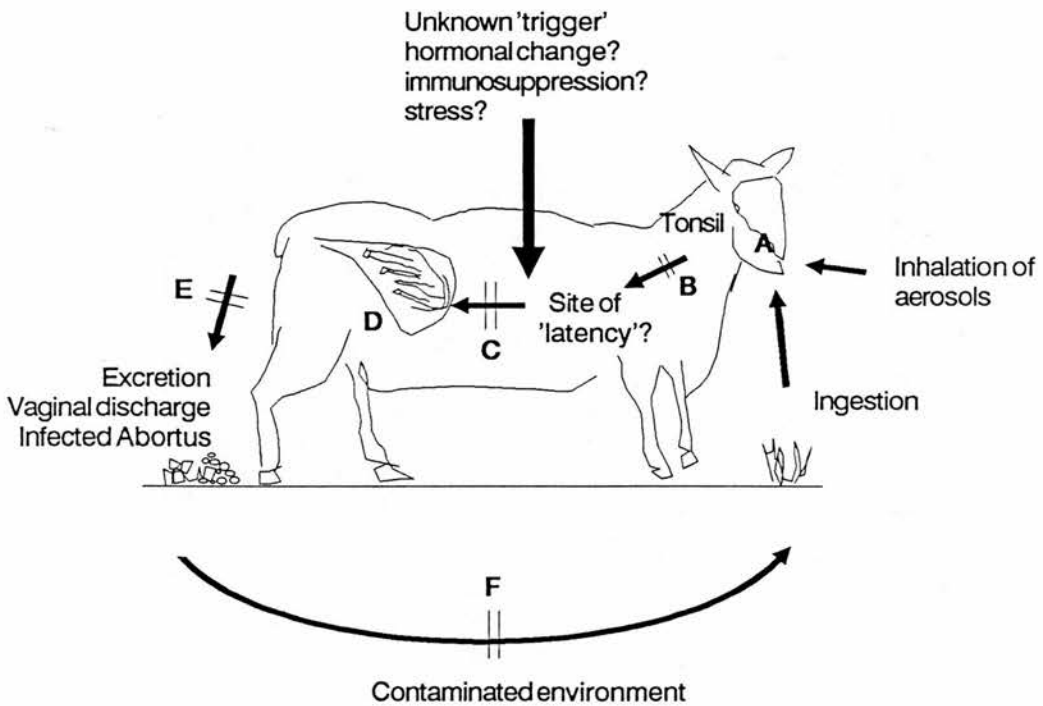


Figure 1.1C. Section of infected human chorionic villi (v) immunostained with anti-chlamydial antibodies. Infected trophoblastic epithelia are laden with stained chlamydial organisms within large inclusion bodies (arrowed) (x180). Inset. Infected epithelial cells and cell debris (x250) immunostained and counterstained with haematoxylin. (Courtesy of Dr David Buxton.)

After cycles of infection, the chorionic epithelial cells appear sloughed. Damage to the basement membrane occurs. Fetal inflammatory cells, predominantly the polymorphonuclear neutrophils and some mononuclear cells, infiltrate the subchorionic mesoderm. The lacunae fill with cell debris, lysed red blood cells, infectious elementary bodies and considerable amounts of inflammatory exudate as the infection spreads laterally into the inter-cotyledonary chorion (Figure 1.1B).

Gross pathology include extensive necrosis of the cotyledons, oedematous and haemorrhagic fetal membranes with large amounts of thick, brown, bloody, mucus-containing exudate adhering to the areas of placentitis on the chorionic surface. Fetuses aborted before 120 days of gestation appear to be retained for several days after death with consequent autolysis. Fetuses from later abortions may show splenic and lymph node enlargement, lesions of the liver, lung, kidney and heart muscle with focal accumulations of hyperplastic reticulo-endothelial cells. Infectious EBs, having penetrated the placental barrier, enter the fetal circulation via the chorionic blood vessels to infect these tissues. Nevertheless, fetal lesions in general are relatively minor and secondary to the primary lesions of the placenta.

Under natural conditions, ewes are believed to be infected with *C.psittaci* either as newborn lambs or as mature ewes during previous or current enzootics of abortion (McEwen *et al*, 1951a; Blewett *et al*, 1982; Wilsmore *et al*, 1984b). A possible portal of entry appears to be the nasal mucosa and the tonsils (Jones and Anderson, 1988), presumably through inhalation of chlamydia-laden aerosols or through ingestion. These routes may account for the observation that outbreaks of the disease are rare in highland extensive sheep farming but common in the lowlands where farming is more intensive. Studies of experimental infection indicate that the agent can remain in the infected lamb or sheep without overt symptoms for various durations until colonisation of the placenta occurs from some



A vaccination may elicit mucosal immunity
B vaccination may inhibit migration to site of 'latency'
C vaccination may inhibit haematogenous migration of EBs to the placenta
D vaccination may induce immunity at the placenta
E proper disposal of infected abortus
F mode of husbandry

Figure 1.2. The infectious cycle in ovine enzootic abortion.

unknown site(s) of "latency" (McEwen *et al*, 1951a; Foggie, 1954; Munro and Hunter, 1981; Blewett *et al*, 1982; Wilsmore *et al*, 1984b).

It has been proposed by Studdert (1968) that release of infectious agent from the site of "latency" may be a periodic or a fortuitous event. The recent work of Buxton *et al* (in press) has demonstrated that no sign of placental pathology could be detected in experimental infection prior to 90 days gestation whereas infection after 60 days of gestation resulted in direct colonisation of the placenta. One interpretation is that periodically released organisms may reach the placenta but they succeed in colonising the trophoblastic cells only after about 60 to 90 days gestation when the target cells become ready for infection. Alternatively, some physiological signal coupled to the progress of pregnancy might trigger the emergence of chlamydiae from its quiescent state to infect the target cells.

Zoonotic infection in women

It had been suspected for many years that ovine *C.psittaci* may be responsible for a number of spontaneous abortions and perinatal deaths but evidence was largely circumstantial (Roberts *et al*, 1967; Beer *et al*, 1982). Since then, it has been established with clinical, serological, histopathological, ultrastructural and molecular evidence that OEA strains of *C.psittaci* have a strong predilection for the human placenta and have caused abortion and severe illness in a number of women in their second or third trimester of pregnancy (McKinlay *et al*, 1985; Wong *et al*, 1985; Johnson *et al*, 1985; Buxton, 1986; Herring *et al*, 1987; Helm *et al*, 1989). Initial symptoms of the zoonotic infection are influenza-like with fever, headache and malaise followed within the next few days by spontaneous contractions and abortion. Following abortion, patients show symptoms of profound thrombocytopenia with disseminated intravascular coagulation (DIC), renal and hepatic dysfunction with myocardial involvement.

Reminiscent of the pathology in sheep, degenerative loss of trophoblast cells in the chorionic villi and inflammation of the mesoderm are prominent histological characteristics with chlamydia-laden trophoblastic cells frequently demonstrable. The tissue damage and toxic products of the chlamydiae may then lead to insufficiency of maternal-fetal transfer, subsequent fetal death and abortion.

In contrast to the placentation in ewes, the human uterine epithelium, the underlying tissue layers and the endothelium are eroded so that the chorionic epithelium is in direct contact with the maternal circulation (haemochorial placentation). Such a placentation facilitates the spread of infectious chlamydiae, chlamydial endotoxin, cellular debris and thromboplastic factors into the maternal circulation which may account for the severity of the sequelae mentioned above.

Concurrent infection with other zoonotic agents associated with sheep farming e.g. *Coxiella burnetii* has also been recorded and may result in abortion in the first trimester (McGivern *et al*, 1988). In suspected cases, early delivery of the infant before serious infection occurs may reduce the severity of post-partal illness, save the infant, and assist a more rapid recovery of the mother (Beer *et al*, 1982).

THE ORGANISM

Taxonomy

Chlamydia psittaci is assigned to the distinct order *Chlamydiales*, which consists of one family, *Chlamydiaceae*, with a single genus, *Chlamydia* (Moulder, 1988 - review). *C.psittaci* and the only other currently recognised species in the genus, *C.trachomatis*, are obligate intracellular prokaryotic parasites of eukaryotes. They share many characteristics with Gram-negative bacteria but possess a unique biphasic developmental cycle which alternates between two forms, the elementary body (EB) and the reticulate body (RB). Recently,

the TWAR strain, a human respiratory pathogen, which was originally classified as a type of *C.psittaci*, has been proposed as a third species to be called *C.pneumoniae* (Grayston *et al*, 1989).

Unlike *C.trachomatis* which consists of 15 human serotypes and one murine strain, *C.psittaci* comprises a large and diverse group of strains that have broad host range and pathogenic potential (Storz, 1971). *C.psittaci* hosts include invertebrates such as molluscs and arthropods, ectothermic vertebrates, wild and domesticated birds, and mammals including man. Diseases associated with *C.psittaci* are as varied, with symptoms depending on factors such as virulence of the strain, host species, age and sex of the animal, environment, farm management, ecology and physiology. Among the ruminants, in particular, the sheep, intestinal infection, diarrhoea, pneumonia, abortion, urogenital infection, mastitis, polyarthritis, encephalomyelitis, hepatitis and conjunctivitis are typical of chlamydial infection (Storz, 1971; Shewan, 1980).

Criteria used to classify *C.psittaci* strains include plaque reduction assay (Schachter *et al*, 1974), inclusion morphology, response to different treatments in cell culture, (Spears and Storz, 1979; Anderson and Baxter, 1986), amino acid requirements (Allan and Pearce, 1983), microimmunofluorescence (mIF) assay (Eb and Orfila, 1982), indirect mIF assay (Perez-Martinez and Storz, 1985), tests using monoclonal antibodies (Toyofuku *et al*, 1986; Delong and Magee, 1986; Fukushi *et al*, 1987; Andersen and van Deusen, 1988), immunoblotting (Fukushi and Hirai, 1988), restriction endonuclease profiles and DNA hybridisation (McClenaghan *et al*, 1984; McClenaghan *et al*, 1986; Herring *et al*, 1986; Timms *et al*, 1988; Fukushi and Hirai, 1989). The difficulty in classifying such a highly heterogeneous species is evident. For instance, up to 9 immunotypes of mammalian strains (Perez-Martinez and Storz, 1985) and up to 7 avian strains (Toyofuku *et al*, 1986) have been proposed; in addition, there are other types from other studies mentioned above which may not belong to these

categories. *C.psittaci* isolated from ruminants have been grouped into immunotype 1 (abortion and some intestinal isolates), immunotype 2 (polyarthritis, conjunctivitis, encephalitis and enteritis isolates) and immunotypes 3 and 9 (strains occurring in bovine and ovine intestinal flora) according to the scheme of Perez-Martinez and Storz (1985).

Life cycle

The EB is the extracellular infectious form. It is metabolically inactive and resistant to osmotic lysis. Since its Gram-negative envelope appears to lack peptidoglycan, it is thought that the structural rigidity of the EB is provided by cysteine-rich outer membrane proteins, highly cross-linked by disulphide bonds (Newhall and Jones, 1983; Bavoil *et al*, 1984). Surface determinants are also thought to assist the attachment and entry of the infectious EB into eukaryotic cells (Wenman and Meuser, 1986; Hackstadt, 1986a). However, the mechanism by which chlamydial EBs enter host cells is not clear. Various modes of entry into host cells have been proposed and these were summarised by Wyrick *et al* (1989) recently. They include phagocytosis that is parasite-directed, microfilament-dependent or microfilament-independent, and pinocytosis via receptor-mediated clathrin-coated pits or non-coated smooth pits. It is possible that chlamydiae can exploit different mechanisms of entry into target cells (Prain and Pearce, 1989) or that the differing modes of entry reported in the literature may be due to differences in the type and state of host cells used, in the multiplicity of infection, in the technique of visualising morphology and ultrastructure, and in the technique of enhancing infectivity such as centrifugation.

Within the cytoplasm, the organism resides in a topologically distinct vesicle and is able to escape destruction by inhibiting fusion of the vesicle with lysosomes (Friis, 1972). In this protected environment, the EB transforms into the metabolically active and non-infectious RB which multiplies by binary fission.

Although fragments of various metabolic pathways are present in chlamydiae, their metabolic capabilities are limited (Hatch, 1988 - review). They cannot generate net ATP and possess an ATP-uptake mechanism instead (Hatch *et al*, 1982); they have been aptly called "energy parasites" by Moulder. Within the cell, these organisms rely on the host cell for basic metabolites and other factors to fuel the synthesis of chlamydial products.

As the RBs proliferate, the vesicle expands, sometimes fusing with other chlamydiae-laden vesicles to form a prominent inclusion within the host cell. The RBs mature and and condense into EBs which are eventually released into the surrounding medium to infect other cells, thereby completing the cycle (Ward, 1988 - review).

Morphology and ultrastructure

Elementary bodies of chlamydiae are coccoid in shape (except TWAR strains which are pear-shaped) with a size close to the limit of the light microscope. Each EB has a dense, eccentric nucleus surrounded by cytoplasm that is composed of ribosomes and moderately dense amorphous material. Scanning electron micrographs show the presence of surface projections hexagonally arranged with a centre-to-centre spacing of 35-50 nm on the surface of the EBs (Matsumoto and Higashi, 1975; Gregory *et al*, 1979). Each projection protrudes from a hole in the middle of a 30nm wide "flower", so-called because of its appearance under freeze-deep-etching (Matsumoto, 1982a). Each flower, also called a rosette, is composed of nine regularly arranged subunits. Measurements from negatively stained membrane preparations reveal each rosette to be 19-20nm in diameter with each subunit 3-5 nm wide surrounding the 10-12 nm hole from which the projection emerges (Matsumoto, 1973). The projections and the rosettes are localised in one patch per EB (Matsumoto, 1982c). Projections are easily broken off the rosettes; isolated projections (6nm diameter; 45nm long) appear like hollow nail-shaped structures, each with a pointed end (Matsumoto, 1988).

Reticulate bodies are larger, more pleomorphic and flexible, ranging in size from 300 to 800 nm in diameter. RBs also possess projections identical to those of EBs. These projections originate from the chlamydial cytoplasm, extend beyond the cell envelope through a rosette in the cell wall and appear to pierce the inclusion membrane, suggesting a role in facilitating intercellular traffic (Matsumoto, 1981 and 1982b).

Chlamydial outer membrane preparations possess on the inner surface a hexagonally arrayed, macromolecular structure composed of particles about 10nm in diameter (Manire, 1966; Matsumoto and Manire, 1970). The periodicity of these hexagonal units was determined to be 16.7nm (Matsumoto, 1979). A three-dimensional model of the hexagonal units has been proposed by Chang *et al* (1982). Protruding from the outer surface of the outer membrane are fine particles 5-6 nm wide with a centre-to-centre spacing of 7 - 8nm. These particles seem to be a major structural component of the outermost surface of the EB cell wall (Matsumoto, 1979; 1982c; 1988).

ANTIGENIC STRUCTURE OF CHLAMYDIAE

Historically, chlamydiae have been associated with viruses because many of their characteristics are virus-like. EBs are typically 200-400 nm in size, close to that of large pox viruses. In comparison to typical bacterial genome sizes of 4000 kbp (*Escherichia coli*), the chlamydial genome is one of the smallest estimated at approximately 1000 kbp, second only to the mycoplasmas (750 kbp). The small genome is characteristic of prokaryotes that live in or on eukaryotic cells such as *Coxiella* (1560 kbp), *Rickettsia* (1650 kbp) and *Neisseria* (1950 kbp) (Moulder, 1988). It corresponds to the capacity to encode 400 to 600 proteins (Stephens, 1988). Typically, more than 100 bands consisting of resolved proteins and lipopolysaccharide may be visualised by SDS-PAGE (Salari and Ward, 1981; Caldwell *et al*, 1981; Hatch *et al*, 1981) or by two-dimensional electrophoretic systems (Batteiger *et al*, 1985). Of these components only a limited number are known to be antigenic. For example, about

16 antigens of *C.psittaci* (Cal10 meningopneumonitis strain) were described by Caldwell *et al* (1975) using two-dimensional immunoelectrophoresis; about 15 bands could be detected by immunoblotting with antisera raised against *C.psittaci* guinea pig inclusion conjunctivitis (GPIC) strain (Batteiger and Rank, 1987); at least 14 bands of *C.trachomatis* were identified with sera from infected human patients (Newhall *et al*, 1982); more than three prominent antigens of *C.psittaci* ovine enzootic strains have been demonstrated by McClenaghan *et al* (1986) in preliminary immunoblotting experiments, including several high molecular mass (M_r) antigens (78-90 kDa).

Several chlamydial components appear to be common to both *C.psittaci* and *C.trachomatis*: chlamydial lipopolysaccharide (Nurminen *et al*, 1983; Caldwell and Hitchcock, 1984), major outer membrane protein (Hatch *et al*, 1981; Caldwell *et al*, 1981), 60kDa and 12kDa cysteine-rich proteins (CRPs) (Newhall *et al*, 1982; Newhall and Jones, 1983), 57kDa protein (Morrison *et al*, 1989; Menozzi *et al*, 1989), 45kDa protein (Morrison *et al*, 1989), the 30-32kDa and 17-19kDa adhesins (Hackstadt, 1986a and 1986b; Wenman *et al*, 1986). Most of these are antigenic and although they are present in both species, they may possess species-, subspecies- or type-specific epitopes (see below). Some of these and other antigens, including a 155kDa *C.trachomatis* species-specific protein, a 30-32kDa *C.trachomatis* type-specific protein and a glycolipid, have been described in a recent review by Newhall (1988). Data pertaining to the more important antigens are detailed below. Recent information will also be dealt with in the discussion sections of the following chapters.

Major outer membrane protein

The most striking component in the SDS-PAGE profiles of EBs of all chlamydial strains is a protein of some 40 kDa. This is the major outer membrane protein (MOMP) which comprises 60% of the protein mass in the outer membrane (Caldwell *et al*, 1981; Salari and Ward, 1981; Hatch *et al*, 1981) and is by far the best characterised chlamydial antigen. This surface-exposed

protein is vital for the structural integrity of the chlamydial outer membrane. Unlike matrix proteins of Gram-negative bacteria which can be solubilised without destroying the basic morphology of the organism, detergent extraction of MOMP from the outer membrane results in the total loss of structure. It is an acidic protein (Batteiger *et al*, 1985) containing several cysteine residues involved in inter-molecular disulphide cross-linkages (see next section). These cross-linkages are critical for the function of MOMP as a porin and for the role of MOMP in the developmental cycle (Newhall and Jones, 1983; Bavoil *et al*, 1984; Hatch *et al*, 1984; Hackstadt *et al*, 1985; Hatch *et al*, 1986; Newhall, 1987). MOMP exhibits variation in M_r among chlamydial strains (Salari and Ward, 1981; Hatch *et al*, 1981; Caldwell *et al*, 1981; Caldwell and Schachter, 1982; Newhall *et al*, 1982; Vitu and Russo, 1984; Fukushi and Hirai, 1988; Herring *et al*, in preparation). These variations are localised on four variable domains in the deduced amino acid sequence of MOMPs (Stephens *et al*, 1986 and 1987; Yuan *et al*, 1989; Zhang *et al*, 1989a); these domains are also the location of antigenic sites.

Antigenically, MOMP is very complex and possesses a hierarchy of epitopes bearing genus-, species-, subspecies- and serotype-specificity (Caldwell *et al*, 1981, Caldwell and Schachter, 1982; Stephens *et al*, 1982; Newhall *et al*, 1986; Batteiger *et al*, 1986). Structural studies of MOMPs from various serovars of *C.trachomatis* and *C.psittaci* by peptide mapping suggest that each MOMP represents a serological mosaic of antigenic determinants located on conserved and variable segments (Caldwell and Judd, 1982; Ma *et al*, 1987). Molecular analyses of the MOMP gene of *C.trachomatis* serotypes have essentially confirmed these findings and have located such epitopes to specific variable domains of the MOMP (Baehr *et al*, 1988; Stephens *et al*, 1988b; Conlan *et al*, 1988).

Some epitopes on MOMP are important for neutralisation of *C.trachomatis* infectivity since anti-MOMP IgG cross-linking of MOMP at the EB surface demonstrably interfered with the

infectious process at a stage after EB internalisation (Caldwell and Perry, 1982). Such *in vitro* neutralisation of infectivity has been shown with an anti-MOMP species-specific monoclonal antibody (mAb) capable of neutralising more than one serovar of *C.trachomatis* (Peeling *et al*, 1984). Neutralisation of infectivity has also been demonstrated by Zhang *et al* (1987a and 1989b) using mAbs specific for immunoaccessible epitopes on MOMP. Recently, Su *et al* (1988) have demonstrated that for *C.trachomatis* serovar B, trypsin cleavage of specific variable domains in the MOMP correlates with inhibition of infectivity. Although MOMP is an important target for the neutralisation of chlamydial infectivity, its value as an immunoprophylactic has only been partially demonstrated (Taylor *et al*, 1988).

The usefulness of MOMP in serotyping and diagnosis is clear from the literature. The antigenic and sequence diversity of MOMPs reflect the diversity among serovars of *C.trachomatis* (Batteiger *et al*, 1986; Yuan *et al*, 1989); whether the analogous situation exists with *C.psittaci* has only been recently studied (Fukushi and Hirai, 1988; Zhang *et al*, 1989a).

Cysteine-rich outer membrane proteins

C.psittaci and *C.trachomatis* possess outer membrane proteins with relatively high cysteine content; in addition to MOMP, they are polypeptides of M_r around 60 kDa and 12 kDa (Newhall and Jones, 1983; Hatch *et al*, 1984; Newhall, 1988 - review). The 60kDa protein exists as a single polypeptide in trachoma biovars of *C.trachomatis* but is found as a doublet in the lymphogranuloma venereum (LGV) biovar of *C.trachomatis* (Batteiger *et al*, 1985) and in *C.psittaci* (Hatch *et al*, 1984). These cysteine-rich proteins (CRPs) are extensively cross-linked by disulphide bonds to form a supra-macromolecular network in the outer membrane that confers structural rigidity and osmotic stability to the EB (Newhall and Jones, 1983; Bavoil *et al*, 1984; Hatch *et al*, 1984 and 1986; Hackstadt *et al*, 1985;

Newhall, 1987). The reduction or oxidation of this structural network is thought to be involved in the respective transitions of EB to RB and RB to EB during the developmental cycle of chlamydiae.

The 60kDa CRPs are highly antigenic and possess both species- and genus-specific epitopes (Newhall *et al*, 1982; Newhall and Basinski, 1986; Newhall, 1988). The N-terminus of the *C.trachomatis* 60kDa protein has been sequenced (Newhall and Basinski, 1986) and recently, the gene has been cloned and sequenced, revealing the presence of 24 cysteine residues (Clarke and Lambden, 1988; Clarke *et al*, 1988; Allen and Stephens, 1989). DNA hybridisation of a *C.trachomatis* 60kDa-CRP gene probe with *C.psittaci* DNA indicated the presence of genus-conserved regions in the gene (Clarke *et al*, 1988). The low molecular mass (12kDa) CRP is also antigenic and possesses biovar- and species-specific epitopes (Zhang *et al*, 1987b; Newhall, 1988). The role of these CRPs in the developmental cycle suggests that antibody cross-linking of these CRPs may interrupt the infectious cycle; the CRPs are therefore potential vaccine candidates. However, protection against infectivity has not been reported with antibodies directed to these antigens.

Adhesins

Investigations of the endocytic uptake of chlamydiae into host cells suggest specific binding of EBs with host receptor molecules (Byrne, 1976; Ward and Murray, 1984). The chlamydial binding molecules or adhesins are likely to be cell wall components (Levy and Moulder, 1982) and proteinaceous (Byrne, 1976). Using radio-iodinated host cell membranes, Wenman and Meuser (1986) have detected chlamydial adhesins, a 30-31kDa doublet and an 18kDa protein, in *C.trachomatis* by ligand blotting. These adhesins were antigenic in rabbits and specific antibodies were able to inhibit chlamydia-host cell association. At the same time, Hackstadt (1986a) also detected similar adhesins for both *C.trachomatis* and *C.psittaci*. In the latter species, only an 18kDa adhesin was found. Further

studies by Wenman and coworkers (1986) revealed a single 30kDa adhesin in addition to a slightly smaller 16kDa adhesin for the meningopneumonitis strain of *C.psittaci*. Antibody neutralisation of infectivity against homologous or heterologous strains have been demonstrated. In other studies, immunoblot profiles commonly show antigens in the 30kDa and 18kDa region for the GPIC agent (Batteiger and Rank, 1987) but whether these antigens recognised in infection are the same as the adhesins has not been shown. The 18kDa adhesin has been cloned (Kaul *et al*, 1987) and this should facilitate further analysis of the exact role of the putative adhesins. Both adhesins were recently shown to be synthesised late in the developmental cycle in relatively small amounts (Newhall, 1987).

Lipopolysaccharide

All members of the genus *Chlamydia* share a common, heat-stable, protease-resistant, periodate-sensitive antigen that is the basis of the complement fixation test for chlamydial infection (Schachter and Caldwell, 1980; Newhall, 1988 - reviews). It is a glycolipid containing a 2-keto-deoxy-sugar as the immunodominant moiety (Dhir *et al*, 1971; 1972). Evidence to show that this carbohydrate antigen is indeed lipopolysaccharide (LPS) include its surface location on the outer leaflet of the outer membrane, its physico-chemical properties during extraction, its mobility and staining in SDS-PAGE gels, detailed analysis of chemical composition, its serological cross-reaction with the LPS of *Salmonella* Re mutants and *Acinetobacter calcoaceticus*, and its endotoxin-like activity in the *Limulus* amoebocyte lysate assay (Nurminen *et al*, 1983, 1984 and 1985; Caldwell and Hitchcock, 1984). No mAb reacting with LPS has been found to possess neutralising activity.

Chlamydial LPS (M_r 3-4 kDa) contains typical bacterial LPS constituents, namely, D-glucosamine, long chain fatty acids, 2-keto-3-deoxyoctonic acid (3-deoxy-D-*manno*-2-octulosonic acid; KDO) and phosphates. Some of the fatty acids are novel 3-hydroxy fatty acids with 18 to 22 carbon atoms (Nurminen *et*

al, 1985; Brade *et al*, 1986). Chlamydial LPS is structurally divided into the lipid A moiety (which is made up of the amino sugars and fatty acids) and the oligosaccharide core component (formed from KDO). Lipid A intercalates with the outer leaflet of the outer membrane while the KDO core is directed towards the exterior. Chlamydial LPS lacks other sugar residues in the oligosaccharide core and the O-antigen structure of "smooth" LPS and appears to be similar to deeply truncated forms of LPS such as that of *Salmonella* Re mutants (Nurminen *et al*, 1983).

The chemical composition of LPS differs between *C.psittaci* and *C.trachomatis*, the fatty acid:D-glucosamine:D-galactosamine: KDO:phosphate ratios being 6:2:1:5:2 and 5:2:0:3:2.6 respectively. The *C.psittaci* LPS contains D-galactosamine (not found in *C.trachomatis* LPS) and more KDO residues suggesting that the oligosaccharide core component of the respective chlamydial LPS may have a different chemical structure (Nurminen *et al*, 1985; Brade *et al*, 1985 and 1986).

Antigenic reactivities of the LPS can be divided into epitopes specific to Chlamydia or epitopes cross-reactive with enterobacterial Re LPS (Caldwell and Hitchcock, 1984). By analysis of recombinant rough mutants expressing a chlamydial KDO-glycosyl transferase, Brade *et al* (1988) have associated an epitope of the first type with a unique α-2,8-linked KDO disaccharide found on the oligosaccharide core of the LPS molecule. Taylor and Prendergast (1987) did not find this structure useful as an immunoprotective antigen. Two other epitopes on the oligosaccharide belonging to the second type have also been chemically characterised as a terminal α-pyranoside KDO residue and an α-2,4-linked KDO disaccharide A third epitope of the second type is thought to reside in the lipid A portion of the LPS molecule because it cross-reacts with enterobacterial lipid A and is unmasked only after acid hydrolysis (Brade *et al*, 1985).

AIMS OF THIS PROJECT

This project formed part of an inter-disciplinary research collaboration, the aims of which are to understand the pathogenic and immune mechanisms operating in OEA and to improve existing OEA vaccines. Initially, the aim of the project was to characterise various antigens of *C.psittaci* already identified by preliminary immunoblotting studies. With the availability of serum and lymph samples from ongoing experiments of the research group, further immunoblotting was also carried out in tandem with the characterisation work to fulfil the following aims:

a. to identify antigens of OEA strains of *C.psittaci* recognised by sheep of different immune status and
b. to identify the potentially immunoprotective antigens in vaccinated and protected ewes (CHAPTER 3);
c. to initiate characterisation of selected antigens and
d. to develop methods of purification (CHAPTER 4);
e. to test purified antigens for immunoprotective epitopes.

As the importance of the major outer membrane protein gradually became evident from the immunoblot results and from the data of other investigators, the characterisation work was focussed on MOMP. An experiment was carried out to test MOMP-enriched outer membrane complexes as an experimental vaccine (CHAPTER 5). This revealed the potential of MOMP as an immunogen. However, more rigorous studies to prove its role in immunity were hampered by the difficulty in producing adequate amounts of the purified protein. A recombinant DNA approach was adopted. Relying on the availability of the MOMP gene cloned from an abortion isolate of *C.psittaci*, subsequent aims were

f. to analyse the sequence of the MOMP gene (CHAPTER 6) and
g. to express recombinant MOMP (CHAPTER 7).

CHAPTER 2

MATERIALS AND METHODS

I. MICROBIOLOGICAL, IMMUNOLOGICAL AND BIOCHEMICAL TECHNIQUES

STRAINS AND ISOLATES OF CHLAMYDIAE USED

Name	Type	Remarks
C.psittaci strains		
A22	ovine abortion	original reference strain used in the commercial vaccine (Stamp *et al*, 1950).
S26/3	ovine abortion	isolated from an outbreak (1979) in a vaccinated flock (Anderson, 1984) - highly virulent.
84/501	ovine abortion	subsequently isolated from the same flock which S26/3 was derived.
H574	ovine abortion	single sporadic abortion in a non-experimental ewe at MRI (Anderson, 1984) -slightly less virulent than S26/3.
S507	ovine abortion	isolated from a vaccinated flock - low virulence.
S152/3	ovine abortion	isolated from vaccinated flock in S.E. Scotland.
S124/3A	ovine abortion	isolated from vaccinated flock in S.E. Scotland.
84/521F	faecal isolate	from a recently aborted ewe; possessed inclusion morphology similar to abortion isolates (Anderson and Baxter, 1986).
84/604	faecal isolate	from a lamb in a flock having had abortion in the same season - inclusion morphology similar to arthritis isolates (Anderson and Baxter, 1986).
P787	ovine polyarthritis	isolated from synovial fluid of a male lamb suffering from arthritis (Anderson, 1984).

CH1287	ovine conjunctivitis	isolated from the eye of a sheep with conjunctivitis in MRI (Anderson and Baxter, 1986).
Cal10	laboratory strain	from a case of presumptive ornithosis (Francis and Magill, 1938).
725	avian strain	isolate from a parakeet (gift of Dr Bob Bevan).
C.trachomatis strain L2	reference LGV strain	gift of Dr I.W. Smith, Department of Bacteriology, University of Edinburgh.

CELL CULTURE OF CHLAMYDIAE AND MEDIA

Mycoplasma-free baby hamster kidney cells (BHK-21 heterodiploid cell line - Wellcome Foundation Laboratories, Beckenham) were used for cell culture of chlamydial organisms. The method of culture was performed according to Anderson (1984 - FIMLS thesis). Briefly, cells were seeded in 25 mls of Growth Medium (GM) at a density of 150,000 - 200,000 cells/ml onto 75 sq cm plastic tissue culture bottles (Nunc) and incubated at 37°C overnight. Growth Medium (pH 7 - 7.2) contained 1 x BHK-21 Glasgow MEM medium (Gibco, Europe) with 10% newborn calf serum (Gibco, Europe), 2.95 mg/ml tryptose phosphate broth (Difco Laboratories, Surrey), 25 μg/ml streptomycin sulphate (Evans Medical Ltd, Middlesex), 25 U/ml mycostatin (E. R. Squibb & Sons, Middlesex), 20 μg/ml of gentimicin (Nicholas Laboratories, Slough), 1.6 mg/ml sodium bicarbonate (BDH Chemicals, Poole) and 0.4 μg/ml phenyl red.

After overnight incubation to obtain confluent monolayers, spent medium was replaced with fresh medium containing 80 μg/ml 5-iodo-2'-deoxyuridine (IDU) (Sigma, Poole) in maintenance medium (GM with 2% instead of 10% newborn calf serum) and the culture incubated at 37°C. After three days, the spent medium was exchanged for the inoculum medium containing 1% (v/v) of chlamydial inoculum in maintenance medium (25ml). The Falcon flasks were then centrifuged (2000 g) for 30 minutes at 20°C using an adapted microplate carrier (MSE Scientific, Sussex) and cultures were then left to incubate at 37°C for 3 - 7 days.

To monitor the progress of a chlamydial culture, flat-bottomed, glass universal bottles containing 16mm diameter coverslips were also similarly seeded (2ml), treated, inoculated and centrifuged (2000 g for 30 minutes). The coverslips were removed daily and stained with Giemsa (see next section) to assess the quality of the culture.

For preparing inocula, the same procedure was followed using stock chlamydia held at the Moredun Institute except that only monolayers with 50% or more cells infected were used and harvested at 3 days post-inoculation (dpi) directly into sterile transport medium (74.6 mg/ml sucrose, 0.512 mg/ml potassium dihydrogen orthophosphate, 1.237mg/ml di-potassium hydrogen orthophosphate, 0.721 mg/ml L-glutamic acid, 10% fetal bovine serum, 150 U/ml mycostatin, 50 μg/ml gentimicin, 100μg/ml streptomycin sulphate, 16 μg/ml phenyl red) at roughly 3 ml per Falcon flask of infected cell monolayer. Inocula were immediately frozen at -70°C until use. For purification, chlamydial cultures were harvested at 7 dpi with the medium and frozen at -70°C until the purification procedure.

HISTOLOGICAL STAINING

Cell monolayers on coverslips were fixed in methanol for 10 minutes and stained in 5% (v/v) Giemsa solution (Gurr's unproved R66, BDH, Poole) for 20 minutes. The coverslips were then washed and dehydrated by sequential rinses in the following 5 solvents: acetone; 2:1; 1:1, 1:2 (v/v) acetone:xylene mixtures; and xylene. Coverslips were then mounted onto microscope slides using DPX mountant. The stained monolayers were then examined under a light microscope for chlamydial inclusions which appear violet against a pink background.

PURIFICATION OF CHLAMYDIAE

The method of purifying chlamydiae was carried out as described in McClenaghan *et al* (1984) with only one modification. Urografin 370 (Schering AG, FRG) was used in centrifugation steps as the density medium instead of

Renografin. Briefly, cell culture harvests were thawed and any intact cells disrupted with a Jencons glass-Teflon homogeniser. The culture was clarified by centrifugation (1500g) for 5 minutes at 4°C. Chlamydiae in the clarified supernatant were pelleted through a 10 ml density cushion of 30% (v/v) Urografin in TrisKCl (20 mM Tris-HCl pH 7.5 and 150 mM KCl) by centrifugation at 53,000g for 45 minutes at 4°C. The crude chlamydial pellet was resuspended in a small volume (about 1ml) of TrisKCl and homogenised where necessary to disrupt aggregates before layering onto a 30%-60% Urografin continuous density gradient. Purification of chlamydial EBs and RBs was achieved by ultracentrifugation at 53,000g for 2 hours at 4°C. Two opalescent bands, the upper containing predominantly RBs and the bottom containing EBs, could be seen after centrifugation. Both bands were collected with a syringe and diluted in TrisKCl and re-pelleted by centrifugation (53,000g at 4°C for 45 minutes). The pellets were then resuspended in TrisKCl (100-500μl) with a homogeniser or by vigorous vortexing where necessary before freezing at -70°C in small aliquots. Preparations were routinely checked by electron microscopy.

ELECTRON MICROSCOPY

The carbon collodion-coated grid was first wetted with 3 μl of 0.01% poly-L-lysine (Sigma) solution for 20 seconds and dried with the edge of a piece of blotting paper. The sample (3μl) was then added to the grid and left for 20 seconds before being similarly dried. The coated grid was washed twice with distilled water (3μl) to remove salts. The washed grid was then treated with 3μl of the negative stain, either 1% phosphotungstic acid (BDH) pH 7.0 or 1% ammonium molybdate (Taab Laboratories, Reading) pH 5.3 for 20 seconds and dried, ready for examination under the electron microscope (JEOL 1200EX). The operating voltage was typically 80 kV.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS (SDS-PAGE)

This procedure was carried out using a modification of the method of Laemmli (1970) as described in Herring and Sharp (1984). For mini-gels (80mm x 60mm), the BioRad apparatus

(MiniProtean II) was used according to the manufacturers' instruction. For large gels (150mm x 140mm), the BioRad apparatus (Protean) was used. Gel loadings were typically 10-20 μg protein per track depending on the number of expected bands and the method of staining. Before electrophoresis, samples were boiled for 90 seconds with an equal volume of 2 x Sample Buffer containing 20% sucrose, 2% SDS, 4% (v/v) 2-mercapto-ethanol and 0.04% (w/v) bromophenol blue as tracker dye.

ELECTRO-TRANSFER AND IMMUNOBLOTTING

The electro-transfer was carried out according to Herring and Sharp (1984) without modification. This procedure was based on the method of Burnette (1981) using a methanol(20%)-Tris(20mM)-glycine(150mM) blot buffer. The immunoblotting procedure of Herring and Sharp (1984) was used with the following modifications. The wash solution and diluent were replaced with phosphate buffered saline pH 7.4 containing 0.5% Tween 20 (PBST). Blocking was achieved simply by a wash in PBST. The membranes were immunologically probed with serum samples diluted 1:100 in PBST for 1 h. Visualisation was either carried out enzymically or by autoradiography. The radio-iodinated rabbit anti-sheep $F(ab)_2$ antibody and radio-iodinated Protein A were obtained from the Moredun Institute (gift of P.Dewar and A.Dawson). This second incubation step was also reduced to 1 h at room temperature. The horse-radish peroxidase-conjugates with antibodies to sheep IgG, sheep IgM, human IgG and murine IgG were obtained from the Scottish Antibody Production Unit (SAPU).

For protein microsequencing of MOMP, SDS-PAGE was carried out as described above on a sample of outer membrane preparation, highly enriched in MOMP. The gel was equilibrated for 10 minutes in transfer buffer (50mM boric acid pH 9 20% methanol) before electro-transfer onto a glass fibre support (GLASSYBOND^R, Biometra Ltd, Manchester) by a semi-dry method (Eckerskorn *et al*, 1988). Briefly, the "Glassybond^R" support was cut to size, pre-wetted with methanol and equilibrated with transfer buffer for 20 minutes with three buffer changes.

Electro-transfer was carried out between pre-rinsed graphite electrodes in a transfer sandwich containing the following arranged from the anode to the cathode: 2 sheets of filter paper (Whatman 3mm) pre-soaked in transfer buffer, 1 sheet of pre-wetted "Glassybond^{R}", equilibrated gel, 2 sheets of filter paper pre-soaked in transfer buffer. Care was taken to eliminate air bubbles between the individual layers. The transfer was performed with a constant current density of 1mA/square cm for 90 minutes. After the transfer, the blotted "Glassybond^{R}" was rinsed with distilled water and stained in Coomassie blue as described below. The MOMP band was identified and excised for sequence analysis.

STAINING OF GELS AND BLOTS

Coomassie Blue staining of protein gels

The gel was incubated in Coomassie Blue stain (0.04% (w/v) Coomassie brilliant blue R250 (Sigma), 50% (v/v) methanol, 7.4% (v/v) glacial acetic acid, 1% (v/v) trichloroacetic acid) for 1 h with gentle shaking. To remove non-specific staining, the gel was incubated a further 1 - 2 h in destain solution (7% (v/v) glacial acetic acid, 23% (v/v) ethanol) with gentle shaking. To assist stain removal, tissue paper was soaked in a corner of the incubation tray to adsorb excess stain.

Silver staining of protein gels

This technique was used when a greater degree of sensitivity was required (at least 10 ng of protein per band detected). The method of Morrisey (1981) was used with slight modifications. The gel was incubated for 20 minutes in each of the following three solutions sequentially: 50% methanol 10% acetic acid (v/v); 5% methanol 7% acetic acid (v/v); and 10% glutaraldehyde (v/v). The gel was then washed in distilled water for 4 hours (5 - 6 changes of 200ml per gel) or overnight (1L).

The washed gel was then incubated sequentially for 30 minutes in each of the following two freshly made solutions: 5 μg/ml dithiothreitol; and 0.1% silver nitrate (w/v). The treated gel was then rinsed quickly with distilled water once

and then twice with freshly made developer solution (3% w/v sodium carbonate containing 0.05% v/v formaldehyde 37-42% solution) before being soaked in developer solution until the desired intensity of staining was achieved. The reaction was then stopped with 2.3M citric acid (5ml per 100ml of developer solution used) and left for 30 minutes before storage. At all stages, all containers used were thoroughly cleaned. Direct contact with the gel was completely avoided by the use of gloves. Typically, 200ml of each solution per stage was used per gel (or 50ml per mini-gel). The gels were subjected to gentle shaking at all incubation stages for even staining.

Preservation of gel

For short term storage (up to 6 months), polyacrylamide gels were sealed in polythene bags. For longer term storage, they were soaked in 4% glycerol for at least 1 h before drying down onto filter paper or between two sheets of clear semi-permeable backing membrane (BioRad) in a BioRad slab gel dryer.

Coomassie Blue staining of nitrocellulose blots

Strips of nitrocellulose blots of SDS-PAGE gels were incubated in Coomassie Blue stain solution diluted 1:40 with blot destain solution containing 30% (v/v) methanol and 5% (v/v) glacial acetic acid for 1 - 2 hours and destained with blot destain solution as described above for gels.

Coomassie Blue staining of "Glassybond^{R}" blots

The blot was briefly rinsed in distilled water and soaked for 5 minutes in Coomassie Blue stain solution (undiluted). The stained blot was rinsed in water and destained in blot destain solution until the background was colourless. The blot was then dried between paper towels and stored until sequence analysis.

AuroDye staining of nitrocellulose blots

For increased sensitivity (10 ng of protein per band), the AuroDye stain (Janssen) was used. Blots were blocked in PBS containing 0.3% Tween for 30 minutes before being soaked for 30 minutes (or until desired colour intensity achieved) in AuroDye.

PROTEOLYTIC PEPTIDE MAPPING

This procedure was carried out according to Cleveland *et al* (1977). The protein sample was subjected to SDS-PAGE and the protein bands stained by Coomassie Blue as described above. The desired protein band was excised and incubated in Cleveland buffer (100mM Tris pH 6.8 containing 0.1% SDS and 1 mM EDTA) for 30 minutes. The gel slice was trimmed and manually inserted onto the bottom of a well of another PAGE gel that was cast with a longer stacking gel (1cm). The gel slice was then overlayed with protease dissolved in Cleveland Buffer containing 10% glycerol to a predetermined concentration. Electrophoresis was carried out until the protease and protein sample were stacked closely at the interface of the stacking gel and resolving gel. Proteolysis was allowed to take place in the gel for 1 hour. The peptide fragments were separated by resuming electrophoresis and the peptide profile visualised by silver staining or immunoblotting.

IMMUNO-DOT BLOTTING

For detection of specific antibodies in serum samples the following method was used. Nitrocellulose paper (Schleicher and Schuell) 0.45 μm wetted in distilled water was mounted on a HybriDot manifold (BioRad Laboratories, FRG). One to five μl of the sample was applied onto the nitrocellulose paper in each well and vacuum applied gently to the manifold. Each well was then rinsed with 200 μl of PBS containing 0.5% Tween 20. The blotting procedure was then carried out according to the manufacturer's recommendations. Serum dilution used was 1:100 in PBST as in the immunoblotting procedure.

RADIO-IODINATION AND LIGAND BLOTTING

Purified chlamydial EBs were labelled using IodoBeads (Pierce Chemical Coy, Rockford), non-porous polystyrene beads derivatised with N-chlorobenzene sulphonamide (sodium salt), as oxidising agent. Five μl of radioactive iodine-125 (0.5 mCi) and 2 Iodobeads were mixed in 100μl of TrisKCl for 5 minutes at room temperature. 100μl (about 80μg) of chlamydial EBs suspended

in TrisKCl were added and incubated for a further 15 minutes at room temperature. The solution was separated from the beads and the reaction quenched with either 2-mercaptoethanol (1% v/v - final concentration) or sodium metabisulphite (0.2% w/v - final concentration). Carrier in the form of potassium iodide (1% w/v) was added followed by sodium thiosulphate (1% w/v) before the mixture was centrifuged at 53,000g for 30 minutes at 4°C to pellet the labelled EBs. The pellet was washed once with TrisKCl and similarly pelleted and finally resuspended in 100μl of TrisKCl.

BHK21 cell monolayers were washed twice with PBS, harvested with a rubber policeman into 10 ml of PBS, washed again three times in PBS by repeated low speed centrifugation (500g) at +4°C before being resuspended in PBS to give $3x10^6$ cells/ml. 200μl of the cell suspension were radiolabelled as described above. Radiolabelled cells were washed four times with PBS and resuspended in 100μl PBS. These cells ($3x10^6$ cpm) were then solubilised with 1% Triton X100 (Sigma) in 100μl PBS for 2h at 37°C with constant agitation. Insoluble material was pelleted (11,000g), washed with PBS again and the two supernatants pooled. The radiolabelled cell material ($3x10^6$ cpm) was diluted in PBST (the Triton X100 concentration was reduced from 1% to 0.03%) and the radioactive solution (3ml) used to probe pre-blocked nitrocellulose strips bearing electroblotted chlamydial proteins (2h at 37°C with gentle shaking). The probed strips were then washed with PBST, dried and exposed to X-ray film as in the immunoblot procedure. Radioactivity of the labelled preparations were measured in a Packard counter 460CD (15-80keV).

PROTEIN MICROSEQUENCING

N-terminus amino acid sequencing was done automatically with an Applied Biosystems gas-phase microsequencer 477A on a piece of "Glassybond^R" containing MOMP. The procedure was carried out according to the manufacturers' recommendations. Briefly, polybrene (2mg - 30μl) was added to the sintered-glass fibre sample disc, which was then subjected to a 3 h pre-cycle

of washes before the "Glassybond^{R}" sample was loaded. The microsequencer automatically carried out sequential Edman degradation (Edman, 1950), conversion of amino acid-thiazolinones to the corresponding phenylhydantoin derivatives and chromatographic separation by high performance liquid chromatography (HPLC) with on-line data collection and analysis by a dedicated computer.

II. RECOMBINANT DNA TECHNIQUES

STRAINS OF *ESCHERICHIA COLI* USED

JM101 *supE*, *thi*, del(*lac-proAB*), [F', *traD36*, *proA*$^{+}$*B*$^{+}$, *lacI*^{q}, *lacZ* delM15]. Restriction (*rk*$^{+}$,*mk*$^{+}$), *mcrA*$^{+}$. Yanisch-Perron *et al* (1985).

JM109 *recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1* (λ^{-}), del(*lac-proAB*), [F' *traD36*, *proA*$^{+}$*B*$^{+}$, *lacI*^{q}, *lacZ* delM15].
A host for growth of the single-stranded phage M13 and its recombinants that is *recA*$^{-}$, modification plus and restriction minus.

TG-1 del(*lac-proAB*), *thi*, *supE*, *hsd*Δ*5* (*rk*$^{-}$, *mk*$^{-}$) [F' *traD36*, *proA*$^{+}$*B*$^{+}$, *lacI*^{q}, *lacZ* delM15]
An *E.coli* strain from LMB (Cambridge) maintained on M19 media plus vitamin B and glucose in order to retain the F' episome.

HB101 *hsd* S20, *supE44*, *ara*l4, λ^{-}, *galK2*, *lacY1* *proA2*, *rspL20*, *xyl*-5, *mtl*-1, *recA13*.
Restriction: (*rB*$^{-}$,*mB*$^{-}$), *mcrA*$^{+}$, *mcrB*$^{-}$.

N99cI^{+} *galK*, *strA*, λcI^{+}. A K12 λ lysogen of N99 containing containing the wild type cI^{+} λ repressor. N99 is derived from MM28 (Pharmacia).

N4830-1 F^{-}, *su*$^{-}$, *his*$^{-}$, *ilv*$^{-}$, *galK*del8, del(*chlD-pgl*), λ, del*Bam*, N^{+}, cI857, delH1. A P1 transductant of N4830-1 screened for *galK*$^{-}$. It carries the temperature-sensitive λ cI857 repressor integrated into the genome. Useful for vectors with λ P_L or P_R promoter (Pharmacia).

Bacteria were propagated and maintained using standard microbiological techniques as detailed in Maniatis *et al* (1982).

RESTRICTION ENDONUCLEASES AND DNA MODIFYING ENZYMES

Restriction endonucleases (REs) and DNA-modifying enzymes were purchased from various manufacturers (Gibco BRL, Boehringer Mannheim, NBL, Pharmacia) and used according to the manufacturers' recommendations where appropriate. The enzymatic reactions were otherwise carried out according to standard protocols detailed by Maniatis *et al* (1982). RE digestions were typically carried out in buffers supplied by the manufacturer and carried out at an enzyme concentration of 3-5 units/μg DNA at the recommended temperatures for at least 2 hours. Reactions were stopped either by phenol extraction or by heating to 60°C for 10 minutes.

PURIFICATION OF NUCLEIC ACIDS

Nucleic acid solutions were deproteinised by extraction with phenol:chloroform (6:4 v/v) equilibrated with 1.0M Tris-HCl pH 8.0. The nucleic acid sample was mixed with an equal volume of phenol:chloroform by gentle vortexing and the organic and aqueous phases separated by centrifugation at 11,000g for 2 minutes at room temperature. The aqueous phase was carefully removed and transferred into a sterile micro-centrifuge tube. Where necessary, the phenol phase was back-extracted for maximum recovery by the addition of an equal volume of TE buffer pH 8.0 (10mM Tris-HCl and 1mM EDTA), mixing and centrifugation. The aqueous phase was removed and combined with the initial sample for concentration by ethanol precipitation.

DNA was precipitated from aqueous solutions by the addition of 0.1 volume of 3M sodium acetate pH 5.2, 2 volumes of ethanol (-20°C) and stored at -20°C for 2-16 hours. For precipitating nanogram quantities of DNA, the same procedure was used except for the addition of mussel glycogen (BRL) to 20μg/ml as a carrier. For selectively precipitating DNA molecules greater than 200bp, 0.1 volume of 3M sodium acetate and 0.6 volume of isopropanol were added to the aqueous DNA solution instead and stored for 1 hour at -20°C. Precipitated nucleic

acid was pelleted by centrifugation at 11,000g for 10 minutes at room temperature. The supernatant was aspirated and the pellet washed by vortexing in 1ml 70% (v/v) ethanol. Washed nucleic acid was re-pelleted as before and the pellet dried briefly in a vacuum desiccator. Dried DNA was resuspended in TE buffer, usually at a concentration of 330μg/ml and stored at -20°C or 4°C.

AGAROSE GEL ELECTROPHORESIS

DNA fragments greater than 200bp were separated by horizontal agarose slab gel electrophoresis according to standard procedures detailed by Maniatis *et al* (1982). A mini-gel system (Pharmacia GNA-100) was used for casting 20ml and 40ml gels containing 0.8% (w/v) nucleic acid grade 'ultrapure' agarose (BRL) dissolved in Loening 'E' buffer pH 7.6-7.8 (36mM Tris, 1mM EDTA and 30mM sodium di-hydrogen orthophosphate) and ethidium bromide at a concentration of 0.5μg/ml. The electrophoresis was carried out in Loening 'E' buffer. DNA samples were mixed with 0.1 volume of loading buffer (50mM EDTA, 0.1% (w/v) Bromophenol blue and 25% (w/v) sucrose or Ficoll), applied to the wells and subjected to electrophoresis for 1-2 hours at 3.75 volts/cm using a Pharmacia EPS 500/400 power supply. Molecular mass standards used were either *Hin*dIII-restricted λ phage DNA or 1kbp DNA ladder (BRL). After electrophoresis, the DNA bands were visualised over an ultraviolet wavelength transilluminator at wavelength 302nm.

GEL PHOTOGRAPHY

Ultraviolet-illuminated, ethidium bromide-stained agarose gels were photographed with a Polaroid MP-4 Land camera, using Polaroid 667 high speed positive or Polaroid 665 positive/negative film through a Kodak Wratten 9 UV filter. Typical settings for 667 film were f8 for 1/2 second and f4.5 for 10 seconds with 665 film. Film was developed for 30 seconds at room temperature.

MEASUREMENT OF [α-^{32}P]- ATP INCORPORATION INTO NUCLEIC ACID

This was performed by the quantitation of trichloroacetic acid (TCA)- precipitable counts described in Maniatis *et al* (1982) with the following modifications. Two μl of the labelling reaction solution was removed and mixed with 10μl of sonicated salmon sperm DNA (500μg/ml in TE pH 7.5 and 0.1% (w/v) SDS). Half the sample was spotted onto a Whatman glass fibre filter, in a gamma counter tube and left to dry. The remainder of the sample was spotted onto a Whatman glass fibre filter and wetted with ice cold 5%(w/v)TCA. After 5 minutes incubation on ice, the filter was given 10 x 2.0ml washes with 5% (w/v) TCA. The washed and unwashed filters were counted in a gamma counter (Packard Autogamma 5650, Packard Instruments, USA). Total counts were compared to acid-precipitable counts and the percentage incorporation calculated.

SYNTHESIS OF RADIOACTIVE RNA PROBES

The BLUESCRIBE vector system (Vector Cloning Systems, San Diego) containing T3 and T7 viral transcriptional promoters in the coding region of the *lacZ* gene of plasmid pUC19 was used to generate specific ssDNA as radiolabelled probes in the hybridisation experiments. A BLUESCRIBE recombinant carrying the MOMP gene was kindly provided by S. Baxter. The procedure was carried out according to the manufacturer's instruction. Radiolabelled MOMP gene probes were used in Southern hybridisations.

HYBRIDISATION OF NUCLEIC ACIDS

Pre-hybridisation blocks non-specific binding sites for the probe on the membrane filter. Baked nitrocellulose filters were briefly washed in 6 x SSC (standard sodium citrate buffer pH 7 - 1xSSC contains 150 mM sodium chloride and 15 mM sodium citrate), then pre-hybridised for 2 hours at 42°C, with gentle rolling, in 500ml or 1000ml Azlon, screw top, wide-mouth polypropylene bottles containing 5 or 10ml of pre-hybridisation fluid (50% (v/v) deionised formamide, 0.25 x Denhardt's

solution (100 x Denhardt's solution is 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone - PVP-40), 1.25 x SSC, 0.001% SDS and 100μg/ml denatured salmon sperm DNA).

The pre-hybridisation fluid was discarded and replaced with an equal volume of fresh pre-warmed (42°C) pre-hybridisation fluid containing denatured probe. Radiolabelled probe was denatured by heating for 5 minutes in 50% (v/v) formamide, at 80°C. Hybridisation was performed overnight at 42°C with gentle rolling of the polypropylene bottles containing probe solution and filter.

Hybridisation fluid was aspirated and the unhybridised probe was removed from the filters by two 15-minute washes in 1xSSC and 0.1% (w/v) SDS at 42°C, followed by two 30-minute washes in 0.1 x SSC, 0.1% (w/v) SDS at 42°C.

Autoradiography of ^{32}P labelled nucleic acids was performed using Fuji RX medical X-ray film. Filters or dried gels were mounted between a piece of de-emulsified X-ray film and a sheet of Saran wrap. The sandwich was exposed in an X-ray exposure cassette fitted with a Dupont Cronex lightning plus HC intensifying screen at -70°C. X-ray film was developed for 2-8 minutes in Photosol CDL8 X-ray developer, washed briefly in water then fixed for 2 minutes in Photosol CF40 fixer.

POLYACRYLAMIDE GEL ELECTROPHORESIS OF NUCLEIC ACIDS AND SILVER STAINING

For greater sensitivity in the detection of low amounts of nucleic acid (<10ng) or low molecular mass nucleic acid (100 bases to 3 kb), polyacrylamide gel electrophoresis and silver staining were used. Typically, 7.5% acrylamide solution in 1 x Loening 'E' buffer was employed using a 'E' buffer system. Gel apparatus used were the same as that for protein SDS-PAGE gels as described above. Nucleic acid bands were visualised by staining with silver (Herring *et al*, 1982). Gels were fixed in a solution of 10% (v/v) ethanol and 0.5% (v/v) acetic acid for 20 minutes followed by a 20-minute incubation in 0.2% (w/v) silver nitrate (BDH). The gel was rinsed in distilled water twice and

developed in degassed 0.15M aqueous sodium hydroxide supplemented with formaldehyde (0.3% v/v) until the desired colour intensity. The reaction was halted by rinsing and soaking the gel in 0.75% (w/v) sodium carbonate solution for 1h.

POLYMERASE CHAIN REACTION (PCR)

This procedure was carried out as described by Saiki *et al* (1985) on a Techne Programmable Heat/Cool Dri-Block (Cambridge) using 30 cycles of the following incubations: 1.5 minutes 94°C; 2 minutes 37°C; 3.5 minutes 70°C. To complete the reaction, a final incubation of 8 minutes at 70°C was carried out.

The reaction mixture (50μl) typically contained 50 pmol of the appropriate synthetic oligonucleotide amplimers (OSWEL service, University of Edinburgh) each, 10-50 ng of target DNA, 200 mM of each dNTP (BRL or Pharmacia), 2-3 units of *Taq* polymerase (Cetus Corp) in buffer containing 10mM Tris pH 8.5 1.5 mM $MgCl_2$ 50mM KCl (ultrapure, Aldrich) and 0.1% (w/v) gelatine (biochemical- BDH). The reaction mixture was boiled for 3 minutes before addition of the dNTPs and polymerase. To prevent evaporation, a drop of mineral oil (400-5 Sigma) was overlayed onto the reaction mixture before beginning the incubation cycles.

SUBCLONING, LIGATION, TRANSFORMATION AND TRANSFECTION

The M13 clone containing the MOMP gene was subcloned for sequencing according to Messing (1983) and Yanisch-Perron *et al* (1985). The essential items of an M13 Cloning Kit (Boehringer Mannheim) were purchased and the cloning procedures recommended by the manufacturer was followed with the exception that the *E.coli* host strain JM101 was used for making competent cells. M13 (+)ssDNA was prepared accordingly for analysis and for sequencing.

For the construction of plasmids expressing MOMP, the following vectors were used: pUC8, pRIT5 and pRIT2T (Pharmacia). Restriction digestions, dephosphorylations and ligations were carried using standard procedures described in

Maniatis *et al* (1982). MOMP gene inserts were isolated by preparative agarose electrophoresis and the excised bands treated with the GENECLEAN kit (Bio101, California) as per instructions. The *E.coli* host strains used for the transformation of MOMP constructs in these vectors were JM101, HB101 and N99cI$^+$ respectively.

MINI-PREPARATION OF DOUBLE STRANDED DNA

A variation of the rapid alkaline-SDS lysis method for preparing dsDNA from the M13 cloning experiments was used (Maniatis *et al*, 1982). Briefly, bacterial pellets (1.5ml cultures) were resuspended in 100 μl ice cold TEG buffer (25 mM Tris pH 8.0, 10mM EDTA, 50 mM glucose) containing 5mg/ml of lysozyme (Sigma) and incubated at room temperature for 5 minutes. 200 μl of freshly prepared alkaline SDS (0.2M NaOH and 1% SDS) was mixed in by several rapid inversions of the tube. The mixture was incubated at room temperature for 5 minutes. Ice cold solution (150μl) of 3M potassium acetate and 11.5% (v/v) glacial acetic acid was then mixed in and the mixture incubated for 5 minutes on ice before centrifugation (15 minutes at 11,000g). The supernatant was extracted with phenol:chloroform (6:4 v/v) and precipitated with ethanol as described above. To remove RNA, the DNA solution was either treated with RNase (20 μg/ml) for 30 minutes at 37°C or purified using GENECLEAN (Bio101, California) according to the manufacturer's instructions.

GEL RETARDATION ASSAY

Equal amounts (100 ng) of test ssDNA and the complementary probe ssDNA was mixed with 2% SDS (1 μl), agarose electrophoresis loading buffer (3 μl) and TBE buffer (0.1M Tris 89mM boric acid 2 mM EDTA pH 8.3) (13 μl). The reaction mix was incubated for 3 h at 65°C before being analysed by agarose gel electrophoresis. The appropriate ssDNA controls were also run for comparison. Test ssDNA containing sequences complementary to the probe formed dsDNA at the relevant regions and migrated at a much slower rate under electrophoresis compared to the controls.

M13 CHAIN TERMINATION METHOD FOR DNA SEQUENCING

The M13 sequencing kits from Boehringer Mannheim (DNA polymerase Klenow fragment) or from Pharmacia (T7 DNA polymerase) were used. The chain termination reactions were carried out exactly according to the manufacturer's instructions. The radioactive label, [α-^{35}S]-dATP (Amersham, UK) was used. Urea (50% w/v)-polyacrylamide (6%) sequencing gels (0.4 mm) were cast and electrophoresis carried out on the Sequencing Gel apparatus Model S2 (BRL) according to the manufacturer's instructions. The electrophoresis buffer used was Tris-borate-EDTA (TBE) buffer (see above section). Gels were pre-run for 20 minutes before samples were loaded. For the resolution of 20 to 200 bp fragments, 2h of electrophoresis at a constant 60 W was sufficient. For larger fragments up to 500 bp, run times of 5 to 6 hours were commonly used. After electrophoresis, the sequencing gel was agitated in a solution containing 5% (v/v) acetic acid and 5% (v/v) methanol for 20 minutes to remove urea and to fix the gel. The gel was then dried onto filter paper for 1 h at 80°C and exposed to X-ray film in a light-tight cassette at room temperature overnight.

COMPUTER ANALYSIS OF DNA AND AMINO ACID SEQUENCE

The sequence analysis software package of the University of Wisconsin Genetics Computer Group (UWGCG) as described in Devereux *et al* (1984) was used extensively for the analysis of DNA and deduced amino acid sequences. This package was available on licence to the Edinburgh Regional Computer Centre (ERCC) and was supported on the ERCC Vax 8500 mainframe (ERCVAX). Programs used other than those mentioned below included COMPARE, DOTPLOT, TERMINATOR, FOLD, MAPPLOT, FIND.

Secondary structure analysis

The deduced amino acid sequence of MOMP was subjected to computer analysis using the algorithms of Chou and Fasman (1978) and Garnier *et al* (1978) which were available in the GCG package PEPPLOT, PEPTIDESTRUCTURE and PLOTSTRUCTURE programs. In

addition, a combined secondary structure prediction incorporating eight prediction methods was used (Sawyer *et al*, 1986 and references therein).

PEPPLOT shows several common measures of protein secondary structures together on one coordinated plot (Gribskov *et al*, 1986). Generally, the curves are the average, sum or product of a particular residue-specific attribute within a window of residues (w). The first part of the plot shows a representation of the sequence according to the characteristics of each residue using coloured vertical lines: red = hydrophilic charged either up (basic) or down (acidic); blue = hydrophilic, uncharged either short (amides) or long (hydroxyl); green = hydrophobic either short (aliphatic) or long (aromatic); black = proline; unmarked = alanine , glycine and cysteine. The next five plots show the propensity measures for α-helix and β-sheet (w=4) (Chou and Fasman, 1978), the regions of the sequence typically found at the amino- or carboxyl- ends of α-helix and β-sheet structures (w=6), and regions typically found in β-turns (w=4).

The seventh part of the plot shows the hydrophobic moment (Eisenberg *et al*, 1984) at each residue if the sequence were forming either helix or sheet structure respectively at that position. The hydrophobic moment measures the amphiphilicity of a protein segment and is useful for predicting sequences that may be located at the interface of the surface and the interior of the protein. In these curves, the magnitude of hydrophobic moment of the protein segment is calculated by the modulus of the Fourier transform of a one-dimensional hydrophobicity function. This hydrophobicity function is the sum of the magnitude of the hydrophobicity vector at each residue for each residue of the protein segment, which is given by the sum of the components of each hydrophobicity vector. The hydrophobicity vector for a residue is the product of the numerical hydrophobicity of the residue and the unit vector in the direction from the nucleus of the α-carbon towards the geometric centre of the side chain. Thus protein segments with the most apolar side chains on one side are characterised by

large values of the hydrophobicity moment. In other words, the hydrophobicity moment can be expressed as a function of the periodicity of the protein segment, ie. the number of residues per turn or alternatively, the inter-residue angle. For a periodic structure, say a strand of beta structure, the number of residues per turn is 2, ie., the inter-residue angle is 180°. If the beta strand twists, then the inter-residue angle will not be exactly 180°. To take into account such possible deviations from the ideal structure, the beta hydrophobic moment in this plot takes the maximum value for inter-residue angles from 140° to 180° and calculates the sum over a window of six residues. Similarly, the helix hydrophobic moment takes the maximum for residue angles 90° to 110° over a window of eight residues (3.6 residues per turn is equivalent to an inter-residue angle of 100°).

The eighth part of the plot is composed of two curves. The first is the hydrophobicity index averaged over nine residues and is based on the method of Kyte and Doolittle (1982). The second is another measure of hydrophobicity averaged over 20 residues called the GES curve (Goldman, Engelman and Steitz) for identifying nonpolar transbilayer helices as reviewed by Engelman *et al* (1986).

PEPTIDESTRUCTURE makes secondary structure predictions for a peptide sequence. The predictions include, in addition to alpha, beta, random coil and turn, measures for antigenicity, flexibility, hydrophobicity and surface probability which PLOTSTRUCTURE displays in a single coordinated plot. Two secondary structure predictions are used: Chou and Fasman (1978) and Garnier *et al* (1978). Hydrophilicity is calculated according to the algorithm of Hopp and Woods (1981) or Kyte and Doolittle (1982). Surface probability is calculated according to a formula of Emini *et al* (1985). Flexibility is calculated according to the Karplus-Schulz method (1985). The antigenic index is calculated according to Jameson and Wolf (1988).

Multiple alignment of MOMP sequences

The multiple sequence alignment program, CLUSTAL, was devised by Higgins and Sharp (1988). A version maintained on the ERCVAX was used to align the amino acid sequences of *C.psittaci* and *C.trachomatis* MOMPs. This method is based on first deriving a phylogenetic tree from a matrix of pairwise sequence similarity scores, obtained using a fast pairwise alignment algorithm. The multiple alignment is then achieved from a series of pairwise alignment of clusters of sequences, following the order of branching in the tree.

Search of current protein sequence databases

Limited searches of the nucleic acid and protein sequence databases (GENBANK, EMBL - European Molecular Biology Laboratory, NBRF - National Biomedical Research Foundation) available via the GCG program WORDSEARCH (Wilbur and Lipman, 1983) was carried out on the *C.psittaci* S26/3 MOMP sequence to pick out any related sequences. Similar searches were carried out using the FASTA and FASTP programs supported on the AFRC network. In addition, an exhaustive search using fast database searching programs based on a parallel processing supercomputer (Active Memory Technology 510-4 32x32 Distributed Array Processor) accessed through the ERCC Sequent (CASTLE). These programs, PROSRCH and PATSRCH, were recently developed by the Biocomputing Research Unit, Department of Molecular Biology, University of Edinburgh (Coulson *et al*, 1987) and perform rapid but exhaustive searches into current versions (September 1989) of the major sequence databanks: NBRF-PIR (Protein Identification Resource), OWL (Leeds University), EMBL, GENBANK and SWISPROT.

CHAPTER 3

IMMUNOBLOTTING ANALYSES

This chapter gathers the results of several immunoblotting studies into two parts: data pertaining to the identification of antigens of ovine abortion *C.psittaci* are presented in the first part and include immunoblots of sera and lymph from sheep; the second part describes the identification of an immunoprotective antigen in ovine enzootic abortion. The third and final part summarises the findings and includes further discussion.

PART 1: IDENTIFICATION OF CHLAMYDIAL ANTIGENS

The humoral immune response to infection by different chlamydial strains has been studied in various hosts: in small laboratory animals such as guinea pigs (Batteiger and Rank, 1987) and mice (Ramsey *et al*, 1989), in cynomolgus monkeys (Caldwell *et al*, 1987), and in humans (Newhall *et al*, 1982). These studies, most of which were reported during the course of this project, used immunoblotting to characterise the antigenic structure of these chlamydial strains, namely, the *C.psittaci* guinea pig inclusion conjunctivitis (GPIC) strain, *C.trachomatis* mouse pneumonitis (MoPn), trachoma and lymphogranuloma venereum (LGV) biovars. Similarly, initial studies to define the antigenic composition of OEA strains of *C.psittaci* with respect to the sheep immune system have been carried out by McClenaghan *et al* (1986; in preparation). Analysis of sera from experimentally infected sheep by immunoblotting revealed mainly high M_r chlamydial components as antigens, including a 40 kDa protein which was thought to be the major outer membrane protein (MOMP). The antibody reactions to MOMP and lower M_r antigens were weak. Furthermore, variability in the antibody response among animals was observed and this caused difficulties in interpreting the immunoblotting data.

The immunoblotting analyses described in this part followed from the work of McClenaghan *et al* and were aimed at confirming the antigenic structure of OEA strains of *C.psittaci*.

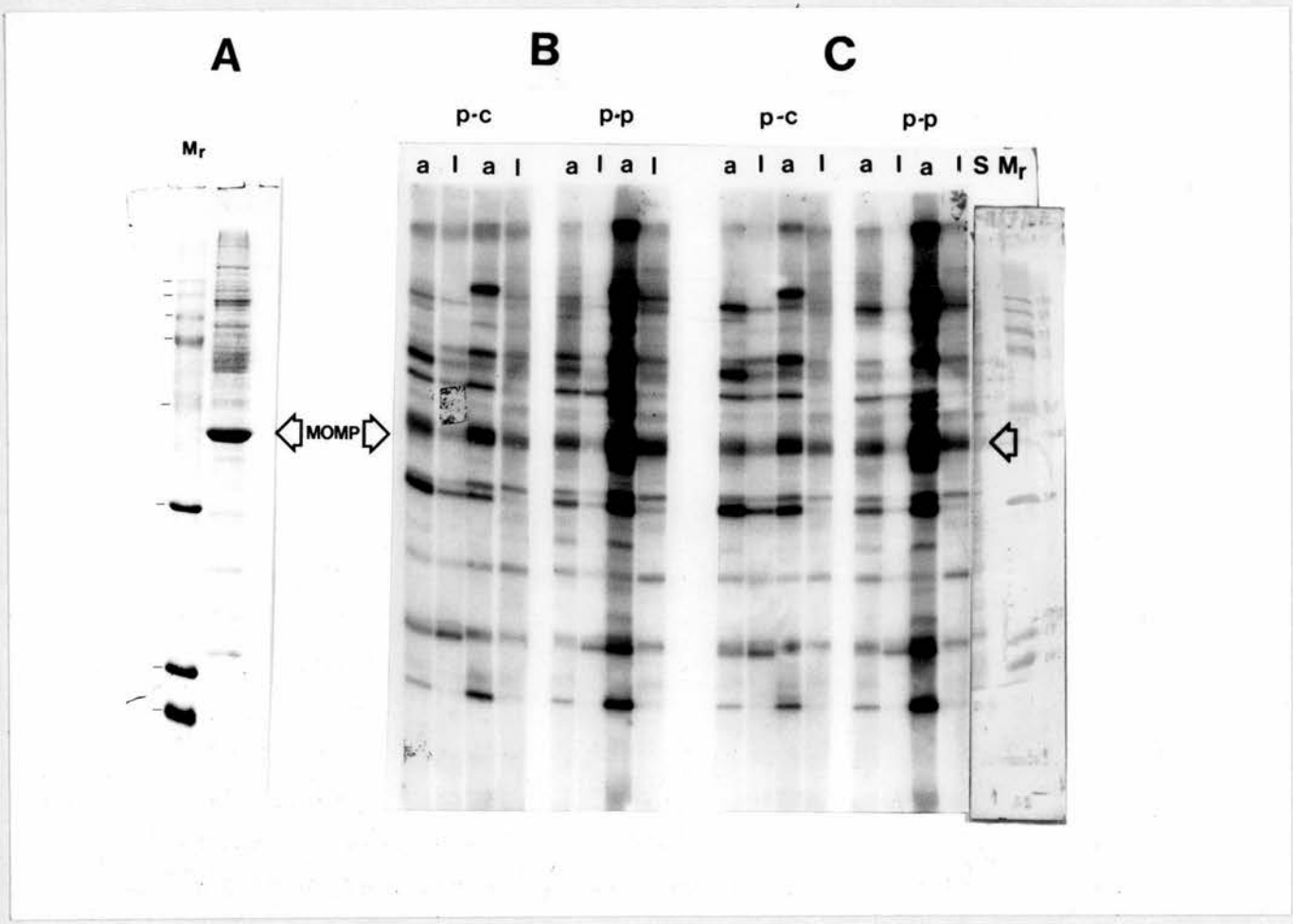


Figure 3.1. A. SDS-PAGE profile of EBs of an OEA strain of *C.psittaci*, S26/3, stained with Coomassie Blue. **B and C.** Autoradiogram of immunoblots of post-challenge (p-c) and post-parturition (p-p) sheep sera from four ewes, two of which subsequently aborted (a) and two of which lambed successfully despite the challenge (l). Panel B shows the blots using A22 antigen and panel C, S26/3. Lane S shows the blot containing the S26/3 profile stained with Coomassie blue. Molecular mass standards (M_r) as listed from the top are β-galactosidase (116kDa) phosphorylase (93) ovotransferrin (76-78), bovine serum albumin (66-67), ovalbumin (45), carbonic anhydrase (30), myoglobin (17) and cytochrome c (12).

A broad spectrum of serum (Section I) and lymph samples from animals with known immune status (Section II) was analysed to determine if any antigenic specificities could be used as markers of the immune status of an animal, that is, whether the animal is susceptible, infected or protected. Sera from zoonotically infected women were also tested (Section III). A simplified and more rapid immunoblotting procedure was developed and used to expedite the process (Chapter 2).

I. ANALYSIS OF SERUM SAMPLES FROM INFECTED SHEEP

A typical SDS-PAGE profile of OEA *C.psittaci* showed a very complex pattern of more than 60 separate bands dominated by an abundant 39-40 kDa protein (Figure 3.1A - arrowed; see also Figure 4.1). This protein has been subsequently shown (Chapter 4) to be the major outer membrane protein (MOMP) of *C.psittaci*, analogous to the MOMPs of other chlamydiae (Caldwell *et al*, 1981; Hatch *et al*, 1981). Silver staining also showed the presence of a rapidly migrating species with an apparent M_r of less than 12 kDa often observed at the dye front of the SDS-PAGE gel. This was presumed to be chlamydial lipopolysaccharide.

To identify the antigens of OEA *C.psittaci*, such SDS-PAGE electrophoretograms were electroblotted onto nitrocellulose paper and probed with sheep sera. Sera collected from control ewes in a previous vaccination-challenge experiment were used (gift of I.E.Anderson and A.J.Herring). These ewes were not vaccinated but were infected with live organisms of OEA *C.psittaci* A22 strain, the original commercial vaccine strain. Figure 3.1B and C show the immunoblot profiles of sera taken post-challenge and post-parturition. Antigens were identified and listed in descending order of the apparent M_r in Table 3.1. (Note that antigens listed in Table 3.1 have been compiled from all immunoblots performed in this project and the approximate apparent M_r are indicated. For convenience in identifying immunoblot profiles, these antigens have been divided into several groups arbitrarily.) Initially, each serum sample was tested against antigens from both *C.psittaci* strains S26/3 and A22, the two strains currently used in the commercial bivalent

Table 3.1 Antigens of ovine abortion isolates of *C.psittaci*

Antigen	apparent M_r (kDa)		Remarks
155kDa	155	(155-170)	155kDa from low percentage gels by extrapolation.
high M_r quartet	120	(117-125)	only one, two or three bands are strongly reactive; the rest very weak.
	105	(98-107)	
	91	(88-95)	
	89	(88-90)	
77kDa		(75-79)	occasionally weak 68kDa also present.
60kDa		(60-62)	strong antigen possibly a cysteine-rich protein (CRP).
mid 50kDa diffuse		(54-58)	sometimes flanked by discrete bands at 54kDa and 57kDa.
50kDa		(49-50)	strong antigen
supra-MOMP bands	48		faint bands above MOMP.
	44		
MOMP	39.5	(39-40)	major outer membrane protein, predominant antigen.
sub-MOMP band	36		weak band below MOMP.
30kDa complex	31		only one band very strong; occasionally two bands seen, difficult to distinguish, possibly one is adhesin.
	30		
	29.5		
inter-adhesin bands	25	(24-27)	generally weak bands.
	22	(22-24)	
18kDa complex	18.4		at least one is strong band; difficult to distinguish; one is probably an adhesin.
	17.5		
	16		
12kDa	12		possibly a CRP.
LPS	6		weak reaction in this study.

OEA vaccine. No significant qualitative or quantitative differences were detected in the immunoblot profiles and this approach to identify any difference between the two strains was discontinued. Subsequently, only the S26/3 strain was used as a source of antigen for immunoblots unless stated.

II. ANTIBODY RESPONSE OF THE SHEEP LYMPH NODE TO *C.psittaci* INFECTION

Immunoblotting was carried out retrospectively on sheep serum and lymph samples taken from a study of the lymph node response to *C.psittaci* infection (gift of H.-S.Huang and D.Buxton). The experimental design and procedure as detailed by Huang (1988 - MPhil thesis, University of Edinburgh) are briefly outlined below. Four different groups of sheep classified by their immune status had been used: **A.** post-abortion ewes; **B.** sheep previously unexposed to chlamydia; **C.** seroconverted sheep; and **D.** vaccinated sheep. The efferent duct of the popliteal lymph node of each sheep had been cannulated by the method of Hall and Morris (1962). Upon subcutaneous deposition of live chlamydial organisms (1 ml of $10^{4.5}$ 50% egg lethal dose) in the draining region of the lymph node, efferent lymph was collected continuously for one to four weeks. Lymph samples were cleared of cellular material by low speed centrifugation and aliquots of clarified lymph sera were frozen at -20°C until analysis by immunoblotting. Some of the data has been included in a paper that has been accepted for publication (Huang *et al*, in press - see Appendix).

Post-abortion sheep - group A

Figure 3.2 shows the lymph node antibody response of three post-abortion ewes. All three ewes possessed chlamydia-specific antibodies at the beginning of the experiment as a result of previous infection and abortion. Reinfection via the lymph node boosted the prevailing antibody response where it had declined. All three ewes produced antibodies to virtually the same set of chlamydial antigens at one time or another. The apparent M_r of high M_r antigens were more accurately estimated by using a low percentage (7.5%) gel for the immunoblotting of lymph from ewe

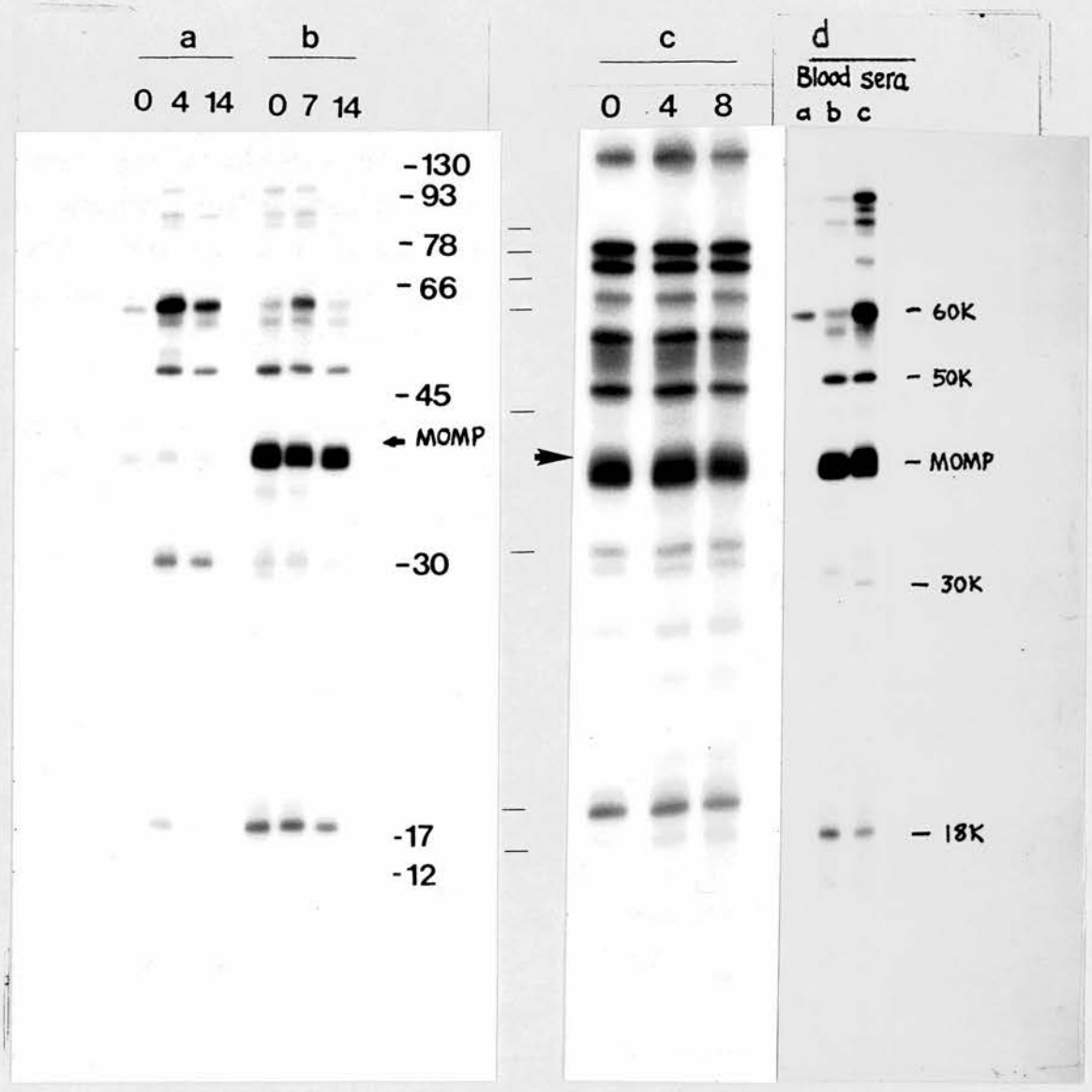


Figure 3.2. Immunoblot profiles of lymph (Panels a,b,c) and blood serum (Panel d) samples of three post-abortion ewes at the various days post inoculation as marked: Naturally infected ewes (1358H - a and 1651J - b). Subcutaneously infected ewe (2123K)(c). d. Day 0 blood sera from each ewe respectively. M_r standards indicated are as given in Figure 3.1.

2123K and correlated with those in blots of other serum samples (data not shown). Low M_r antigens were estimated directly from the profiles of Figure 3.2.

Ewe 2123K (c) was subcutaneously infected with OEA *C.psittaci* and had aborted some 2 months prior to cannulation. This animal had a high antibody titre throughout the experiment and this was reflected in the immunoblots. Lymph and blood serum samples at the beginning of the experiment gave strong antibody reactions to most of the listed chlamydial antigens. No boost in antibody response was detected by immunoblotting either because of limiting antigenic mass on the blots or because the antibody response of the animal was already maximal.

Ewe 1651J(b) was a naturally infected post-abortion animal Antibody response to certain antigens were not as strong as in 2123K. Besides some quantitative differences, the profile was essentially the same as that of 2123K. The anti-62kDa response was boosted at day 7.

Ewe 1358H (a) had the weakest immunoblot reactions. Reinfection triggered a rapid and more dramatic secondary response in the lymph node by day 4. This ewe had suffered from a concurrent infection with *C.psittaci* and toxoplasma (H.-S.Huang, personal communication). Toxoplasmal infection can cause partial immunosuppression in sheep (Buxton *et al*, 1981) and may account for the weaker immunoblot reactions at day 0.

The antigens identified from this group of animals were consistent with those from the previous section and allowed a more comprehensive list of chlamydial antigens to be compiled (Table 3.1). The blots showed that recently aborted animals maintained circulating antibodies in the vascular and lymphatic systems specific to many chlamydial antigens. Where the immune response was weak or had weakened possibly due to concurrent infection, reinfection via the lymph node with *C.psittaci* could induce a rapid secondary response to some but not all antigens.

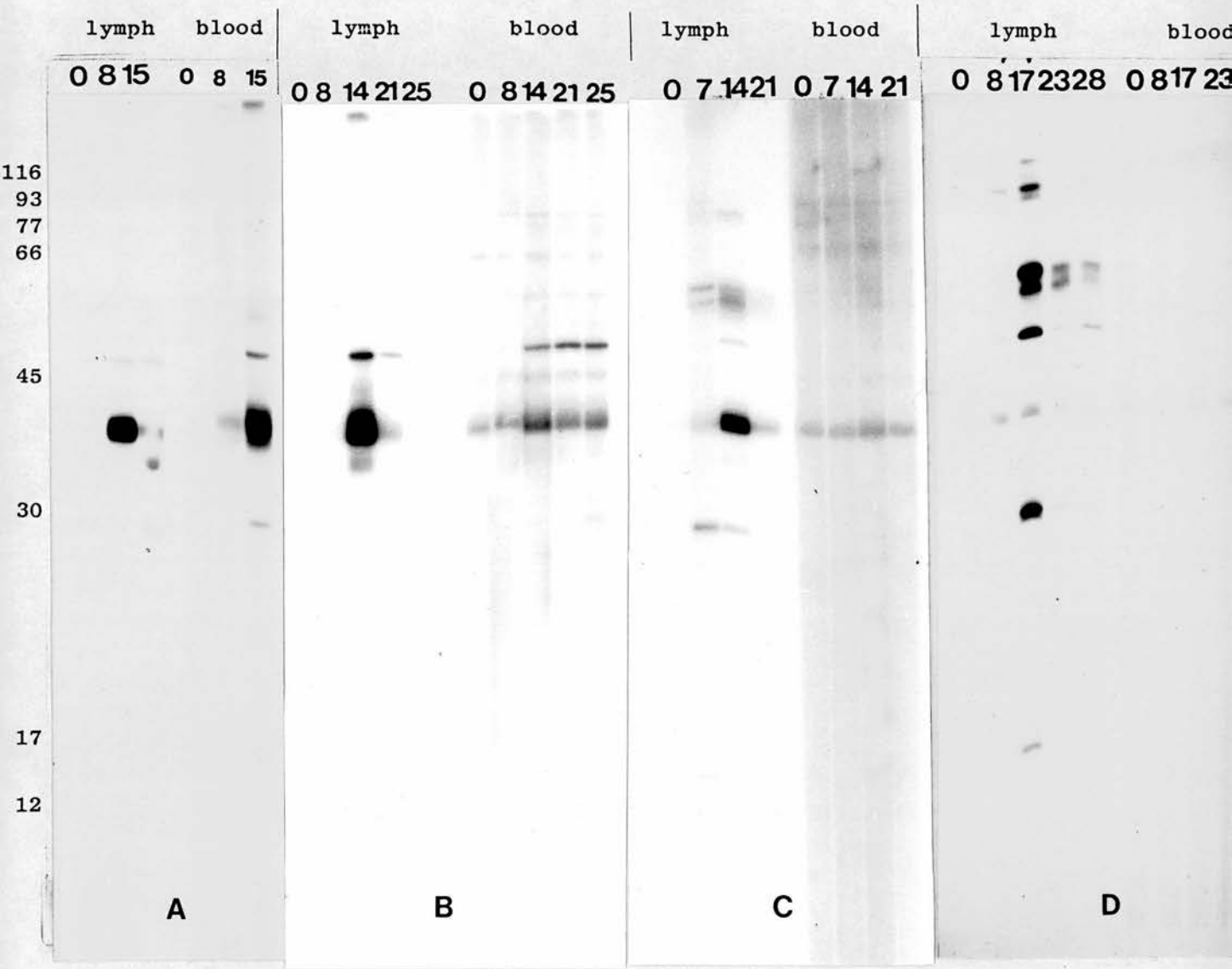


Figure 3.3. Immunoblot profiles of lymph and blood sera from four seronegative sheep at the days post-infection indicated. A. Sheep 1877J. B. Sheep 1778J. C. Sheep 1258J. D. Sheep 1889J. M_r standards are marked in kDa.

None of the three sheep in group A yielded chlamydiae from the lymph throughout the experiment, or from the spleen or lymph nodes samples at the end of the experiment (H.-S.Huang, personal communication). The presence of circulating antibodies, particularly those found at the beginning of the experiment, namely anti-MOMP, -60kDa, and -120kDa antibodies, may be partially responsible for inhibiting the spread of the infectious chlamydiae via the lymphatics. Although the possibility that these circulating antibodies were merely coincident with an effective cellular response exists, the role of their corresponding antigens in protection needs to be investigated.

Seronegative non-immune sheep - group B

Figure 3.3 shows the immunoblot profiles of lymph and sera from seronegative sheep in response to subcutaneous infection with *C.psittaci*. In three sheep 1877J, 1778J, 1258J, there was a strong but transient lymph node response to MOMP and a few other antigens of apparent M_r 50 kDa and 30 kDa. Depending on the sheep, the MOMP response was present beginning d8 to d14 post-infection. By d15 to d21, the response was much reduced. The antibody response in the sera lagged behind the lymph node response by about one week and might indicate lymphocyte traffic by routes other than the efferent duct. Alternatively, infectious organisms deposited subcutaneously could have eventually spread elsewhere other than via the popliteal lymph node to evoke the delayed antibody response systemically.

In contrast to the first three sheep, sheep 1889Jgave very strong but transient antibody reactions to the 91, 60, 58, 50 and 30 kDa antigens but only weak reactions to MOMP and 18K antigens. The relatively strong reactions to other antigens declined by 28 days. While the lymph node response was detectable, no reactions above background were detected for the serum samples.

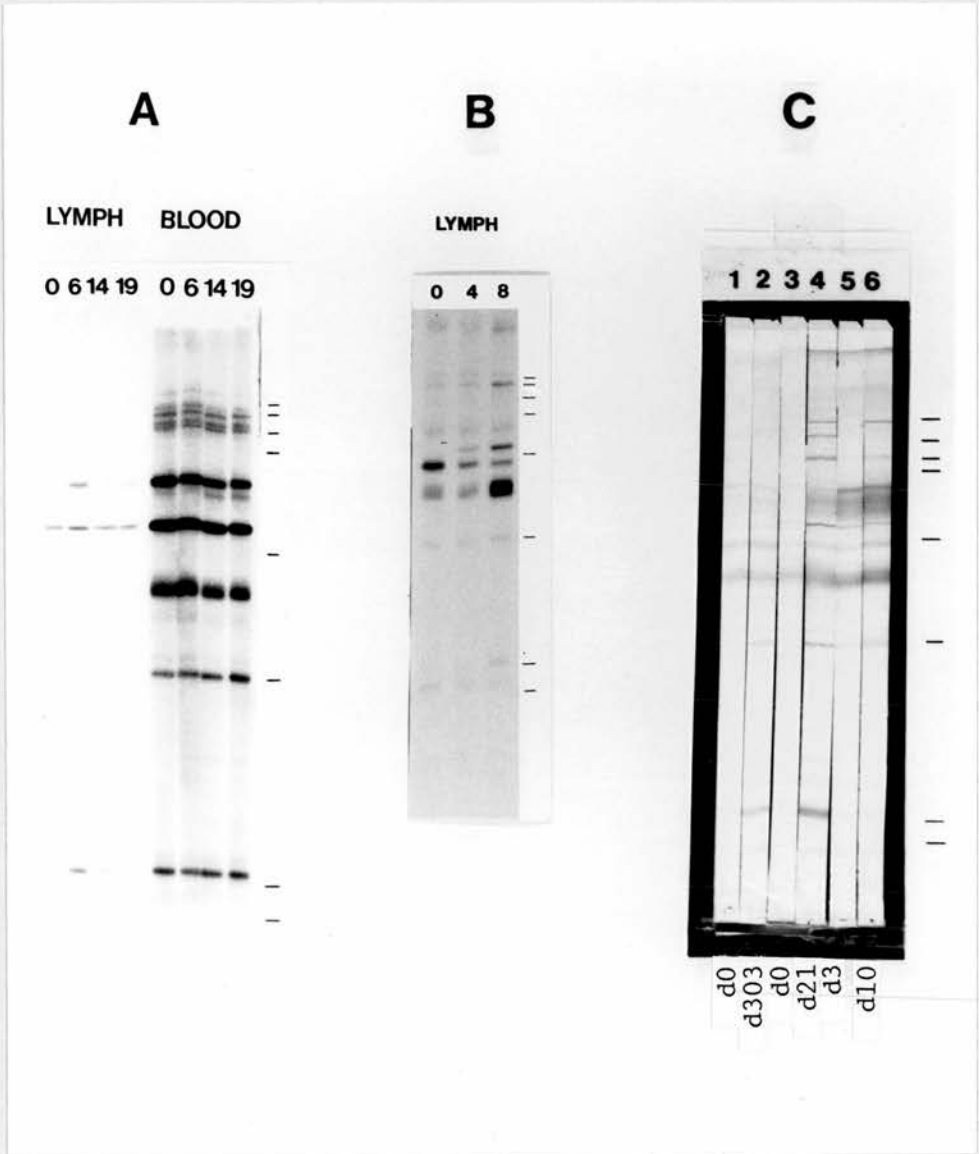


Figure 3.4. Immunblot profiles of lymph and blood sera from seroconverted (Panel **A**) and vaccinated sheep (Panel **B**) taken at the indicated days post-infection. M_r standards indicated are the same as that in Figure 3.1, namely, 116, 93, 77, 67, 45, 30, 17 and 12 kDa from the top. Panel **C** shows the immunoblot profiles of serum samples from women who suffered zoonotic abortion, taken at the indicated days post-abortion. The reactive antigens of each profile is given within the parentheses:

Case a. - lane 1 (60,43,40,18kDa) and
lane 2 (43,40,30,18kDa,LPS);
Case b. - lane 3 (43,40kDa) and
lane 4 (120,100,89,78,58,54-56,50,43,40,32,30,23,18,LPS);
Case c. - lane 5 (60,58,54-56,50,43,40,30,18kDa) and
lane 6 (120,78,60,58,54-56,50,43,40,30,18kDa).

A comparison of the IgG and IgM response of the lymph from sheep 1778J was also carried out (data not shown). High background with the HRP-conjugated anti-IgM probe was observed. MOMP was the most clearly identified antigen. At day 14, reactions were also visible to the high M_r quartet, the 60, 50, 43, 18 and 12 kDa antigens. The IgG response was similar to that observed when radio-iodinated anti-$F(ab)_2$ antibodies were used.

Lymph and blood samples collected from a fifth sheep 1764J did not give any detectable antibody reactions by immunoblotting (data not shown). The total absence of antibody reactions was also observed in another experimental sheep (909H) which had been vaccinated.

Seropositive infected sheep - group C

One sheep was found to have seroconverted by the CF test before the cannulation experiment presumably because of a natural infection. The serum antibody profile (Figure 3.4A) resembled the post-abortion sera profiles and suggested that it would not be easy to distinguish between infected and post-abortion sheep by serological methods. It is not known why the antibody profile of the lymph showed a slightly limited range of antibody specificities compared to that of the serum at day 0. On reinfection, the lymph node reacted to a more restricted range of antibody responses, possibly because of a limited repertoire of B-cells at the site.

Vaccinated sheep - group D

Two sheep 157J and 909H vaccinated with a commercial vaccine were also used for the cannulation experiment. The initial immunoblot profile of sheep 157J showed a relatively limited range of antigens recognised by circulating antibodies. (Figure 3.4B). Infection was able to boost the antibody response but this did not reach the levels seen with post-abortion ewes. No detectable antibody response whatsoever could be found in sheep 909H (data not shown) despite a detectable cellular

response, an observation similar to that of sheep 1764J. Error in sampling is unlikely since multiple samples were carried out over a week in both cases. Moreover, blood samples also did not contain chlamydia-specific antibodies. Failure to deposit live chlamydiae or vaccine is unlikely because of the detectable histological and cellular changes (Huang, personal communication). Repetition of the immunoblotting also gave negative results despite positive blots with control sera. The reason for the failure to respond serologically to chlamydial vaccine is not known.

III. ANALYSIS OF SERUM SAMPLES FROM ZOONOTICALLY INFECTED WOMEN

Serum samples from three women suffering from zoonotic infection with OEA strains of *C.psittaci* were obtained from Dr I. W. Smith, Department of Bacteriology, University of Edinburgh. These were probed using anti-human IgG-horse radish peroxidase conjugates (SAPU). The results are shown in Figure 3.4C. Blots showed that the chlamydial components that are antigenic in women were essentially the same as those antigenic in sheep although the 43kDa antigen appeared more frequently.

The chlamydia-specific reactions were weak at the day of abortion but became much stronger up to 21 days post abortion. In case A, it appeared that the antibodies can last up to 10 months after the disease episode. The antibody response, as measured by the immunofluorescence test, was also low prior to abortion but peaked at or just after abortion (data from I.W.Smith). Owing to the unavailability of prenatal serum samples, it is not known whether specific antibodies can be easily detected by immunoblotting before the crisis. Further immunoblotting analyses of other serum samples were also done but these gave a high non-specific background which has been observed in previous studies on human sera (Newhall *et al*, 1982). The amount of each serum sample available was not sufficient to permit the determination of optimal blotting conditions that may reduce the background for interpretable results.

PART 2: IDENTIFICATION OF MOMP AS A POTENTIALLY IMMUNOPROTECTIVE ANTIGEN

The serum samples of sheep used in a vaccination-challenge experiment (gift of I.E.Anderson, G.E.Jones and A.J.Herring) were tested by immunoblotting to analyse the specific antibody responses to an experimental vaccination procedure and to identify antigens that might correlate with protection for further evaluation as potential subunit vaccines. Details of the experiment have been submitted for publication (Anderson *et al*, submitted - see Appendix) and are briefly outlined below.

Seventeen ewes free from CF antibodies were selected for mating, ten for the test group and seven as controls. Purified EBs (800 micrograms) of *C.psittaci* S26/3 ovine abortion strain were inactivated by glutaraldehyde treatment, precipitated with aluminium hydroxide as an adjuvant and formulated into a water-in-oil emulsion to make sufficient 1ml doses of vaccine for the ten animals. Each ewe in the test group was vaccinated subcutaneously with two doses of the vaccine preparation three weeks apart. The ewes were bled at regular intervals and the sera frozen at -20°C for subsequent analysis. Of the ten vaccinated ewes, six became pregnant and were subsequently challenged via a subcutaneous route with $10^{5.5}$ 50% chick embryo lethal doses (CELD50) of live *C.psittaci*, S26/3 (1 ml each). The seven control unvaccinated ewes were similarly challenged.

All six vaccinated sheep were protected against chlamydial abortion and produced eight healthy lambs. No clinical signs of OEA were detected. Chlamydiae could not be isolated from vaginal swabs or fetal membranes. In contrast, chlamydiae could be isolated from five of the seven unvaccinated sheep, four of which suffered abortion. Five of the ten lambs produced were dead. Thus administration of the experimental vaccine had significantly reduced the incidence of abortion ($P < 0.05$ in the Fisher's Exact one-tail test) and infection ($P<0.02$), and significantly improved the lamb survival rate ($P<0.03$).

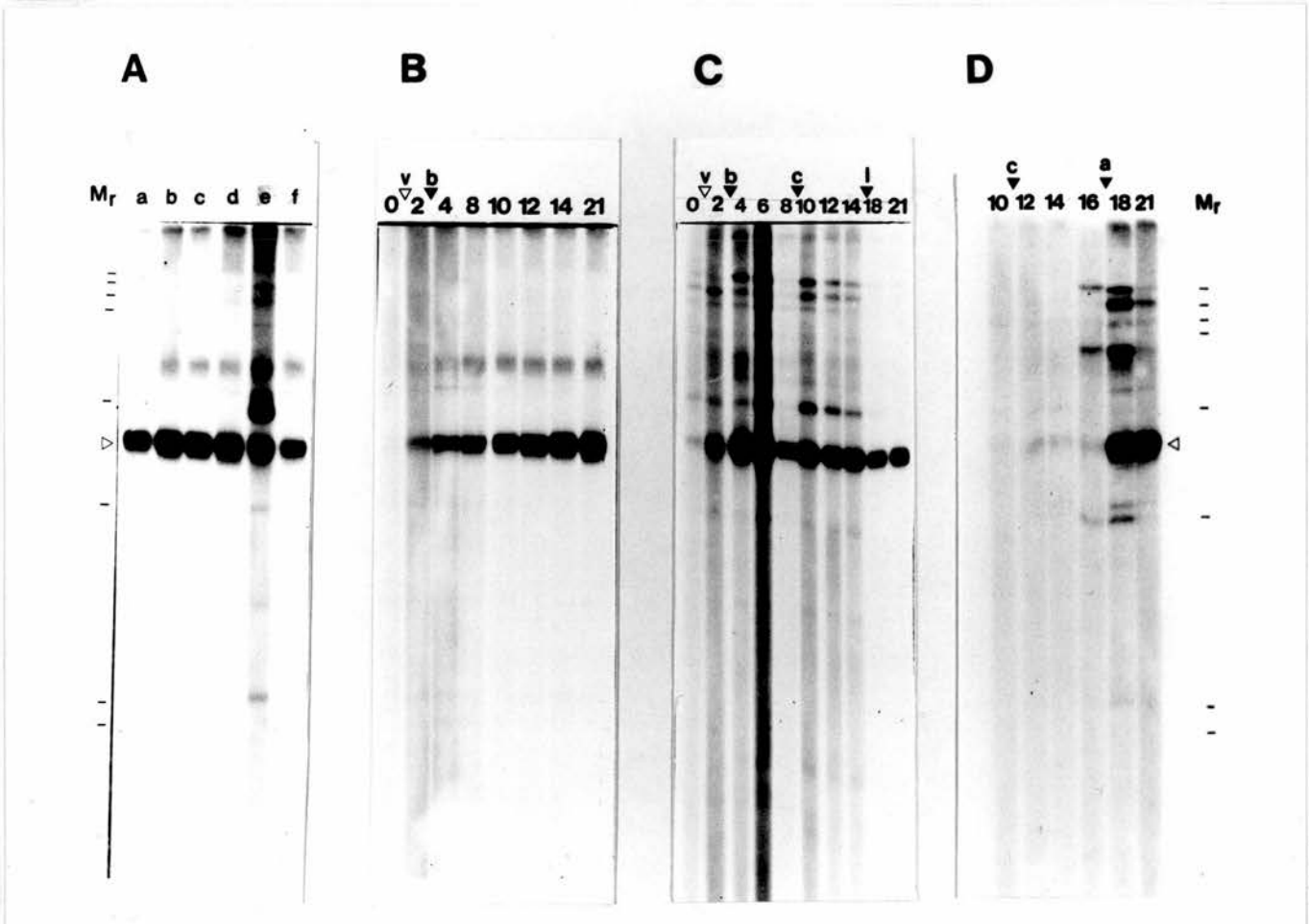


Figure 3.5. Immunoblot profiles from purified EB vaccination-challenge experiment. A. Sera from six vaccinated sheep (a-f) taken 8 weeks post-vaccination but before challenge. B. Antibody response of a non-pregnant unchallenged ewe. C. Antibody response of a pregnant, vaccinated and protected ewe. D. Antibody response of an unvaccinated, challenged and aborted ewe. The numbers indicate the weeks post-vaccination. The arrows marked indicate the time of vaccination (v), boost (b), challenge (c), abortion (a) and lambing (l). M_r standards are marked (from the top in kDa - 116,93,77,67,45,30,17,12). MOMP is arrowed.

Immunoblotting analysis was thus carried out on the serum samples taken from these ewes to determine if any immunoreactive antigen correlated with protection.

RESULTS AND DISCUSSION

Of the chlamydial antigens identified in Part I (Table 3.1), sera from the vaccinated sheep produced antibodies predominantly against the putative major outer membrane protein, MOMP (Figure 3.5A). This striking anti-MOMP response was consistently found in the vaccinated sheep. Although antibody responses to other antigens were also present, they were weak reactions (50-60kDa diffuse band) and/or were not consistent (for example, the 43kDa antigen). The response from a non-pregnant and unchallenged ewe showed that this MOMP response increased throughout the period of the experiment (Figure 3.5B). Typical time-course profiles of pregnant ewes which were subsequently challenged showed similar kinetics although the increase in anti-MOMP reaction was not so pronounced (Figure 3.5C).

Pre-challenge sera from the control group showed that all unvaccinated sheep were relatively free from chlamydia-specific CF antibodies (I.E.Anderson, personal communication); post-infection sera showed very weakly reactive bands (data not shown). A few days prior to or immediately after abortion, a strong antibody response was mounted (Figure 3.5D), a finding similar to that of McClenaghan *et al* from previous infection studies (in preparation). Although the post-abortion response included a reaction with MOMP, it was also strongly directed to other chlamydial antigens, typically observed for post-abortion sheep sera described in Part 1. Thus there was a difference in the kinetics and in the nature of the immune response to chlamydial infection between vaccinated and unvaccinated ewes (see also CF data in Anderson *et al*, submitted - Appendix).

The immunoblotting results showed that the anti-MOMP antibody response in vaccinated sheep correlated with protection against chlamydial abortion under the vaccination-challenge

procedure described. The circulating anti-MOMP antibodies lasted throughout the period of the experiment without diminution (Figure 3.5B and 3.5C). It is speculated that in this experiment, anti-MOMP antibodies may have had a role in protection. The data indicated strongly that the immunogenic and protective properties of the MOMP ought to be investigated further.

PART 3: FURTHER DISCUSSION AND SUMMARY OF THE IMMUNOBLOT ANALYSES

Similarity of immunoblot profiles between OEA isolates, S26/3 and A22

The initial immunoblotting experiments that used both OEA isolates, S26/3 and A22, showed no difference in any pair of immunoblot profiles for each serum sample tested (more than a dozen of either sheep or human serum samples). Consequently, the data presented in this chapter only showed blots carried out on S26/3 antigens. The similarity in these profiles suggested that if there were any distinguishing epitopes between the two isolates, these epitopes could not be resolved by SDS-PAGE. The similarities in band intensities also suggested that if there were any epitopic differences residing at each protein band, these did not differ sufficiently in antigenicity or in affinity. Alternatively, such differences could have been obscured by common epitopes on the same band. McClenaghan *et al* (1984) have already demonstrated that restriction endonuclease analysis profiles of various *C.psittaci* isolates from ovine abortions including S26/3 and A22 do not show any significant differences. No differences between the SDS-PAGE profiles of S26/3 and A22 elementary bodies has been detected (Herring *et al*, manuscript in preparation; see Chapter 4) suggesting that differences between the two isolates, as indicated by the vaccination-challenge experiments of Aitken *et al* (1981), are difficult to detect using biochemical or serological techniques. Data on peptide mapping of MOMP presented in the next chapter further illustrate this point.

The role of antibody in protection

In a previous study, strong anti-MOMP antibody responses could not be detected in post-abortion sheep (McClenaghan *et al*, 1986). However in this study, it was noted that several post-abortion sheep maintained a high level of anti-chlamydial antibodies, notably to MOMP, which persisted for some weeks or even months after abortion and can also be detected in the lymph of post-abortion sheep. Sheep once aborted are known not to abort again from chlamydial infection, not at least from the same strain (Stamp *et al*, 1950; McEwen *et al*, 1951a). It is possible that antibodies against MOMP may play a role in protection by neutralising infectious EBs in the circulation thereby preventing the haematogenous spread from the unknown "latent" site to the placenta. How important antibodies are in immunity may be assessed by experiments involving passive transfer of antibodies from protected animals (see further discussion in Chapter 5 and Chapter 8).

Caution in the interpretation of immunoblotting analyses

The immunoblotting data presented in this chapter should be interpreted in the light of the fact that the electroblotted antigens used in this study were treated by incubation in SDS and 2-mercaptoethanol at 100°C. Therefore, any immunoreactive epitope sensitive to this procedure will not be detected by immunoblotting in the absence of renaturation. It is likely, however, that each antigen carries linear epitopes that are resistant to the denaturing treatment and that renaturation and reformation of some conformational epitopes do occur, both of which should be detected by this method.

Chlamydial lipopolysaccharide is a commonly detected antigen (Batteiger and Rank, 1987; Caldwell *et al*, 1987; Ramsey *et al*, 1989). In this study, it was not found to be a strong antigen and in some cases, no reaction to LPS was detected. It is possible that the immunoblotting procedure used does not favour the detection of non-proteinaceous components such as chlamydial lipopolysaccharide (Batteiger *et al*, 1982; Newhall

et al, 1982). Nevertheless, it is generally thought that chlamydial lipopolysaccharide bears epitopes reactive with CF antibodies which do not appear to have a role in preventing ovine abortion (McEwen and Foggie, 1954; Dawson *et al*, 1986a and 1986b). LPS itself is not thought to be an important antigen in protective immunity, for example, in ocular infection (Taylor and Prendergast, 1987; see discussion in Chapter 5).

Since MOMP is present in greater abundance than other chlamydial components, it could be argued that at the level of sensitivity which the immunoblotting procedure was carried out, antigens other than MOMP are at sub-optimal levels. Hence the dominant MOMP response detected, particularly in the vaccination-challenge experiment, could be due to an antigenic mass effect. However, post-vaccination sera showed a lack of strong reaction to other bands compared with post-abortion sera probed under the same conditions. This would suggest that the vaccinated ewes responded almost only to MOMP.

These caveats apart, the data reported here strongly implicates MOMP as having a possible role in protection. The weak diffuse antigen migrating between the 50kDa and 60kDa antigens was also detected in sera from protected animals; it may be analogous to the 54-64kDa chlamydial outer membrane complex (COMC) antigen possessing cross-reactive epitopes between strains of *C.psittaci* reported by Fukushi and Hirai (1988). It is not certain if this diffuse band or the occasionally observed 57kDa band (Table 3.1) is the same antigen as the 57kDa antigen described by Morrison *et al* (1989) and cloned by Menozzi *et al* (1989). More investigations to characterise its properties are needed.

Summary

In summary, ovine abortion *C.psittaci* has been shown to possess many antigenic bands in the immunoblotting analyses, including a predominant band with an apparent M_r of 39kDa to 40kDa. This protein band is thought to be the major outer

membrane protein (MOMP), an outer membrane protein which is analogous to those of other chlamydial strains reported in the literature (Salari and Ward, 1981; Caldwell *et al*, 1981; Hatch *et al*, 1981; Newhall *et al*, 1982; Fukushi and Hirai, 1988; Newhall, 1988 and references therein). A list of chlamydial antigens has been compiled from immunoblotting data based on a variety of lymph and blood serum samples from sheep and humans. This list should form a basis for future investigation of antigens from OEA *C.psittaci*.

The immunoblot profiles of sheep showed a degree of variability in response within each immunological category such that differentiation of immune status based on immunoblot profiling may not be possible. The extent of variability, however, should be confirmed with a larger sample size in each category. Retrospective analysis of serum samples from vaccinated and demonstrably protected ewes in a vaccination-challenge experiment showed a remarkable lack of reaction to chlamydial antigens other than to the 39-40kDa antigen, the putative MOMP. This result has strongly implicated the putative MOMP of OEA *C.psittaci* as a potential immunogen. In view of the known properties of chlamydial MOMP, it was considered a primary target for vaccine testing. The following two chapters report on the characterisation of chlamydial antigens, particularly MOMP, and on the adaptation of reported techniques to prepare sufficient MOMP for a vaccination-challenge experiment.

CHAPTER 4

SEVERAL CHARACTERISTICS OF MOMP AND OTHER PROTEINS FROM OVINE ABORTION *C.PSITTACI*

INTRODUCTION

All chlamydial strains reported in the literature possess a major outer membrane protein (MOMP) which dominates the SDS-PAGE and immunoblot profiles by its abundance and immunodominance. MOMPs from different strains are heterogeneous in mobility in SDS-PAGE gels (Caldwell *et al*, 1981; Salari and Ward, 1981; Fukushi and Hirai, 1988) and are the sites of antigenic variation (Caldwell and Schachter, 1982; Stephens *et al*, 1982; Newhall *et al*, 1982). MOMP is resistant to extraction with detergents such as sodium N-lauroyl sarcosinate (Sarkosyl) and remains with the outer membrane (OM) fraction. It is also surface-exposed and can be radio-iodinated (Salari and Ward, 1981; Caldwell *et al*, 1981; Hatch *et al*, 1981; Caldwell and Judd, 1982). At the beginning of this project, such data were not available for ovine enzootic abortion (OEA) isolates of *C.psittaci*. Moreover, in view of the increasing number of reports on the functional, developmental and potentially protective role of chlamydial MOMP, it was considered essential that a better understanding of the characteristics of MOMP from ovine abortion strains of *C.psittaci* should be attained.

Experiments presented in this chapter show that the 39 to 40 kDa putative MOMP antigen of *C.psittaci* S26/3 described in the previous chapter bears characteristics similar to MOMPs from other chlamydial strains. With a knowledge of these properties, procedures could then be developed to purify MOMP for further assessment of its role in immunoprotection against OEA. Some preliminary data regarding other antigens such as the putative adhesins are also presented.

Figure 4.1. Comparison of the components of various *C.psittaci* and *C.trachomatis* strains. Lane 1. Standards with M_r indicated in kDa. Lane 2. *C.trachomatis* LGV serovar L2. Lanes 3 to 15 are all *C.psittaci* strains: Avian 725 (3), Call0 (4), CH1287 ovine conjunctivitis (5), P787 ovine arthritis (6), ovine abortion isolates S26/3 (7), A22 (8), 84/501 (9), S124/3A (10), S152/3 (11), S507 (12), H574 (13), ovine intestinal isolates from faeces 84/S604 (14) and 84/521F (15).

RESULTS

Comparison of SDS-PAGE profiles of *C.psittaci* and *C.trachomatis*

Elementary body preparations of various strains of *C.psittaci* and *C.trachomatis* were solubilised in SDS and 2-ME and their components separated by SDS-PAGE. Figure 4.1 shows the MOMP of *C.trachomatis* and its analogues in *C.psittaci* migrating with an apparent M_r ranging from 39 to 42 kDa. Owing to their abundance, this band dominates the protein profiles. No variations in the apparent M_r of the putative MOMPs (39.5 kDa) from the OEA isolates were detected. These MOMPs were distinct from that of other ovine strains such as P787 (ovine arthritis) and CH1287 (ovine conjunctivitis). The two ovine faecal isolates possessed MOMPs of different apparent M_r, one of which (84/521F) co-migrated with the MOMPs from the OEA isolates.

The SDS-PAGE profiles of all tested OEA isolates generally appeared identical whereas differences with that of other ovine strains and with *C.trachomatis* L2 were clearly visible. Besides the difference in the mobility of MOMPs, there were complex variations in the protein bands among the *C.trachomatis*, avian *C.psittaci*, meningopneumonitis (Mn) *C.psittaci* (laboratory strain Cal10) and other ovine *C.psittaci* strains. For example, there are slight variations in apparent M_r in a protein band migrating at about 60kDa. Other striking differences include protein bands below 20kDa for these representative groups (Figure 4.1).

Comparison of immunoblot profiles of *C.psittaci* and *C.trachomatis*

Electrophoretograms of solubilised EBs from the various chlamydial strains used in the previous section were transferred onto nitrocellulose paper and probed with a gnotobiotic lamb antiserum raised by infection with S26/3 EBs (gift of Dr J.M.Sharp). Initial immunoblotting profiles with the homologous antigen showed that much of the reactivity was directed against the 39.5 kDa MOMP antigen (data not shown). Figure 4.2A shows that the polyclonal serum reacted with all ovine abortion MOMPs

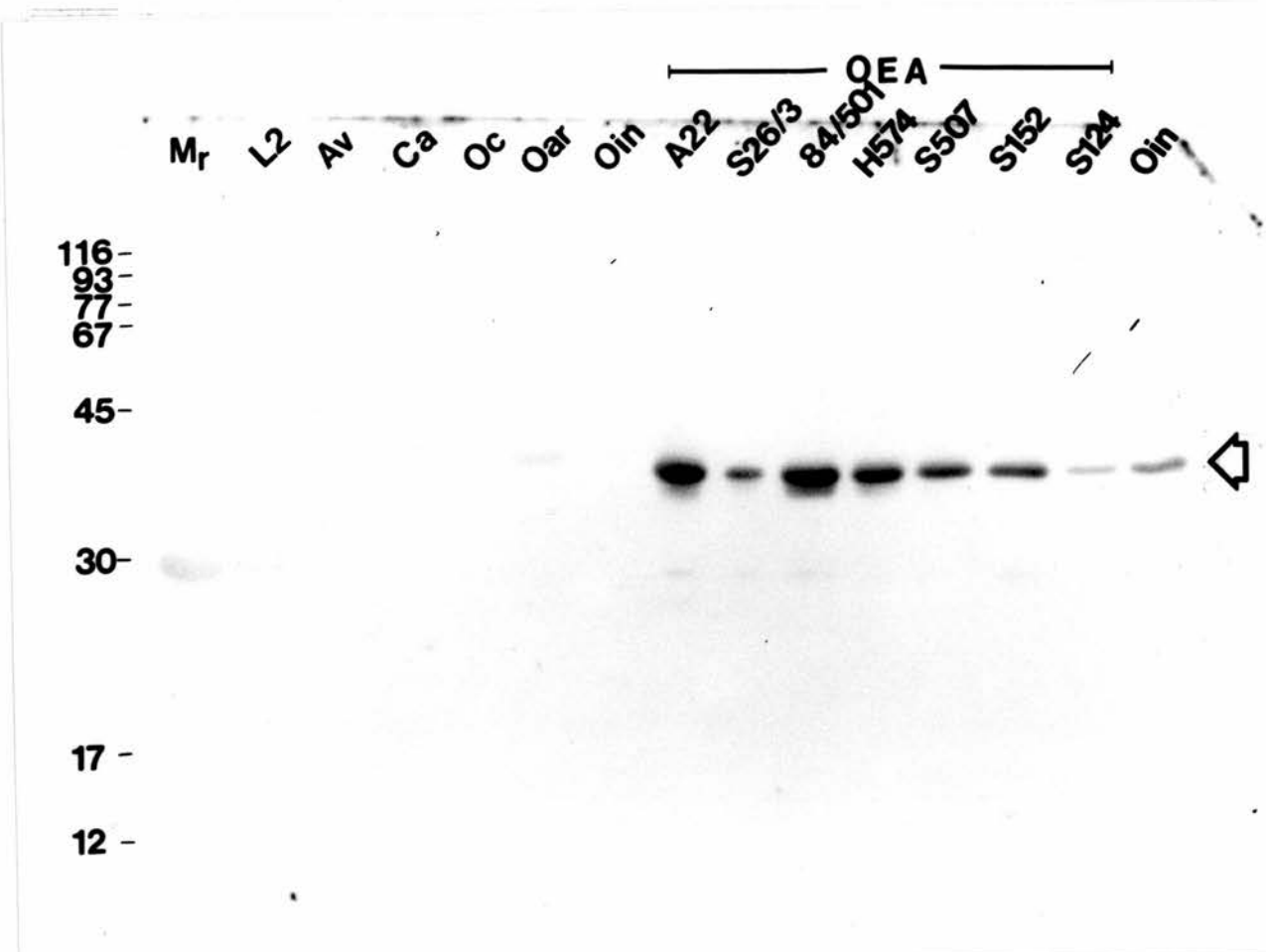


Figure 4.2A. Antigenic profile of various *C.psittaci* and *C.trachomatis* strains by immunoblotting with a gnotobiotic lamb anti-S26/3 antiserum. (From left to right) Lane 1. M_r standards are given in kDa. Lane 2. *C.trachomatis* LGV serovar L2. Lanes 3 to 15 are all *C.psittaci* strains: Avian 725 (3), laboratory strain Cal10 (4), CH1287 ovine conjunctivitis (5), P787 ovine arthritis (6), ovine abortion isolates A22 (8), S26/3 (9), 84/501 (10), H574 (11), S507 (12), S152/3 (13), S124/3A (14), ovine intestinal isolates from faeces 84/S604 (7) and 84/521F (15).

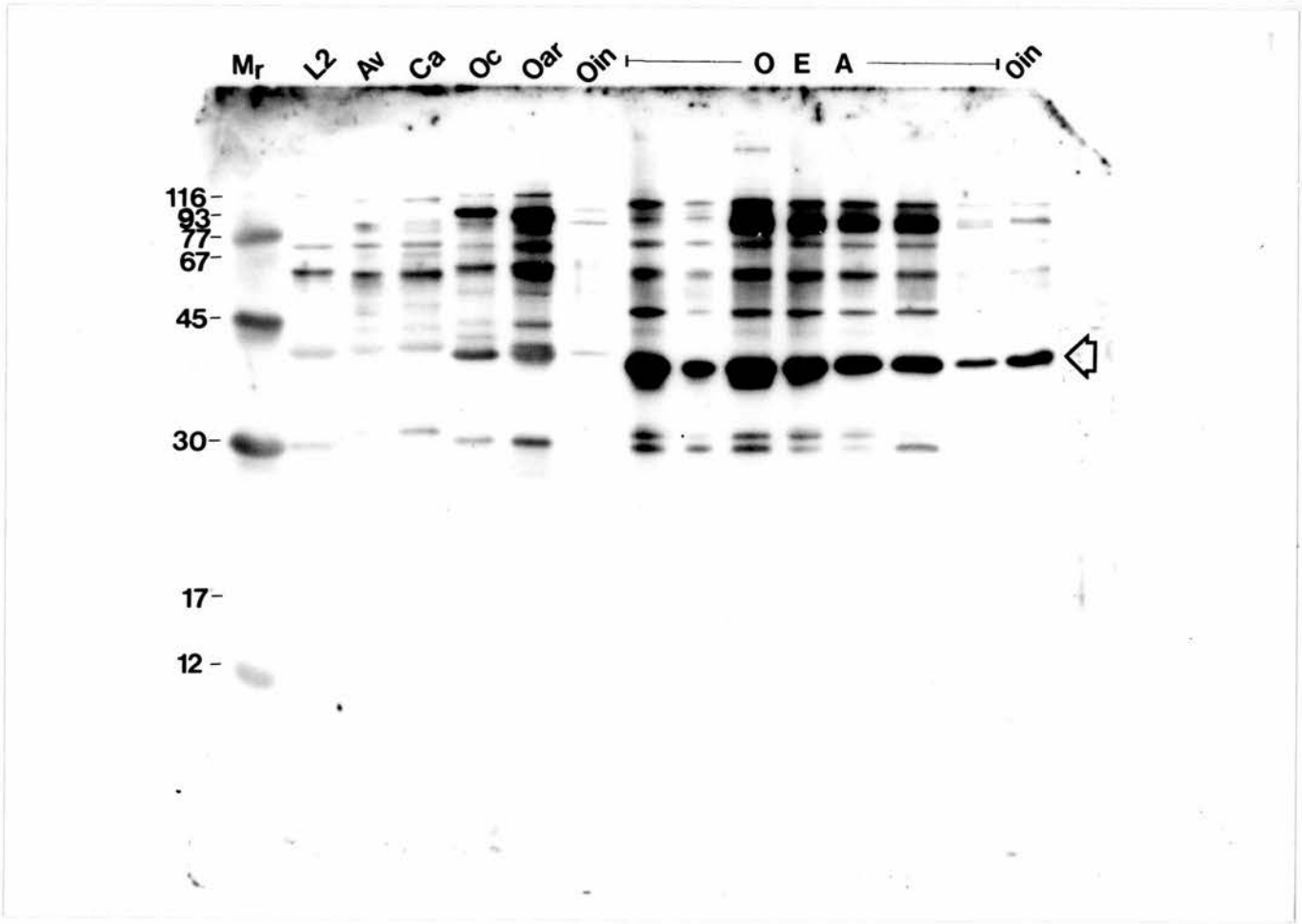


Figure 4.2B. Antigenic profile of various *C.psittaci* and *C.trachomatis* strains by immunoblotting with a replica of the blot in Figure 4.2A using a post-abortion sheep (2123K) anti-S26/3 antiserum. All annotations are as given in Figure 4.2A.

including S26/3 MOMP. Other antibody reactions which were directed against the MOMP bands of other chlamydial strains were barely detectable and indicated the variation in M_r. The loadings in the lanes for the two intestinal strains and one OEA strain (S124/3A) were much lower than for other strains because of poor yields in culture. Consequently, some bands which are probably present in their profiles were not clearly seen.

A replica of this blot was probed with a post-abortion antiserum (2123K) from an ewe experimentally infected with a yolk-sac inoculum of EBs (S26/3). Typical bands characteristic of the OEA chlamydial antigens listed in Table 3.1 were observed (Figure 4.2B). These bands were common throughout the ovine abortion strains although their relative intensities differed. This antiserum cross-reacted weakly with the MOMPs of other ovine strains but reactions with that of *C.trachomatis* L2, avian *C.psittaci* and Call0 were hardly detectable. The blot also showed that the MOMP of one intestinal strain, 84/521F, co-migrated with OEA MOMPs while the other, 84/S604, co-migrated with ovine arthritis or ovine conjunctivitis MOMPs.

Two or three bands of the high Mr quartet (see Table 3.1) were visible in the OEA strain profiles (Figure 4.2B). One or two were present in other chlamydial strains. The 77 kDa antigen of S26/3 was also present in all strains with the same mobility. The 60kDa bands appeared to cross-react throughout the genus although there were variations in M_r as described in the previous section. The 50 kDa antigen of OEA *C.psittaci*, however, did not appear to have an analogue in the other strains. A 30 kDa doublet of OEA *C.psittaci* was detected but only one band was present in the corresponding region of the profiles of other strains. The polyclonal serum of the ewe also cross-reacted with marker proteins namely myosin (17kDa), carbonic anhydrase (30kDa), ovalbumin (45kDa) and ovotransferrin (77kDa) presumably because these were present in the yolk-sac inoculum of EBs.

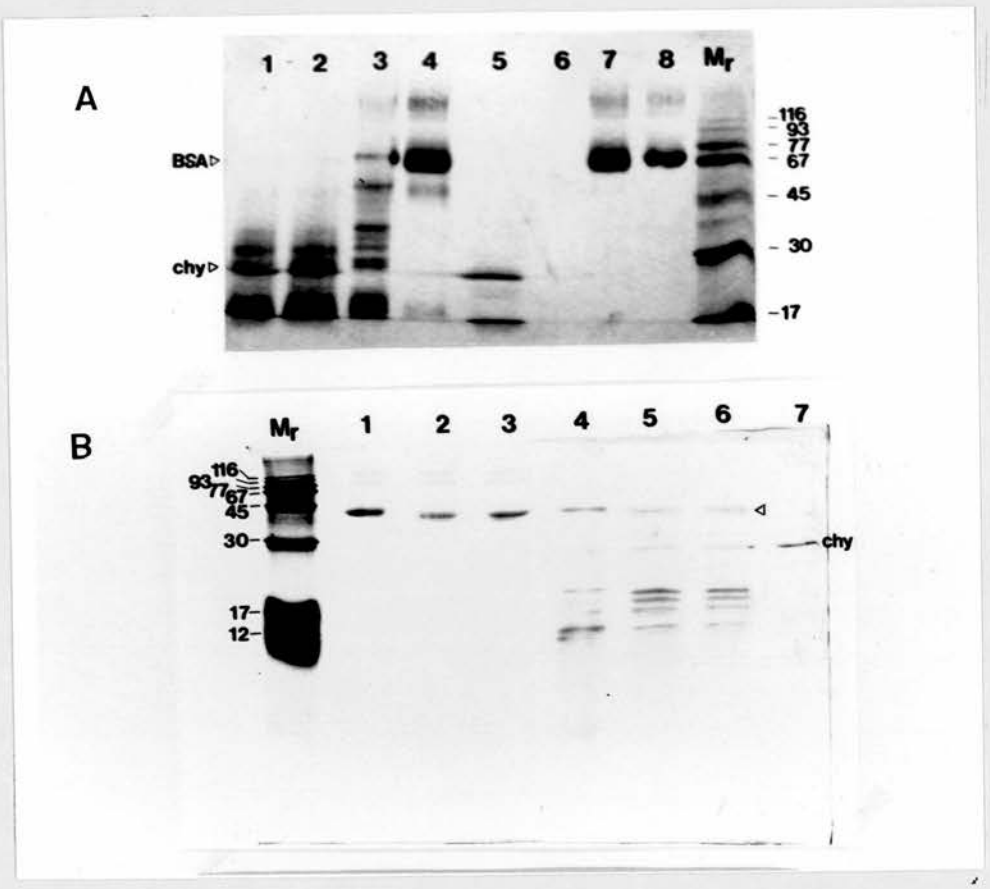


Figure 4.3A. Partial proteolytic digestion of bovine serum albumin (BSA) with a range of chymotrypsin concentrations. BSA (2μg) was incubated with chymotrypsin at 10μg (lane 1), 5μg (lane 2), 500ng (lane 3), 50ng (lane 4), 5ng (lane 7) and no chymotrypsin (lane 8). Lane 5 contained only chymotrypsin (10μg). Lane 6 was a buffer blank. The M_r standards are given in kDa. BSA and chymotrypsin are arrowed. The SDS-PAGE gel (12.5%) was stained with silver.

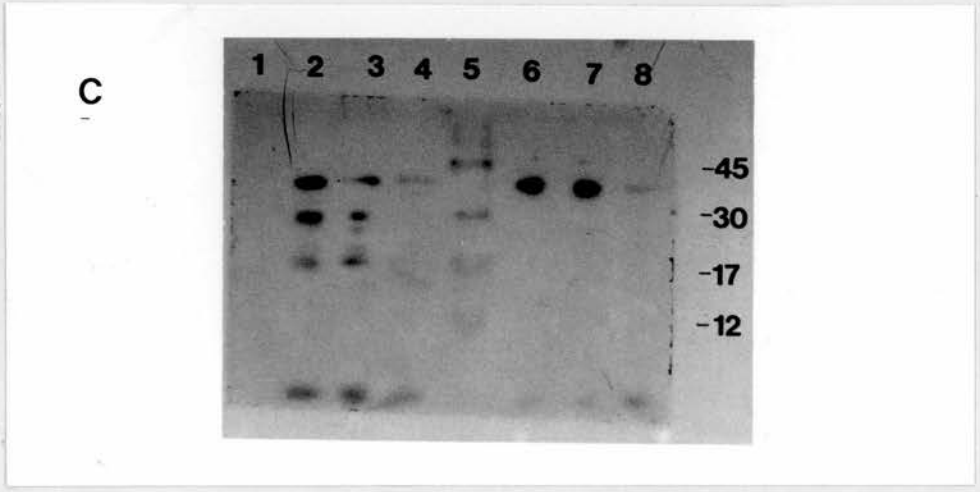
Figure 4.3B. SDS-PAGE comparison of partial chymotryptic profiles of MOMP (arrowed) from *C.psittaci* ovine arthritis strain P787 (lane 4) and *C.psittaci* OEA strains S26/3 (lane 5) and A22 (lane 6). Lanes 1 to 3 contained the respective MOMPs without chymotrypsin. Lane 7 contained chymotrypsin - 25kDa (1μg) only. The M_r standards are given in kDa. The gel (15%) was stained with silver.

Peptide mapping of MOMP using the method of Cleveland *et al* (1977)

Although MOMP is a main locus of heterogeneity among chlamydial strains, no differences in the mobility of MOMP was detected within OEA isolates of *C.psittaci*. Fragmentation of MOMP peptides by partial proteolysis was carried out to determine if any differences existed within the polypeptide. This experiment was carried with S26/3 and A22 MOMP in comparison with the MOMP from the ovine arthritis strain P787 which was already known to be different.

Typical results of the one-dimensional proteolytic mapping experiment is shown in Figure 4.3. Panel A shows the determination of a useful concentration range of chymotrypsin that will result in partial proteolysis of a polypeptide such as bovine serum albumin (BSA - 67 kDa) under the conditions of the experiment described in Chapter 2. Lane 5 shows that chymotrypsin migrated as a single dominant species at 25 kDa. Lanes 8, 7, 4, 3, 2 and 1 show the effect of increasing amounts of chymotrypsin on BSA. The results suggest that a useful range of chymotrypsin to use for profiling MOMP would be about half a microgram per lane.

Panel B (lanes 4, 5 and 6) of Figure 4.3 shows the partial chymotryptic profile of MOMPs from P787, S26/3 and A22 (ovine arthritis). Lane 7 shows that the 25 kDa band in the chymotryptic profile is due to chymotrypsin. Lanes 1, 2 and 3 are the substrate controls, containing the respective MOMP slices without the addition of chymotrypsin. Comparison of lanes 4, 5 and 6 shows that the A22 MOMP and S26/3 MOMP peptide profiles were identical while that of P787 was different. A 30 kDa band was missing in the P787 lane. The 18 kDa fragment in the OEA MOMP profiles may be equivalent to the 20 kDa band in the P787 profile.



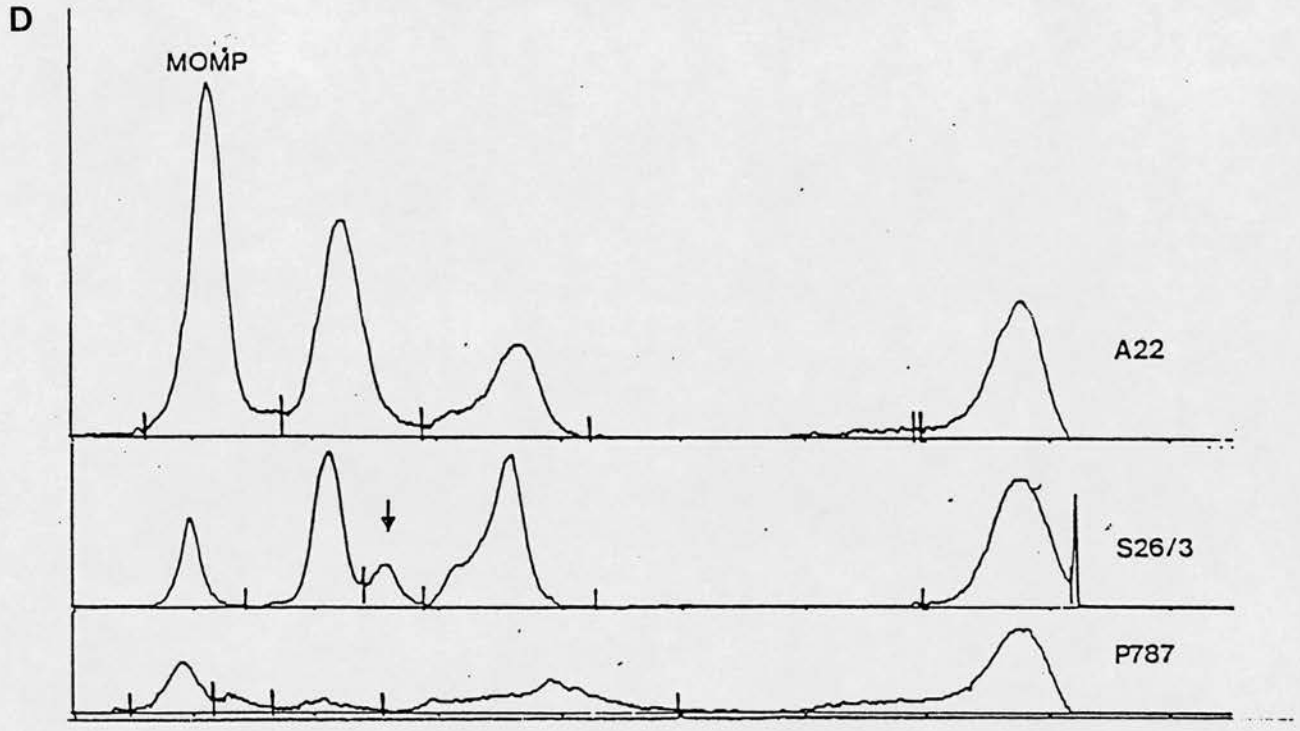


Figure 4.3C Immunoblot comparison of partial chymotryptic profiles of MOMPs from *C.psittaci* OEA strains A22 (lane 2) and S26/3 (lane 3), and ovine arthritis strain P787 (lane 4) using a gnotobiotic lamb anti-S26/3 antiserum. Lanes 6 to 8 contained the respective MOMPs without chymotrypsin. Lane 1 contained chymotrypsin only. The M_r standards are marked in kDa (lane 5).

Figure 4.3D Densitometric scans of the autoradiogram shown in Figure 4.3C - lanes 2, 3 and 4. The extra band is marked by arrow.

Similarly, these MOMPs were also digested with papain and proteinase K (Sigma Chemical Co.). With both enzymes, the same conclusion as that for chymotrypsin was reached, that the S26/3 MOMP profile was identical to the A22 MOMP profile but significantly different from that of P787. These proteolytic fragments are presented in Table 4.1.

Table 4.1. Proteolytic fragments of MOMPs (kDa) from papain and proteinase K digestions.

Papain			Proteinase K		
A22	S26/3	P787	A22	S26/3	P787
39.8	39.8	41.6	39.8	39.8	40.3
31.6	31.6	-	38.3	38.3	-
28.1	28.1	-	35.0	35.0	33.5
22.9	22.9	-	31.0	31.0	-
19.0	19.0	19.0	28.0	28.0	25.5
17.4	17.4	-	23.8	23.8	24.8
13.2	13.2	14.8	21.4	21.4	23.4
10.5	10.5	10.0	19.5	19.5	19.5
9.5	9.5	-	17.2	17.2	17.2
			12.6	12.6	13.2
			9.5	9.5	12.0
			7.9	7.9	10.0

A preliminary immunoblot of a replica of the chymotryptic profiles (Figure 4.3B) using the gnotobiotic lamb anti-S26/3 serum showed that the serum reacted strongly with both OEA MOMPs but gave a weak reaction to P787 MOMP (Figure 4.3C). Peptide fragments of P787 did not react very well compared with those of OEA MOMPs. Although the blot suggested that the mark at 28 kDa was present in the S26/3 profile and not in the A22 profile, it was difficult to decide whether it was an authentic band. Densitometric scans of these profiles are shown in Figure 4.3D.

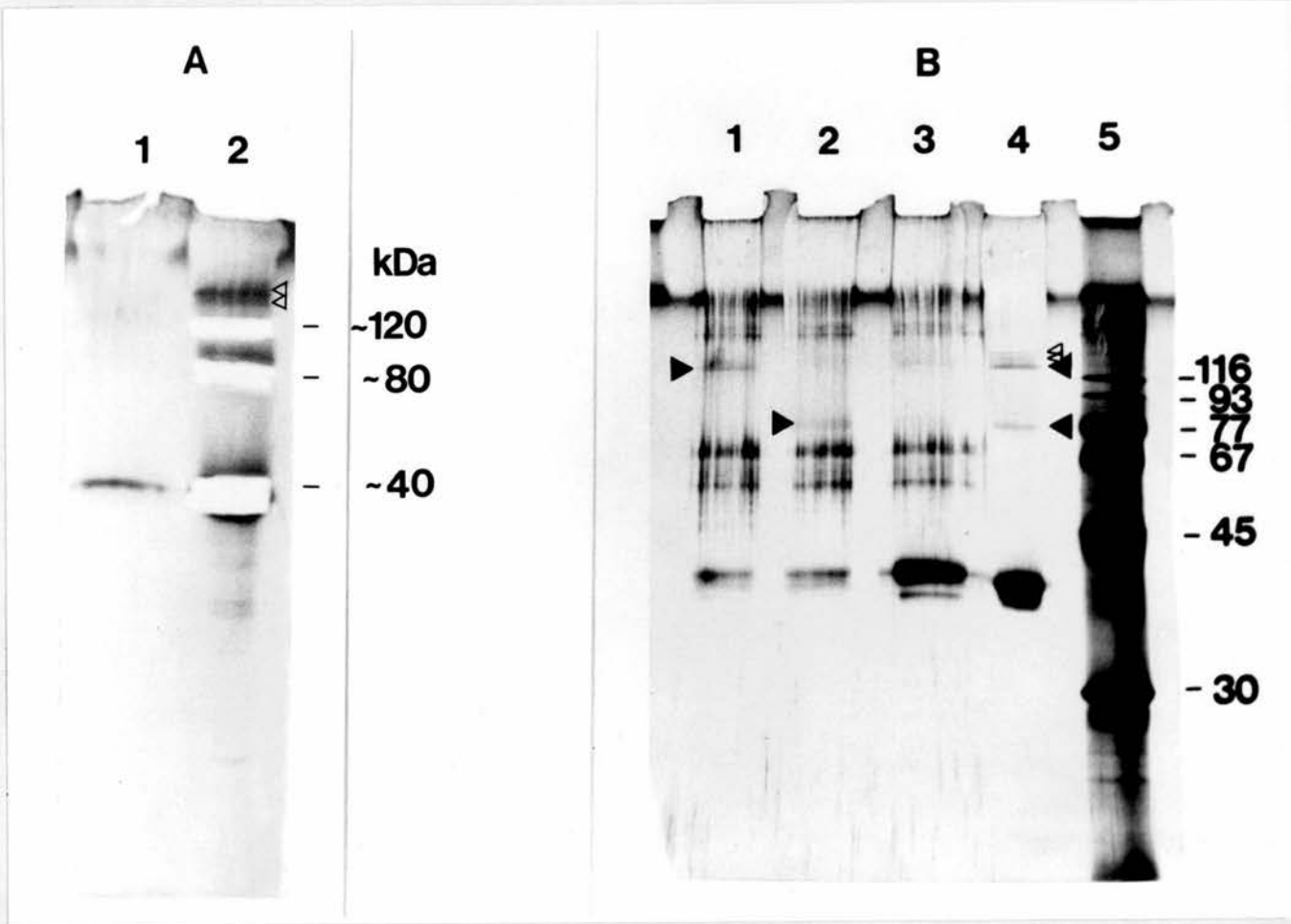


Figure 4.4. Oligomerisation experiment. A. Silver-stained SDS-PAGE profile of MOMP-containing gel slices equilibrated in Cleveland buffer, boiled in sample buffer (lane 1) and untreated (lane 2). The 120, 80 and 40 kDa bands in lane 2 were excised as shown. B. Silver-stained SDS-PAGE profile of re-equilibrated gel slices containing the 120 kDa band (lane 1), 80 kDa band (lane 2) and 40 kDa band (lane 3) boiled in sample buffer to reduce the protein. Lane 4. The same material from lane 3 was not treated in sample buffer. Lane 5. M_r standards in kDa.

Oligomerisation of MOMP *in vitro*

In the course of the peptide mapping experiments, it was noticed that specific high M_r bands always appeared in control lanes containing undigested MOMPs from all three strains (Figure 4.3B lanes 1, 2, 3). These undigested MOMPs had been equilibrated in Cleveland buffer (Chapter 2) which did not contain any reducing agent. These bands possessed an apparent M_r of approximately 80kDa and 120kDa. Further experiments are described in this section to test the hypothesis that these bands are MOMP oligomers, formed as a result of disulphide cross-linking between MOMP chains.

MOMP from A22 or S26/3 EBs was isolated by excision from SDS-PAGE gels. The gel slices containing MOMP were allowed to equilibrate in Cleveland buffer overnight. These slices were then subjected to SDS-PAGE. Besides the MOMP monomer migrating at the expected 39-40 kDa region, other bands were also observed including the 80kDa and 120kDa bands. These three bands were excised from the gel (Figure 4.4A - lane 2), boiled in sample buffer containing (2% w/v) SDS and 4% (v/v) 2-ME for 5 minutes, manually re-inserted into separate gel wells and subjected to SDS-PAGE. Figure 4.4B showed that both the 120kDa and 80kDa gel slices produced only the 40kDa monomeric MOMP band after boiling in reducing agent (lanes 1 and 2). Residual bands corresponding to 120 kDa and 80 kDa (solid triangles) in the respective lanes showed that the reduction was incomplete. The 40kDa gel slice was divided into two, one boiled in sample buffer (lane 3) and the other not treated (lane 4). The profiles showed that the untreated slice reproduced the 80kDa and 120kDa bands (solid triangles) and a few higher M_r bands (small open triangles) while the treated slice did not.

The extra bands at about 60-70 kDa in lanes 1, 2 and 3 were due to 2-ME. These bands were not in lane 4 because the gel slice was not boiled in sample buffer. It was also noted that migrating just in front of each 40kDa MOMP band was a weaker band, particularly in lanes containing boiled gel slices.

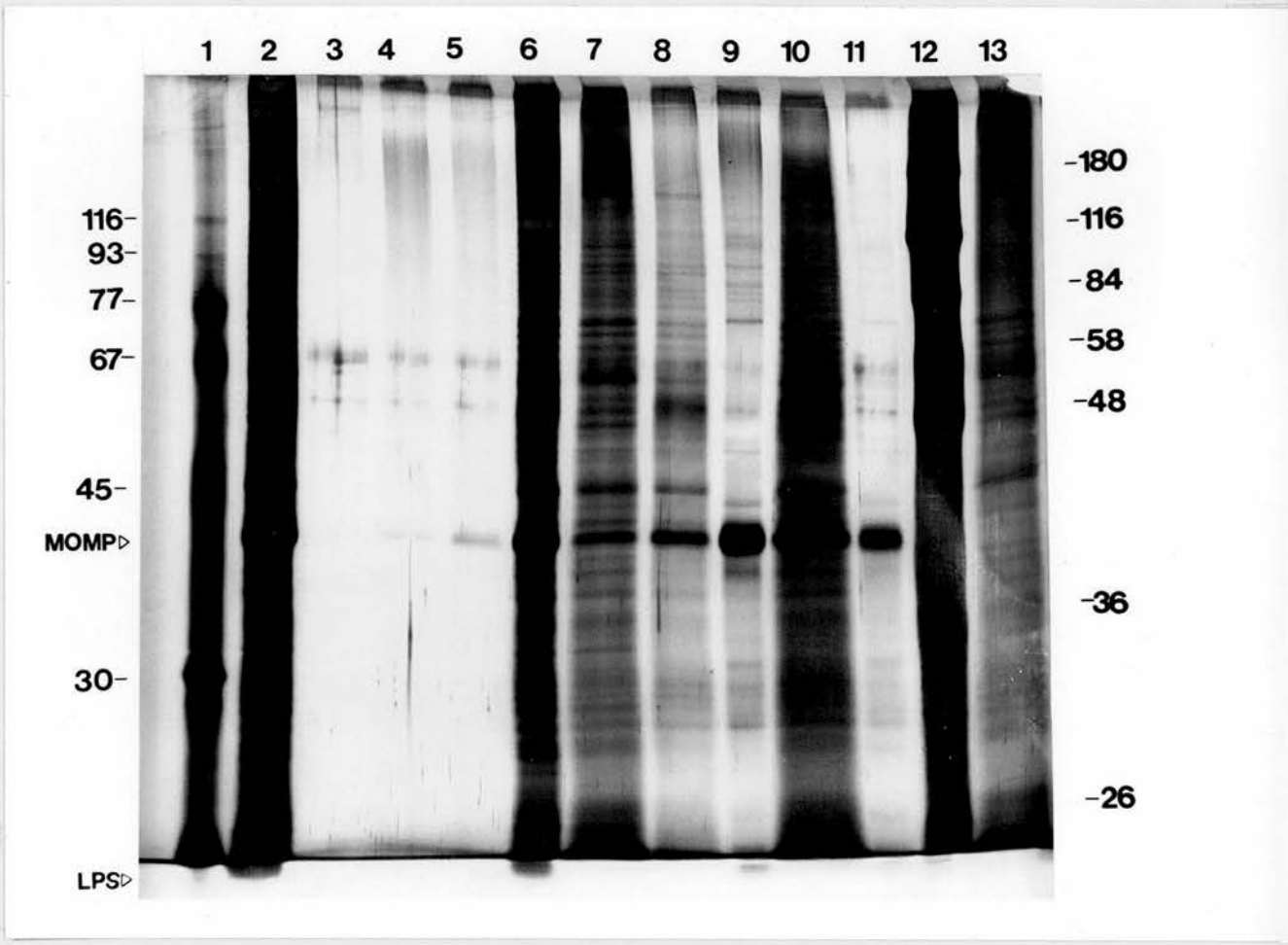


Figure 4.5A. Distribution of chlamydial components and antigens during detergent-reducing agent extractions. SDS-PAGE profiles (10% gel) of M_r standards (lane 1 - from the top, 116, 93, 77, 67, 45, 30, 17, 12 kDa), whole EBs (lane 2), pooled washes (lane 3), PBE wash 1 (lane 4), PBE wash 2 (lane 5), mock extracted EBs (lane 6), PBES supernatant - extraction 1 (lane 7), PBESD supernatant - extraction 2 (lane 8), two-step extracted OM preparation (lane 9), one-step PBESD extraction supernatant (lane 10) and one-step extracted OM preparation (lane 11). Lane 12 contained prestained M_r standards (Sigma) namely α_2-macroglobulin (180), β-galactosidase (116), fructose 6-phosphate kinase (84), pyruvate kinase (58), fumarase (48.5), lactate dehydrogenase (36.5),triose phosphate isomerase (26.6 kDa) from the top. Lane 13 contained the material from lane 7 not boiled in sample buffer (see Discussion - oligomerisation). Figure 4.5B may be found two pages forward.

Fractionation of EBs and the enrichment of MOMP

Purified chlamydial elementary bodies (600μg) were resuspended in 6 ml of 100mM phosphate buffer pH 7.4 containing 1mM EDTA (PBE) and divided into three aliquots. EBs were pelleted (all centrifugation steps at 100,000g for 1h) and the wash supernatants pooled for SDS-PAGE analysis (Figure 4.5A -lane 3). One EB pellet was resuspended in PBE-1% Sarkosyl (PBES) to a final concentration of 0.3mg protein/ml, sonicated in a bath sonicator for a 30 second burst, and incubated for 2 h at 37°C with occasional mixing. Detergent-soluble components (lane 7) were separated from insoluble material by centrifugation. The insoluble pellet was resuspended in PBES-1mM DTT (PBESD), sonicated, incubated and centrifuged as described to give a PBESD-soluble supernatant (lane 8) and the insoluble outer membrane (OM) preparation (lane 9). The second EB pellet was similarly treated except that PBE was used for both incubation steps and yielded two PBE supernatants (lane 4 and 5) and the mock-treated EBs (lane 6). The third EB pellet was subjected to only one incubation step in PBESD for 2 h instead and yielded a PBESD supernatant (lane 10) and an OM preparation (one-step extraction - lane 11).

The SDS-PAGE profiles showed that the mock-treated EBs were identical to untreated EBs (lane 2). Some MOMP was lost into the PBE supernatants (lanes 4 and 5). In the first step of the two-step extraction, most chlamydial components were solubilised (lane 7) and more, including some MOMP, were solubilised in the second (lane 8) leaving MOMP highly enriched in the OM preparation (lane 9). Other protein bands, as well as LPS which migrated at the dye front, were also present in this OM preparation. Lanes 10 and 11 showed that MOMP could also be enriched in the OM in a one-step extraction.

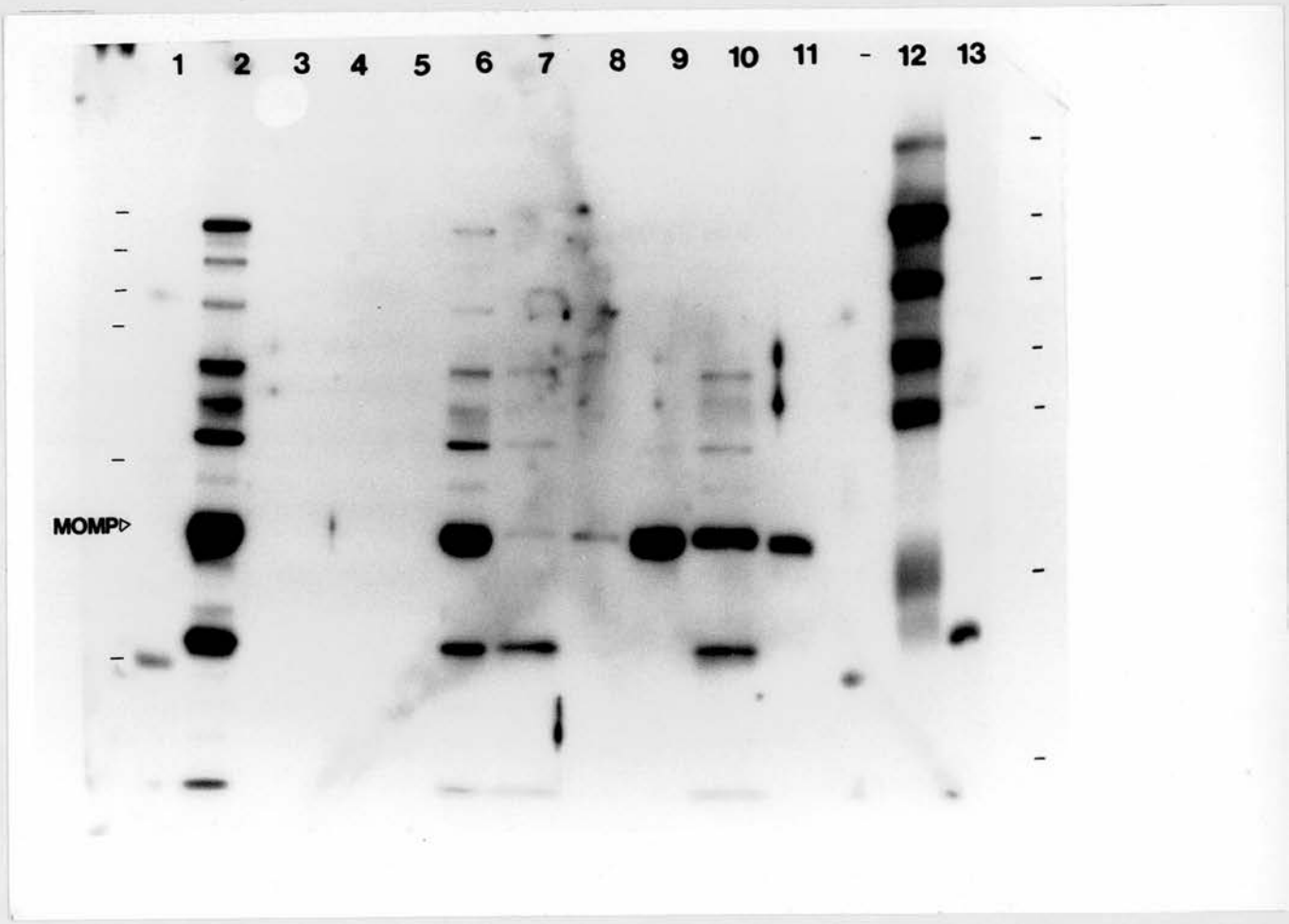


Figure 4.5B. Distribution of chlamydial components and antigens during detergent-reducing agent extractions. Immunoblot of a replica of the gel in **Figure 4.5A** probed with pooled sera from post-abortion ewes. The M_r standards are identical to that in **Figure 4.5A** (two pages before).

Immunoblotting of a replica of the electrophoretogram in Figure 4.5A with a mixture of sera from two post-abortion ewes (2123K and 303H) showed the distribution of chlamydial antigens when subjected to detergent extraction (Figure 4.5B). Lanes 3, 4 and 5 showed that no detectable antigens were released into the washes. The profiles of untreated EBs and mock-treated EBs did not show that any antigen was lost. In contrast, both extraction procedures solubilised most chlamydial antigens leaving MOMP as the only detectable antigen in the OM preparation. Further experiments showed that optimal enrichment of MOMP in the OM could be achieved by 1%-2% Sarkosyl and 10 mM DTT with bath sonication to assist the resuspension and disruption of EBs (Chapter 5).

Electron microscopy of EBs and outer membrane preparations

Chlamydial OMs were prepared by the two-step extraction procedure described in the previous section using concentrations of Sarkosyl and DTT at 2% and 10 mM respectively. At each stage, the OM pellets were sampled for electron microscopic examination. Intact chlamydial EBs were used as a positive control.

Chlamydial EBs negatively stained and viewed under the electron microscope showed a typical coccoid shape about 200 nm to 400 nm across. In Figure 4.6A, a granularity of the surface can be observed. On the edges of the EB, the particulate nature of the general outline can be seen (Figure 4.6A). After the one-step Sarkosyl-DTT extraction, broken membrane fragments may be seen edge on with knob-like particles protruding from the convex face (Figure 4.6B - arrowed). The electron micrograph of the OM preparation after the two extractions (Sarkosyl and DTT) revealed smaller membrane fragments with tightly packed, fine particulate structures (4 nm diameter) on the surface (Figure 4.6C). In some fragments, a hexagonally packed array of such particles may be detected. The centre-to-centre spacing of the particles forming such arrays are approximately 5-6 nm. On

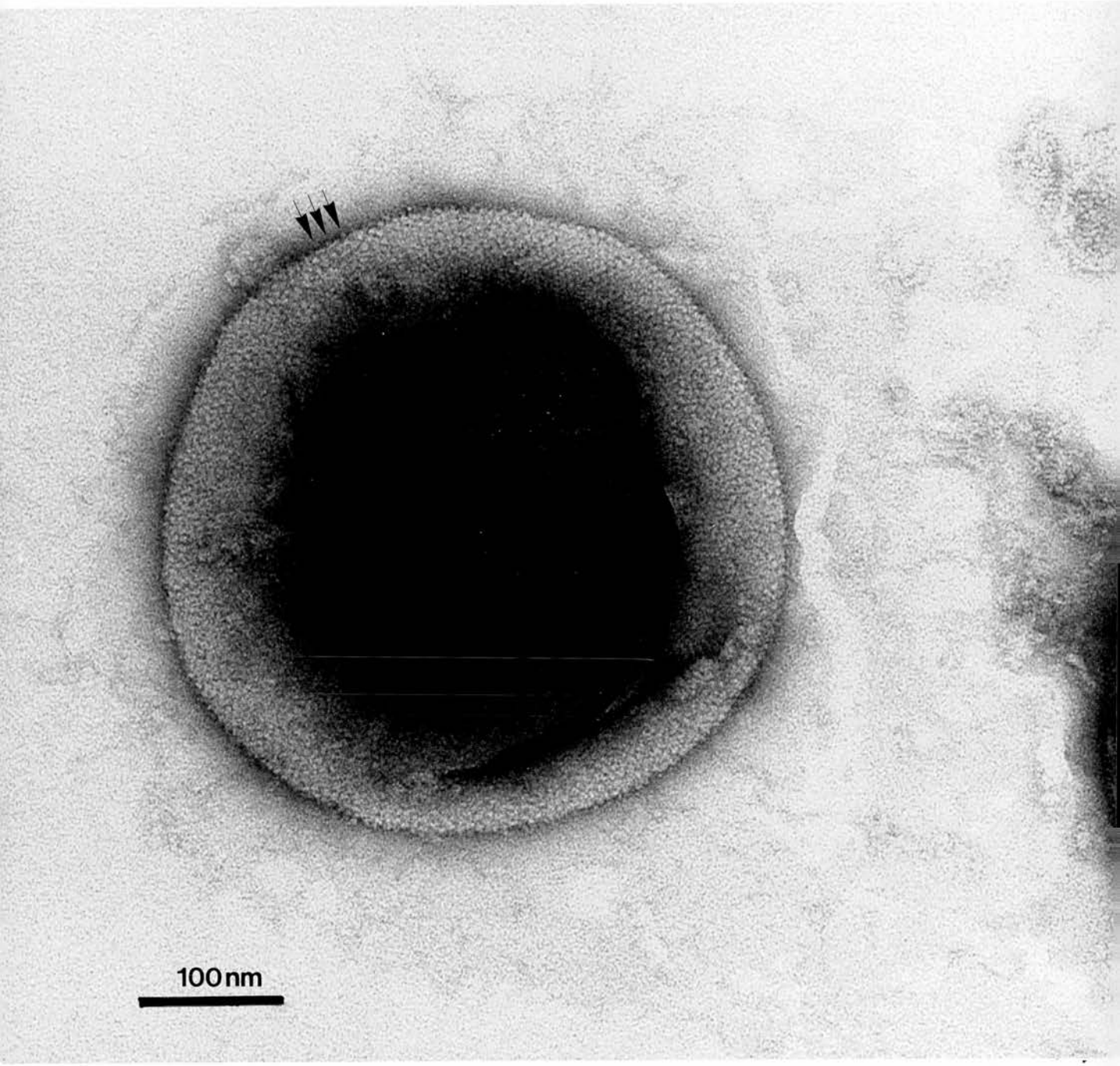


Figure 4.6A. Electron micrographs of negatively stained intact EBs a
OM preparations. Panel **A**. An intact EB. Magnification: x240,00
Bar: 100nm. Knobs on the outer surface of the intact EB (solid arrow
fine particles densely packed on the surface of the OM clearly visibl

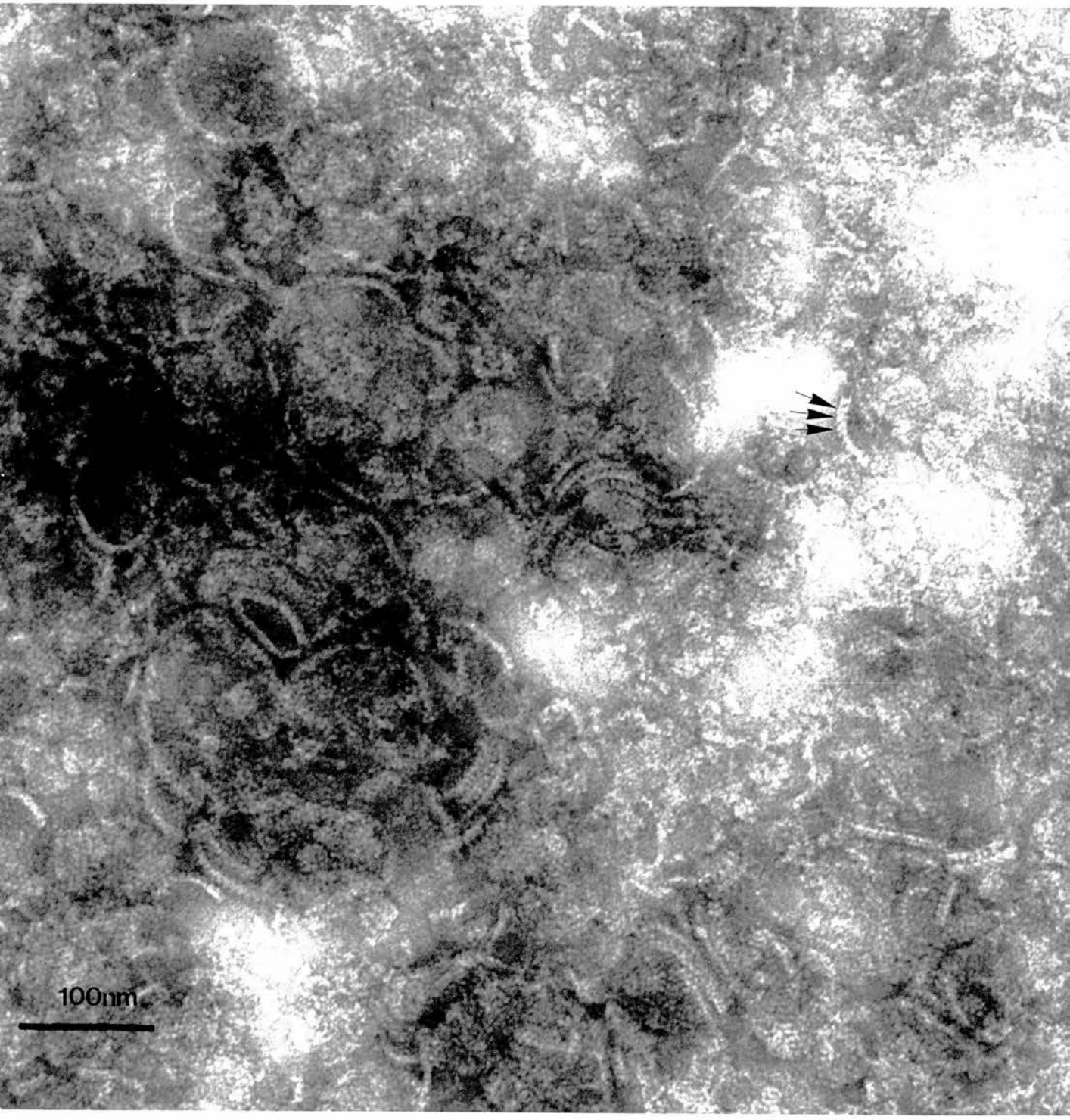


Figure 4.6B. Electron micrographs of negatively stained intact EBs and OM preparations. Panel B. Insoluble OM preparation obtained after the one-step PBE-Sarkosyl-DTT (10mM) extraction. Magnification: x240,000. Bar: 100nm. Large fragments of OMs of chlamydial EBs showing some in profile. Knobs protrude from the convexity of some fragments (arrows).

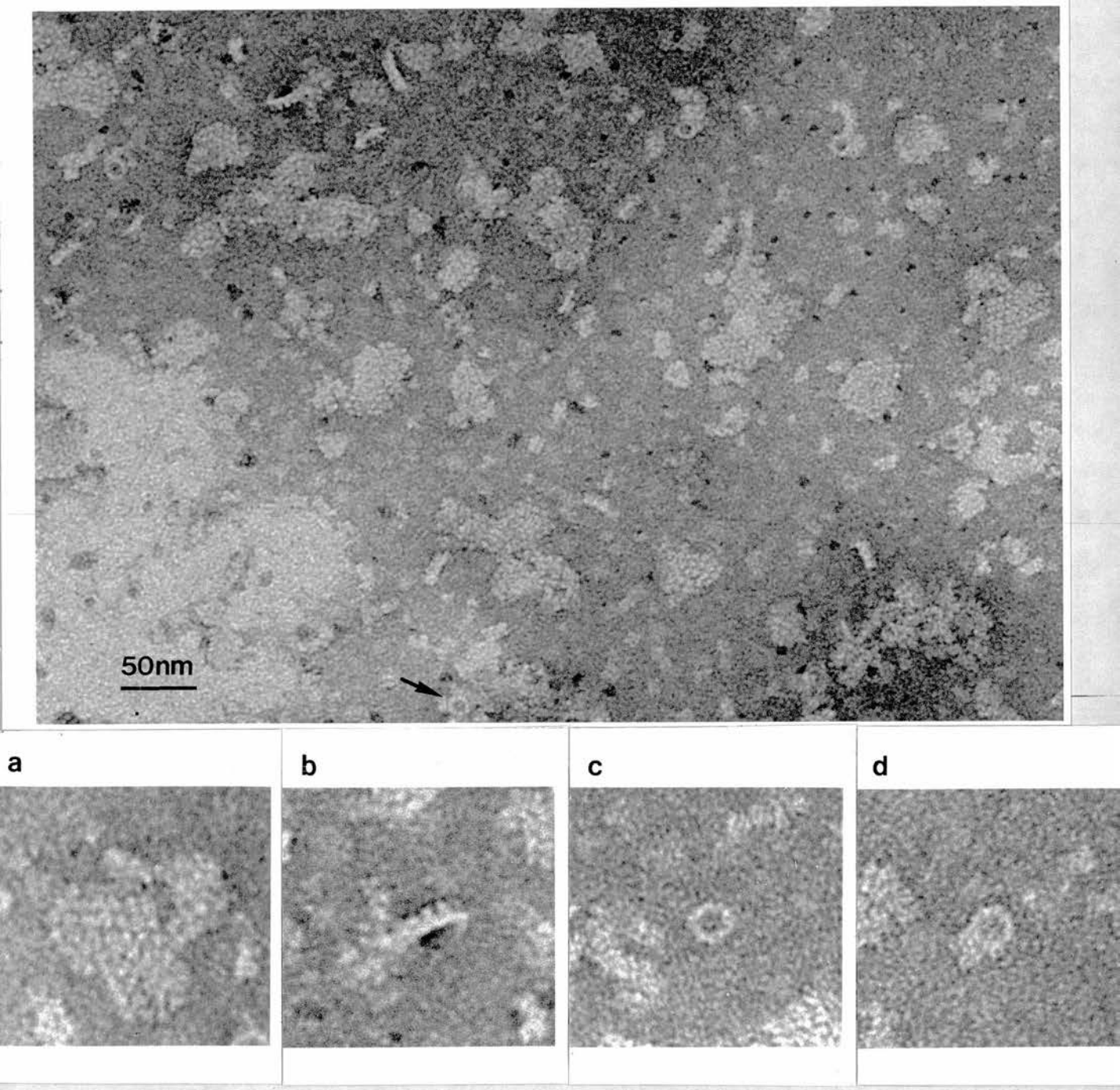


Figure 4.6C. Electron micrographs of negatively stained intact EBs and OM preparations.. Panel C. Insoluble OM preparation obtained after the two-step treatment. A nine-membered rosette is arrowed. Magnification: x240,000. Bar: 50nm.

Insets show a. small fragments of OM bearing fine particles densely packed in hexagonal array; b. profile of membrane fragments showing knobs protruding from the convex edge. c. ring-like structure or rosette ; d. rosette attached to membrane fragment . Magnification: x480,000 each.

other fragments, however, no such regularity could be detected. Close inspection revealed a central dot of electron opacity in some of the fine particles (see Figure 4.6C:inset a).

Some membrane fragments were visible edge on and knob-like particles about 4 nm wide may be observed, (Figure 4.6C) identical to those seen in Figure 4.6B. These knob-like particles have a centre-to-centre spacing of 5 - 6 nm as measured from the side elevation and projected from a continuous substratum (6 - 8 nm thick). No central channel containing electron-dense material which might correspond to the central dots could be detected (see Figure 4.6C: inset b).

Attached to fragments of membrane, or in some cases completely detached, were ring-like structures measuring about 14.5 nm in diameter (Figure 4.6C - arrowed) similar to those described by Matsumoto (1973). These rosettes appeared to be made up of 7 to 9 regularly arranged subunits about 3 - 4 nm in diameter surrounding a central cavity (7 - 8 nm diameter) filled with the electron dense material from the stain (inset c and d).

Protein microsequencing of MOMP

Towards the end of this project, the opportunity arose to perform protein microsequencing of MOMP on an automatic protein sequencer (Applied Biosystems). S26/3 MOMP was prepared by the two-step Sarkosyl and DTT extraction procedure of a previous section. (This preparation of MOMP was the same as that used for the vaccination-challenge experiment used in Chapter 5.) About 10μg of the material was resolved by SDS-PAGE and electrophoretically transferred onto "Glassybond^{R}" (Chapter 2) glass fibre support. Coomassie blue staining of the blot revealed a dominant band at 40kDa relatively free of other protein contaminants, similar to the profile in Figure 4.5 - lanes 11. This band was excised and used for protein sequencing as described in Chapter 2. After a calibration cycle with phenylthiohydantoin-amino acid standards, the sample was loaded and subjected to 10 cycles of Edman degradation.

The N-terminal sequence obtained was as follows (chromatograms not shown):

NH2-L(D)P(G)VGNPAEPS...

In the first two cycles, there appeared to be two alternative residues, Leu or Asp, followed by Pro or Gly. Comparison of this sequence with the N-terminus sequence of *C.trachomatis* L2 MOMP (Nano *et al*, 1985) showed that residues 3 to 9 were identical and that residues 1 and 2 were Leu and Pro respectively. This sequence was in complete agreement with the amino acid sequence deduced from the nucleotide sequence of the MOMP gene as described in Chapter 6. Further inspection of the deduced amino acid sequence suggested that the two residues Asp and Gly could have arisen from an alternative N-terminus downstream of Leu and Pro. This N-terminus may have been generated as a result of mild acid hydrolysis during the staining procedure but the possibility of contamination could not be excluded.

Surface labelling of elementary bodies

Chlamydial elementary bodies and BHK-21 host cells were surface-labelled with radioactive iodine-125 using Iodobeads (Chapter 2). Radiolabelled proteins were analysed by SDS-PAGE and autoradiography. Figure 4.7A shows that a 40 kDa protein band (arrowed) in the EB profile was the most strongly labelled. Other bands were also present but in weaker amounts. Unreacted radio-isotope that was not washed away migrated at the dye front. The radiolabelled BHK-21 cells were subsequently used for ligand blotting experiments to detect chlamydial adhesin which is described next.

Identification of OEA C.psittaci adhesins

The method of identifying adhesins was based on the method of Wenman and Meuser (1986). BHK-21 cells, routinely used for the culture of OEA strains of *C.psittaci*, were surface-labelled by the Iodobead method (Chapter 2). Labelled cell components

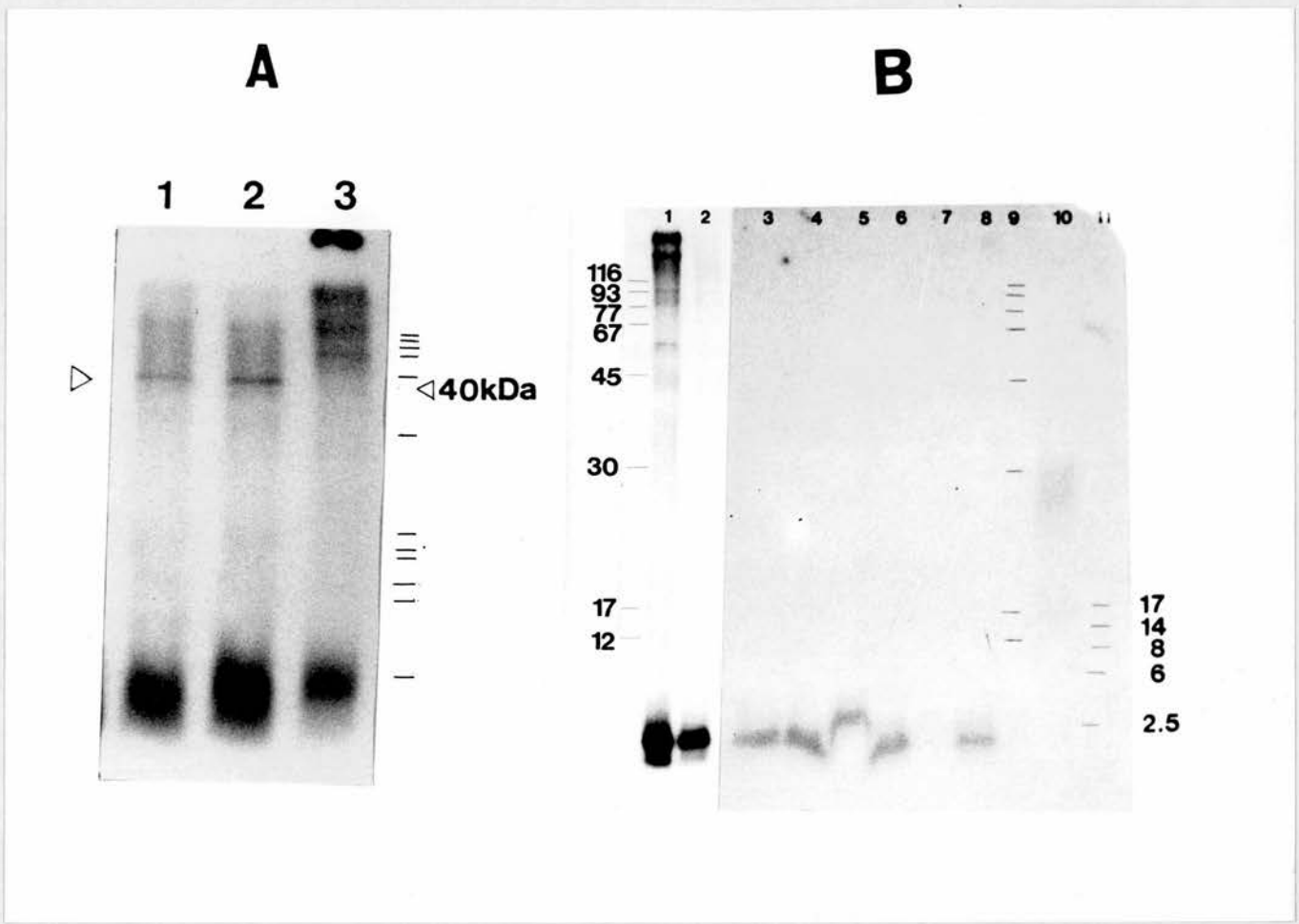


Figure 4.7A. Surface radio-iodination of intact chlamydial EBs (lanes 1 and 2) and BHK-21 cells (lane 3). Autoradiogram of SDS-PAGE profiles (15% gel). Lane 1. Reaction quenched with sodium metabisulphite. Lane 2. Reaction quenched with 2-mercaptoethanol. Lane 3. Reaction not quenched but cells washed extensively. M_r standards are marked from the top in kDa, 116, 93, 77, 67, 45, 30, 17, 14, 12, 8, 6, and 2.5. The position of MOMP is arrowed.

Figure 4.7B. Identification of cell ligand that bound to putative chlamydial adhesins. Composite autoradiograms of SDS-PAGE profiles (15% gel) of radio-iodinated samples. Total BHK-21 cell lysate (1); Triton X100 soluble supernatant from cell lysate used in binding chlamydial adhesins in the ligand blot (2). The radioactive 18kDa (lanes 3 to 5) and 32kDa bands (lanes 6 to 8) were excised from the ligand blot and boiled in sample buffer (3 minutes) to elute the radioactive ligand bound to the putative adhesins for SDS-PAGE analysis. Lane 10 contained a similar eluate of a 32kDa band from an immunoblot probed with radio-iodinated rabbit anti-sheep $F(ab)_2$ antibody. The M_r standards are marked in kDa (also in lane 9).

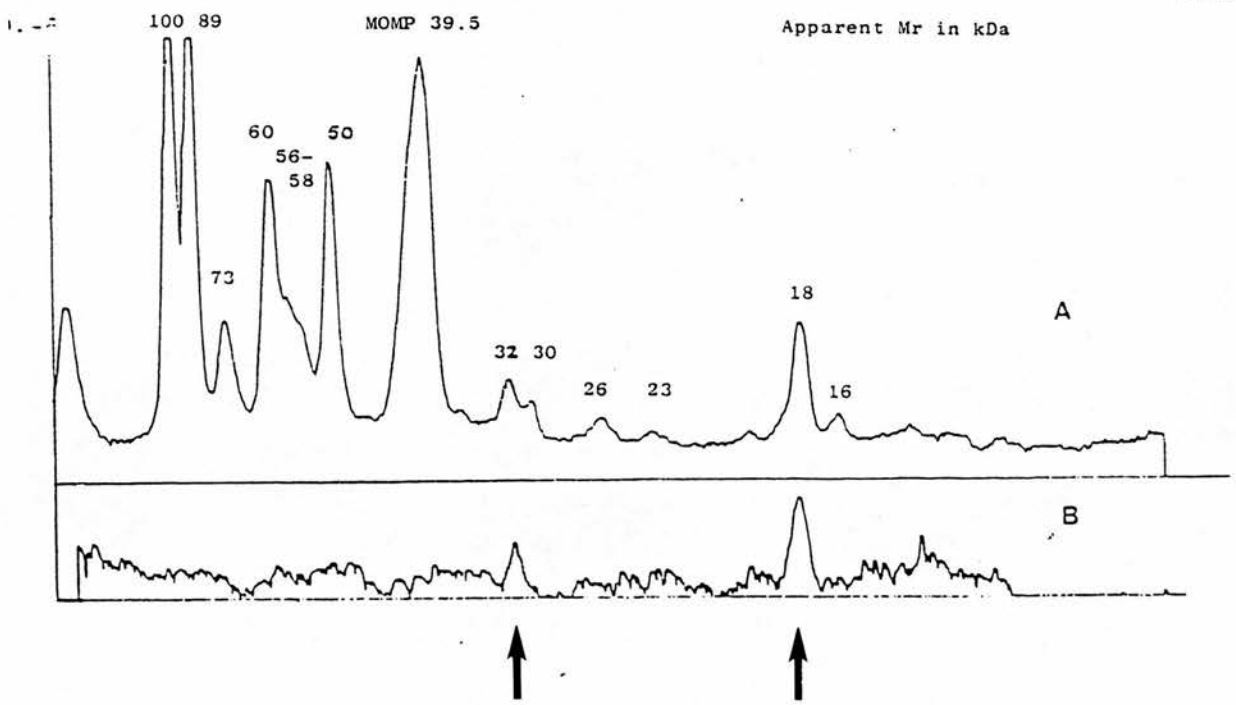


Figure 4.8. Identification of putative chlamydial adhesins (arrowed) from OEA *C.psittaci*. Alignment of densitometric scans of A. an immunoblot using post-abortion sheep serum and B. a ligand blot using Triton X100 soluble supernatant from radio-iodinated BHK-21 cells.

were solubilised in PBS containing 1% Triton X100 and the soluble material ($3x10^6$ cpm) was diluted 1:30 in PBS containing 0.5% Tween 20 (PBST). This labelled solution used to probe strips of nitrocellulose membrane bearing an electrophoretogram of chlamydial EBs in a similar way to immunoblotting, a method also known as ligand blotting (Chapter 2). The ligand blots were washed as with the immunoblotting procedure and exposed to X-ray film to identify any chlamydial adhesin that bound labelled BHK-21 components. An adjacent strip of nitrocellulose was immunoblotted with post-abortion sheep serum (2123K) as a comparison.

Figure 4.8 shows a densitometric scan of the autoradiogram of the ligand blot aligned with that of the immunoblot. The peaks of the immunoblot are annotated to indicate the M_r. Two bands of M_r 32kDa and 18kDa were identified on the ligand blot which appeared to co-migrate with the corresponding antigens. These were the putative adhesins of OEA strains of *C.psittaci*.

Attempts to identify the host cell ligand that bound to these putative adhesins included excision of the radioactive bands at 32 kDa and 18 kDa for elution and SDS-PAGE analysis (Figure 4.7B), and fractionation of labelled cell components by gel filtration and using each fraction as probe in ligand blots (data not shown). In the first approach, only a band migrating close to the dye front was detected as the component(s) that might have possibly bound to the adhesins (lanes 3 to 8). The constituent(s) of this band has not been characterised. The positive control in lane 10 showed that a smear at 25 to 30 kDa had been eluted, probably the light chain of the antibody probe. None of the fractions obtained in the second approach bound to the putative adhesins.

Screening monoclonal antibodies for MOMP specificity

As part of a wider team, the author was, at one stage of the project, involved the screening of anti-chlamydial monoclonal antibodies by the rapid immunoblotting method

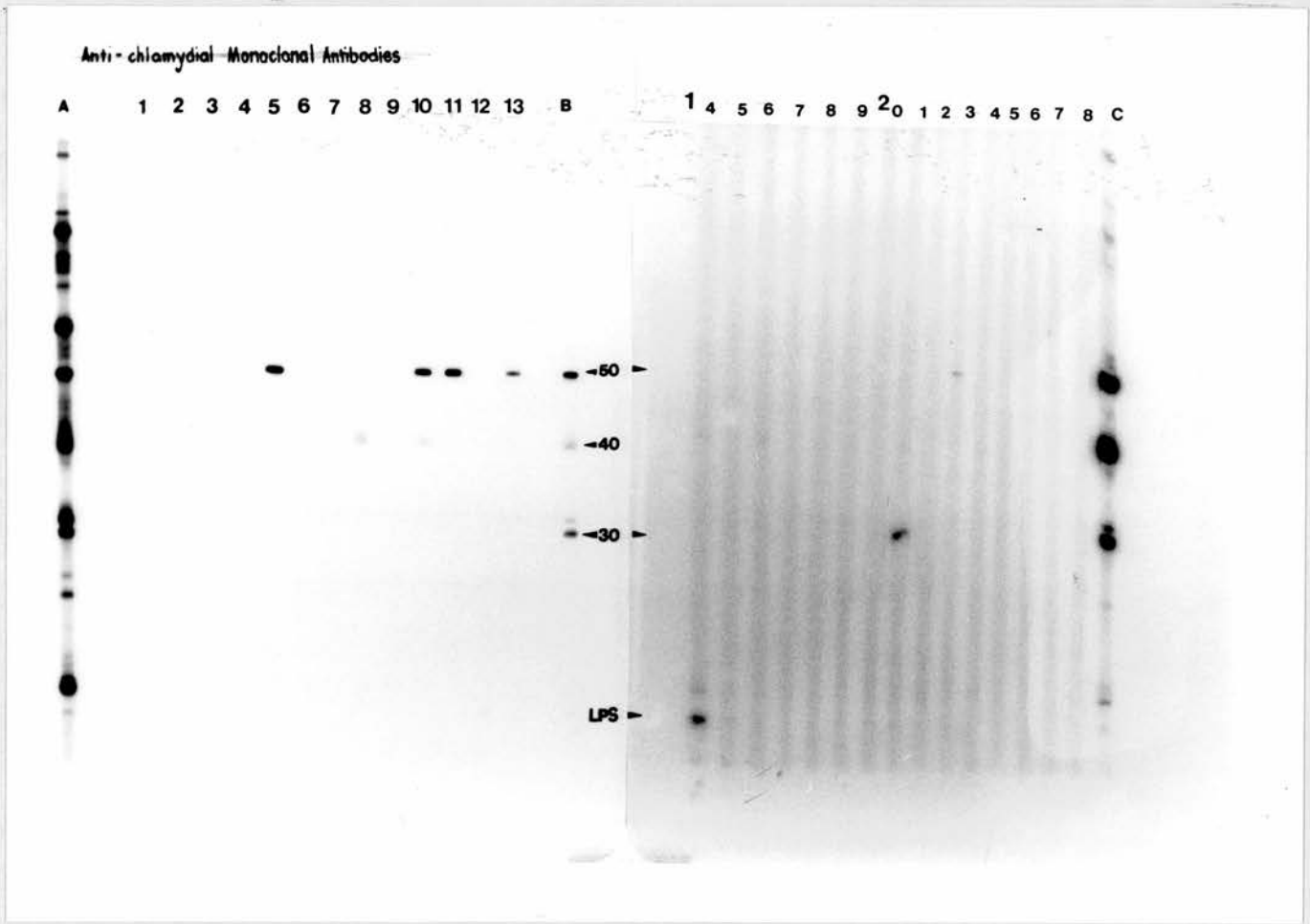


Figure 4.9. Screening of monoclonal antibodies for anti-MOMP specificity by immunoblotting. Lanes 1-13 and 14-28 are immunoblot profiles of hybridoma supernatants made up to the concentration of PBST. Secondary probe was radio-iodinated Protein A. Positive controls were immunoblot profiles of post-abortion sheep serum (A) and murine anti-S507 antiserum (B,C). MAb specificities include 50kDa, 40kDa (MOMP), 30kDa and LPS.

developed for the studies described in Chapter 3. Initial screens yielded several clones producing anti-50kDa, -MOMP, -30kDa and -LPS mAb specificities by immunoblotting (Figure 4.9). All of these clones with the exception of the anti-LPS mAb were eventually lost in the process of cloning stable mAb-producing cell lines (C.Wilson and J.Dooley, personal communication). Consequently, no probe specific for MOMP was available for further characterisation of the properties of MOMP.

DISCUSSION

SDS-PAGE analyses

The SDS-PAGE profiles of OEA *C.psittaci* showed the presence of a dominant 40 kDa band, similar to all *C.psittaci* and *C.trachomatis* profiles published so far except those of Rodolakis and co-workers (1989). In that report, the cell wall or "surface" protein profiles of S26/3 and other *C.psittaci* strains shown in a gradient SDS-PAGE gel did not possess this dominant protein band. However, the published gel was reproduced in another report with two lanes removed (Buzoni-Gatel *et al*, 1989) but the profiles were referred to as those of chlamydial lysates instead of cell wall proteins. The method of lysis indicated that the lysates were soluble supernatants of purified EBs incubated with PBS containing 4% SDS and 3 mM EDTA; the insoluble material was removed by centrifugation at 11,000g for 40 minutes. The latter interpretation appears to resolve the apparent discrepancy because under such conditions, it is unlikely that MOMP can be extracted in any large quantity from the intact outer membrane (OM) (Caldwell *et al*, 1981; Bavoil *et al*, 1984). It is also favoured because the profiles resembled the supernatants of EBs incubated in Sarkosyl and EDTA, shown in Figure 4.5A - lane 7 (or Figure 5.1 - lane 5). The band pattern, however, could not be directly compared because the authors used a 8%-20% gradient SDS-PAGE system. For convenience, the 40kDa protein is referred to as MOMP in the discussion that follows.

The heterogeneity in M_r of MOMPs from different strains is a well-documented phenomenon (Caldwell *et al*, 1981; Salari and Ward, 1981; Newhall *et al*, 1982; Batteiger *et al*, 1985; Fukushi and Hirai, 1988). The M_r of OEA MOMP was estimated to be 39.5kDa, slightly more than that of GPIC MOMP (Batteiger and Rank, 1987) but well below the *C.trachomatis* MOMPs and avian *C.psittaci* MOMPs (40-43kDa). The apparent lack of heterogeneity of MOMPs from different OEA isolates and indeed the overall lack of difference in the other proteins suggested that OEA isolates were either identical or very closely related. This similarity will be discussed further in Chapter 8.

The two ovine faecal isolates 84/S604 and 84/521F, which were also analysed, were known to possess different inclusion morphology and virulence characteristics, the former resembling arthritis strains and the latter more like abortion isolates (Anderson and Baxter, 1986). Their protein profiles showed distinct differences particularly in the mobility of their respective MOMPs. The 84/521F profile is similar to that of abortion isolates (lanes 7-13, Figure 4.1) whereas the 84/S603 profile resembled that of P787 (lane 6), thus supporting the conclusions of Anderson and Baxter (1986). In addition, the difference in the profiles of ovine abortion and ovine arthritis strains demonstrated here agreed with other reports that these two strains can be distinguished (see below). These observations suggest that SDS-PAGE can be used as a method for differentiating ovine *C.psittaci* strains of different biological characteristics and suggests that the MOMP may be conveniently used as a distinguishing marker either at the protein level or at the nucleic acid sequence level (see discussion in Chapter 6 and 8, and also Fukushi and Hirai, 1988; Baxter *et al*, manuscript in preparation).

Further experiments analysed the partial proteolytic profiles of MOMP from one ovine arthritis and two ovine abortion isolates. These isolates represented two immunotypes of *C.psittaci* as mentioned in Chapter 1. The ovine arthritis and

abortion isolates have been distinguished by mAbs (Delong and Magee, 1986; Anderson and van Deusen, 1988), and by restriction endonuclease assay (McClenaghan *et al*, 1984; Timms *et al*, 1988). The proteolytic profiles showed that the protein sequence of the two ovine *C.psittaci* types are significantly different such that all three proteases generated different profiles. In contrast, if there are any differences between the two ovine abortion isolates and if these differences are located on MOMP, these are likely to be minor and may require two-dimensional (2-D) gel electrophoresis or 2-D peptide mapping to reveal any differences (Su *et al*, 1988).

Antigenic structure of *C.psittaci*

The cross-reaction of anti-S26/3 sera with antigens of other chlamydial strains showed that certain chlamydial components are conserved between types and species. Some components such as the 75 kDa antigen do not show detectable variation in M_r and appeared to cross-react in the immunoblot with all representative chlamydial strains tested, suggesting that it is a common genus-antigen. The 60 kDa antigens cross-reacted well in the immunoblot but possess a slight M_r variation suggesting that while most of the immunoblot-resistant antigenic epitopes may have been conserved, the other structural domains have diverged. Others such as the 50kDa antigen appeared to contain type-specific epitopes exclusively. Alternatively, this antigen does not have an analogue in other strains. Within OEA *C.psittaci* strains, some of the high M_r antigens appeared to react more strongly with the heterologous S26/3 antiserum. The reason for this is not known although it might be speculated that the animal (2123K) may have been exposed to these strains before. There were faint bands at the 57kDa and 45kDa region but it is not known whether they are the 57kDa genus-specific antigen which elicited ocular hypersensitivity, and the 45kDa genus-specific antigen, respectively, described by Morrison *et al* (1989). The partial cross-reactivity exhibited by all the chlamydial MOMPs tested in this study is consistent with the well-documented presence of

genus-, species-, sub-species- and serotype-specific epitopes on chlamydial MOMPs (Stephens *et al*, 1982; Ma *et al*, 1987; Stephens *et al*, 1988b; Baehr *et al*, 1988; Conlan *et al*, 1988).

As a cautionary note for the interpretation of all immunoblots, the differences in band intensity discussed above need not necessarily suggest that there are epitopic variations because variation in antigenic mass can give rise to such differences. Post-staining of such bands would be useful but does not conclusively prove that a putative band present on the blot is indeed the same antigenic band in the autoradiogram because of the complexity of the profile and also because highly antigenic bands may not be present in quantities detectable by post-staining procedures.

Disulphide linkages and MOMP oligomerisation

Chlamydial MOMPs are cysteine-rich proteins containing up to nine cysteine residues per polypeptide chain (Hatch *et al*, 1984; Stephens *et al*, 1986; 1987). They form disulphide-linked oligomers in the chlamydial OM (Newhall and Jones, 1983; Hatch *et al*, 1984). The extensive network of disulphide cross-linkages is thought to have a structural role in the OM and is involved in permeability (Bavoil *et al*, 1984) and in the developmental cycle (Hatch *et al*, 1984; Hackstadt *et al*, 1985; Hatch *et al*, 1986; Newhall, 1987). However, the way in which MOMP and other cysteine-rich proteins interact to form such a network is not clearly understood.

The requirement for reduction and denaturation of EBs before OEA MOMP can be solubilised suggested that it possessed cysteine residues like other chlamydial MOMPs and formed part of the network of disulphide linkages. Addition of reducing agent in the detergent extraction procedure resulted in the solubilisation of a trace amount of MOMP, suggesting that some MOMP polypeptides may be more susceptible to reduction and more loosely attached to the OM. Such solubilised MOMP when analysed by SDS-PAGE under non-reducing conditions, however, were not

detected as MOMP monomers (Figure 4.5 lane A13 and B13). Further experiments to discover the state of these soluble MOMPs are required.

A preliminary liquid chromatographic separation of the PBESD supernatant in a hydroxyapatite column was able to resolve several peaks (R.Davies, personal communication). SDS-PAGE of these fractions boiled in sample buffer (SDS and 2-ME) showed that MOMP co-eluted with a 58kDa, 60kDa band and other faint but high M_r bands in one broad peak (this work has not been repeated and the data were consequently not shown). It is not known whether the 58kDa/60kDa doublet is the 59/62kDa cysteine-rich proteins described for other *C.psittaci* and *C.trachomatis* (Hatch *et al*, 1984; Batteiger *et al*, 1985). When this fraction was not boiled in the presence of 2-ME, only one strong band migrating with apparent M_r of 85 to 90 kDa was observed. Upon storage at +4°C, this band disappeared and a band reappeared at very high M_r (> 200kDa); a similar observation was also reported by Bavoil *et al*, 1984). These observations taken as a whole suggest that OEA MOMP and other components are able to reform hetero-oligomers *in vitro*.

Several studies have shown that MOMP can form homo-oligomers by disulphide cross-linkages. Bavoil *et al* (1984) observed that MOMP, when solubilised by a three-step detergent-DTT extraction procedure, was able to reform large aggregates (size not disclosed) when reducing agent was removed by dialysis. Similarly, Hackstadt *et al* (1985) demonstrated a similar reaggregation effect of MOMP within the OM of DTT-reduced intact EBs; DTT-reduced MOMPs formed monomers (a compact conformation migrating at 36kDa), dimers, trimers and higher order oligomers. Alkylating agents were able to inhibit the oligomerisation.

The fortuitous observation in the peptide mapping experiment in which MOMP gel slices exposed to non-reducing conditions produced oligomers suggested that OEA MOMP is also predisposed to aggregation. This property was manifested by

MOMPs of two ovine *C.psittaci* types. The oligomerisation experiment using OEA MOMPs showed that denatured and reduced MOMP monomers can aggregate *in vitro* and form putative dimers and trimers of 80 and 120 kDa respectively. Higher order oligomers were also detected but the sizes were not quantitated. Such oligomers were linked by disulphide bridges since the addition of 2-ME was able to separate the oligomers into monomeric form. The compact oxidised monomeric MOMP species of apparent M_r 36kDa (Hackstadt *et al*, 1985) was also observed in this study.

It would be interesting to determine if oligomerisation of MOMP and other CRPs in the chlamydial OM followed specific assembly pathways. For example, it has been reported that *E.coli* OmpF porin trimers appear to be assembled into the OM from monomeric forms via a metastable dimer intermediate within a minute of the processing of the precursor polypeptides (Reid *et al*, 1988). Newhall (1987) has already suggested that intracellular cross-linking mechanisms, possibly enzymatic, may be involved in this process of cell wall formation in chlamydial RBs.

Two types of approaches have been outlined above. One took a reductionist approach from the *in vivo* situation, asking the question: what are the fragments of the natural structure made of? The other relied on taking each component as building blocks and asking the questions: which components can be reconstituted and to what extent do such reconstituents resemble the *in vivo* situation? As will be discussed in Chapter 7, the expression of rMOMP and its truncated form may provide additional tools for elucidating the structure and assembly of a unique form of OM in Gram-negative organisms. Other techniques, in particular, electron microscopy, will be dealt with next.

Electron microscopy

Several structures of the OM of chlamydial organisms can be detected by various electron microscopic techniques. Models of the OM integrating and interpreting these findings have been proposed by Matsumoto (1979; 1982c; 1988; see Chapter 1). The

EM data presented in this chapter provide further evidence that the Sarkosyl-DTT insoluble preparations, of which MOMP was a major constituent, were OM preparations.

The 4 nm fine particles protruding from the OM as observed in the OM preparations are likely to be the same as the 5 - 6nm particles observed by Matsumoto (1979) which appear to protrude from folded edges of the EB cell wall. These particles appeared to be present as a fine granularity on the intact EB surface (Matsumoto and Manire, 1970), supporting Matsumoto's (1988) suggestion that such particles are a major structural component of the outermost surface of the EB cell wall.

SDS-PAGE analysis showed that the protein composition of the preparation is predominantly MOMP indicating that it is the major constituent of these fine particles. The points of electron opacity in the middle of these particles suggest a depression or possibly a hole in each particle. The side elevation of these particles however do not show any evidence that these particles are hollow. Since MOMP is thought to possess a porin-like function (Bavoil *et al*, 1984), it is speculated that these particles might be formed from macromolecular aggregates of MOMP with certain domains protruding from the OM and that the holes mark the openings of [illegible] macromolecular pores. Whether these protruding domains could be formed by immunoaccessible epitopes analogous to that found for *C.trachomatis* (Zhang *et al*, 1987a) remains to be tested.

The dense packing of these fine particles is reminiscent of hexagonal structures that have been observed in reconstituted *E.coli* membranes and thought to be formed by multimers of a major outer membrane protein (OmpC porin) associated with LPS (Yamada and Mizushima, 1978 and 1980). Birkelund and others (1988) have shown that MOMP can be cross-linked to chlamydial LPS by a comparatively short bifunctional cross-linking agent suggesting that MOMP and LPS are closely located in the intact

membrane. The detection of LPS in the OM preparations suggests that these particulate membrane fragments are formed by the close association of LPS and MOMP.

Rosettes of a similar size described by Matsumoto (1973) were also observed in the OM preparation. They were found either attached to small bits of membrane or totally detached indicating that while the rosettes are usually found on the membrane, they can exist stably as a separate structure. It is possible that these rosettes might be formed from the high molecular weight proteins detected by SDS-PAGE in the preparation. It may be possible to optimise the conditions of detergent extraction and differential centrifugation to separate these rosettes from the larger membrane fragments so that the biochemical composition of these structures can be determined.

It was noted that surface projections described in various EM studies (Matsumoto and Higashi, 1975; Matsumoto *et al*, 1976; Stokes, 1978; Gregory *et al*, 1979; Matsumoto, 1981a and 1981b; Matsumoto, 1982a and 1982b) were not detected in these preparations. One reason could be that these structures were susceptible to sonication and were broken into structures too small to be sedimented by centrifugation at 100,000g. Alternatively, they could have been solubilised by the extraction buffer which contained detergent and reducing agent.

In summary, electron microscopic examination of the MOMP-enriched OM preparation showed a retention of some ultrastructural features of the intact outer membrane namely the fine particles covering the outermost surface of the outer membrane and rosettes as reviewed by Matsumoto (1988). This approach of combining ultrastructural studies with biochemical analysis complements the approaches outlined in the previous section for the elucidation of the structure of the chlamydial outer membrane. In the next chapter, the immunoprotective role of these preparations will be described.

Fractionation of chlamydial organisms and purification of chlamydial MOMP

The success of the purified EB vaccine of Anderson *et al* (submitted) has implicated MOMP and possibly a sub-60kDa diffuse band as potential immunogens through the immunoblotting experiments described in Chapter 3. To test the efficacy of individual antigens for immunoprophylactic properties entails the isolation of these antigens from whole organism preparations. The experiments described in this chapter adapted the methods of Caldwell *et al* (1981), Caldwell and Schachter (1982) and Bavoil *et al* (1984) for fractionating chlamydial EBs to obtain different mixtures of antigens which may be tested for efficacy. These fractions could subsequently serve as starting points for the purification of specific antigens such as MOMP. Many mechanisms of chlamydial pathogenesis are thought to be associated with the bacterial surface and hence the immunoprotective antigens are most likely to be found there. As a result, much work in the field has concentrated on the OM of *C.trachomatis* and, to a lesser extent, on that of *C.psittaci*. MOMP, being the predominant component in the OM, was thus an obvious target in terms of purification compared to other antigens.

Both Gram-negative bacteria and chlamydiae have OMs that respond similarly to treatment with ethylenediamine tetraacetic acid (EDTA) under alkaline conditions (Narita *et al*, 1976; Narita and Manire, 1976). Detergents have been used as standard reagents for the solubilisation of the inner membrane of Gram-negative bacteria and the preparation of bacterial cell envelopes. Various detergents have been applied to the preparation of chlamydial envelopes including sodium deoxycholate (Jenkin, 1960; Bavoil *et al*, 1984), SDS, sodium N-lauroyl sarcosinate (Sarkosyl) (Hatch *et al*, 1981; Caldwell *et al*, 1981; Caldwell and Schachter, 1982; Bavoil *et al*, 1984; Batteiger *et al*, 1985), Triton X100, Zwittergent and octylglucoside (Bavoil *et al*, 1984). By comparing the ability of several detergents to extract MOMP from the OM, Caldwell *et*

al (1981) reported that sequential anionic detergent treatment of intact *C.trachomatis* EBs with Sarkosyl followed by SDS was the most desirable method for isolating MOMP. They observed that Sarkosyl, a detergent previously used for the solubilisation of cytoplasmic proteins and cytoplasmic membrane proteins of Gram-negative bacteria (Filip *et al*, 1973), effected extensive solubilisation of proteins from the intact EBs with the exception of MOMP. Electron microscopically, Sarkosyl-insoluble fractions appeared as empty sacs of rigid OM which they termed chlamydial outer membrane complexes (COMC). These results were confirmed by later experiments by Bavoil *et al* (1984) who succeeded in preparing purified MOMP in a relatively undenatured state.

The detergent extraction procedure described in this chapter was able to enrich for MOMP in the OM fraction considerably. Further attempts to achieve a greater degree of purity by solubilisation of MOMP with the non-ionic detergent, octylglucoside, followed by liquid chromatography resulted in negligible yields (data not shown). Alternative approaches at obtaining pure MOMP required the use of strongly denaturing detergents such as SDS, and were not considered appropriate for vaccine efficacy studies. Consequently, the Sarkosyl-DTT-insoluble OM preparation was used in the next stage of a logical progression of vaccine testing at the Moredun Research Institute as part of this project (see Chapter 5). In retrospect, this was partly justified in view of the recent report that MOMP purified by SDS extraction did not provide good protection against *C.trachomatis* infection (Taylor *et al*, 1988).

Putative adhesins

It has been postulated that the surface components of *C.trachomatis* play a role in the stimulation of phagocytosis (Byrne and Moulder, 1978) and in the inhibition of phagosome-lysosome fusion (Friis, 1972). The putative chlamydial adhesins of Wenman and Meuser (1986) and Hackstadt (1986a) are of particular interest since the attachment of the pathogen to its host cell represents a critical point of interaction and is

essential for successful entry. Moreover, antibodies directed against these adhesins inhibit association of EB to host cells and neutralise infectivity of homologous or heterologous strains (Wenman and Meuser, 1986; Wenman *et al*, 1986), suggesting that these adhesins should be characterised for immunoprotective properties against chlamydial infections.

Two putative chlamydial adhesins of approximately 32 and 18 kDa were identified for OEA *C.psittaci* using the ligand blotting method of Wenman and Meuser (1986). The adhesins were similar in M_r to those of *C.trachomatis* (30-32 and 18 kDa)(Wenman and Meuser, 1986; Hackstadt, 1986a) and *C.psittaci* Mn strain (30 and 16 kDa)(Wenman *et al*, 1986). Hackstadt (1986a;b), however, could only identify one 17-19kDa adhesin for a *C.psittaci* GPIC strain. These OEA adhesins co-migrated with two antigens of similar M_r, a finding consistent with the immunological properties of the *C.trachomatis* adhesins mentioned above. Further work is essential to determine if these two antigens are the putative adhesins.

Allan and Pearce (1987) have shown that EBs can bind cells lacking sialic acid moieties. A recent report by Kaul *et al* (1989) indicated that host cell receptors are likely to be proteinaceous. Allan and Pearce (1987) suggested that EB adhesion to host cells may involve either non-specific mechanisms or different receptors depending on the mode of entry. Experiments to identify the BHK-21 host cell receptor(s) that bound these adhesins were also carried out. However, only low M_r components, possibly degradation products, were identified. Although further work in identifying the receptor was subsequently discontinued as the focus of the project shifted to MOMP, identification of the receptor and further characterisation of the adhesins are needed to assess their role in pathogenicity and immunity in OEA.

Surface labelling and monoclonal antibodies

Radio-iodination of intact EBs labelled MOMP as the primary target, indicating that MOMP possessed reactive moieties which were exposed on the OM surface. The Iodobead method used for iodination is catalysed by N-chloro-benzenesulphonamide groups coupled to polystyrene spheres (Markwell, 1982). Radioactive iodinating species released into the solution, although minimal as demonstrated by Markwell (1982), may penetrate the OM and prevent vectorial labelling. More rigorous methods are therefore required to demonstrate surface exposure. One way is to isolate anti-MOMP monoclonal antibodies to allow surface tagging with electron-dense or fluorochrome conjugates (Clark *et al*, 1982; Kuo and Chi, 1987). Since the initial attempt to isolate such mAbs failed, it was not possible to provide additional evidence that OEA MOMP moieties project from the OM. The reason for the failure to isolate mAbs directed against non-LPS epitopes was attributed to the dominance of anti-LPS hybridoma clones (G.E.Jones, personal communication).

SUMMARY

In arriving at the conclusion that the 39 to 40 kDa antigen identified in the previous chapter is unequivocally the chlamydial MOMP of OEA isolates of *C.psittaci*, several lines of evidence showing that it possessed properties of other chlamydial MOMPs have been adduced.

(1) Firstly, it was shown that this 40 kDa antigen from OEA isolates is the most dominant component in SDS-PAGE profiles.

(2) The antigen could be enriched, together with chlamydial LPS, in the outer membrane fraction by a detergent extraction method typically used for preparing outer membranes of Gram-negative bacteria.

(3) Electron microscopy revealed that this antigen-enriched outer membrane fraction possessed ultrastructural features similar to the outermost layer of the intact elementary body.

(4) Surface radio-iodination labelled a major 40 kDa protein.

(5) This polypeptide was only partially sensitive to the reducing agent, dithiothreitol. Boiling in 2-mercaptoethanol and sodium dodecyl sulphate was required to solubilise it, suggesting that it possessed considerable inter-polypeptide disulphide cross-linkages. Additional experiments showed that reduced and denatured protein could re-form oligomers that were sensitive to reducing agent.

(6) This antigen partially cross-reacted with *C.trachomatis* L2 MOMP and other *C.psittaci* MOMPs in immunoblots. The cross-reactivity was stronger with related strains such as ovine arthritis and ovine conjunctivitis *C.psittaci* but very weak with *C.trachomatis*. This observation was consistent with the general observation that MOMP is the site of antigenic heterogeneity, bearing genus-, species-, sub-species and serotype-specific epitopes.

(7) This heterogeneity was also reflected in the partial proteolytic profiles within two types of ovine *C.psittaci* MOMP although these MOMPs did not appear to be the site of heterogeneity (if any) among OEA isolates.

(8) Finally and most conclusively, the N-terminus sequence of this antigen was shown to be identical to that of *C.trachomatis* MOMP.

Having accumulated satisfactory evidence as to the nature and identity of the antigenic protein implicated as a potential immunoprotective component in OEA vaccines, that it is the OEA

C.psittaci equivalent of the major outer membrane protein in other well-characterised chlamydiae, an experiment to test its efficacy as a protective immunogen in the sheep was performed and is described in the following chapter.

CHAPTER 5

PRODUCTION, ANALYSIS AND ASSESSMENT OF A SUBCELLULAR VACCINE CONTAINING MOMP

INTRODUCTION

Evidence which suggested that the major outer membrane protein (MOMP) of *C.psittaci* may be useful for protection in sheep has been described in previous chapters and reported in the literature (Tan *et al*, 1988, Huang *et al*, in press, Anderson *et al*, submitted). For testing the hypothesis that MOMP is an immunoprotective antigen, it would ideally require large quantities of pure MOMP to vaccinate sheep in a vaccination-challenge experiment. It was, however, difficult to obtain pure MOMP in sufficient quantities without using strongly denaturing procedures. A detergent extraction method was used instead to produce an outer membrane preparation containing enough MOMP in a highly enriched and undenatured state for a vaccination experiment. To extend the previous observation that an experimental two-dose vaccine comprising purified organisms could protect sheep from OEA (Chapter 3; Anderson *et al*, submitted), a similar preparation given in a single dose has also been used. Owing to the prevalence of *C.psittaci* infection in the sheep population, steps were also taken to ensure that animals obtained for this study were immunologically naive.

MATERIALS AND METHODS

Preparation of Vaccines and Placebo

Chlamydial culture and purification of S26/3 organisms were carried out as described in Chapter 2. Purified organisms were divided into two equal aliquots of 2 mg each. One aliquot (1ml) was made up to 2 ml with standard phosphate buffered saline pH7.4 (PBS) and inactivated by incubation for 5 minutes at room temperature with an equal volume of PBS containing 0.15% (v/v) glutaraldehyde (BDH). The reaction was quenched with 2 ml of PBS containing 0.1M glycine (Sigma). The mixture was centrifuged at 100,000g for 30 minutes at 4°C and the EB pellet obtained was resuspended in physiological saline (0.15M NaCl).

This preparation was then adsorbed with a 0.3% aluminium hydroxide solution (Alhydrogel, Miles Laboratories, Slough) until the mixture of chlamydial components and aluminium hydroxide turned flocculent, typically 2:1 to 3:1 (v/v). The suspension was emulsified with an equal volume of 9:1 (v/v) of Bayol 82 and Arlacel A (Esso Petroleum Co.) to make the purified EB vaccine of approximately 160μg of protein per 1ml dose).

The other aliquot was subjected to a two-step detergent extraction procedure, similar to a previously reported method (Caldwell *et al*, 1981; Caldwell and Schachter, 1982; Bavoil, *et al*, 1984). Elementary bodies of the second aliquot (2mg/ml) were first washed in 12 ml of 100mM phosphate buffer pH 7.4 containing 1 mM EDTA (PBE) and collected by centrifugation at 100,000g at 4°C for 30 minutes. The pellets were then resuspended in PBE containing 2% Sarkosyl (PBES) to a final protein concentration of 0.3mg/ml and incubated for one hour at 37°C with occasional mixing and bath sonication (2x30s bursts) to prevent aggregation. The mixture was then centrifuged at 100,000g at 20°C for 60 minutes to pellet the insoluble material. The PBES supernatant was removed and the pellet resuspended in 5.6ml of PBES containing 10mM dithiothreitol (PBESD) using a Teflon homogeniser. The suspension was incubated at 37°C for 2 hours with occasional sonication as in the previous step. The PBESD insoluble material was collected by centrifugation as before and resuspended in PBS to make 1.5 ml containing about 200μg of protein. This subcellular outer membrane preparation was added to 1.5 ml of PBS containing 0.15% (v/v) glutaraldehyde (BDH) and incubated for 5 minutes before quenching with 1.5 ml of PBS containing 0.1M glycine. This mixture was centrifuged as before. The supernatant was removed and the pellet was resuspended in saline and formulated into a vaccine as described for the purified EB vaccine. Each dose of this subcellular vaccine contained approximately 20μg of protein.

To produce the placebo vaccine, uninfected mycoplasma-free BHK-21 cell monolayers from two Sani Glass bottles were harvested in a similar way as chlamydial purification, concentrated by low speed centrifugation (2000g at 4°C for 5 minutes) and resuspended with a Teflon homogeniser in PBS (1ml). This solution (2 mg/ml) was inactivated and quenched with equal volumes of PBS-glutaraldehyde and PBS-glycine respectively, and formulated into a placebo vaccine as described above for the purified EB vaccine. Each 1ml dose was estimated to contain about 0.15 - 0.2 mg.

Protein estimation was carried out using the Bio-Rad Protein Assay kit according to the manufacturer's recommendations (Bio-Rad Laboratories, FRG). Electrophoresis, immunoblotting, immunodot-blotting, and electron microscopy were carried out as described in Chapter 2.

Animal procedure

Ewes were obtained from a flock with no known history of OEA. These were screened by the CF test, immunodot-blot and immunoblot analyses for chlamydia-specific antibodies as a crude indicator for previous exposure to chlamydiae. Following synchronisation of oestrus, selected ewes were mated and penned separately in three groups. Within a month of mating, ewes from each group were vaccinated subcutaneously with a single dose of either the placebo (A), purified EB (B) or subcellular (C) vaccines. Serum samples were taken at regular intervals for analysis by the CF test (Stamp *et al*, 1952) and by immunoblotting. At seventy days gestation, all animals were challenged by subcutaneous injection with 1 ml of live S26/3 organisms ($10^{5.5}$ chick embryo lethal doses). At parturition, vaginal swabs or placental tissues were taken for isolation of chlamydiae as previously described (Anderson, 1986b; Chapter 2; Anderson *et al*, submitted).

RESULTS

Production of the vaccine

The procedure described in the methods section of this chapter for producing the subcellular OM preparation was arrived at after preliminary experiments were done to optimise conditions of enrichment for MOMP. Several attempts were made to solubilise the MOMP in these insoluble outer membrane preparations using the non-ionic detergent, octylglucoside, and dithiothreitol. Yields were low and further purification by gel filtration column chromatography resulted in even lower recovery. Consequently, no further attempt to purify MOMP from the OM preparation was made.

The results of SDS-PAGE analysis of the vaccine preparations and the intermediate stages of preparation were shown in Figure 5.1. The two-step extraction procedure was able to remove almost all low M_r proteins below 40kDa leaving MOMP in a highly enriched state. Some high M_r components were still present and were particularly visible when the well was overloaded. Densitometric scanning showed that in the final detergent insoluble preparation, MOMP comprised more than 90% of total protein. Chlamydial lipopolysaccharide (LPS) was also stained by silver in this gel and migrated as a band ahead of the 14 kDa marker, just behind the dye front.

Kinetics of the antibody response

The serum samples of all experimental animals were taken and monitored by the CF test at regular intervals during the course of the study (Figure 5.2; Table 5.1). No antibody response to chlamydial antigens was detected after control ewes were vaccinated with a placebo vaccine (Figure 5.2A; Table 5.1A). In contrast, most control animals responded with $\log_2$ CF antibody titres ranging from 4 to 6 within four weeks of homologous challenge. Although three ewes had responded slowly, all control animals except one eventually seroconverted (Table 5.1B). Only a weak CF response was detected in this sheep. It was resistant to infection.

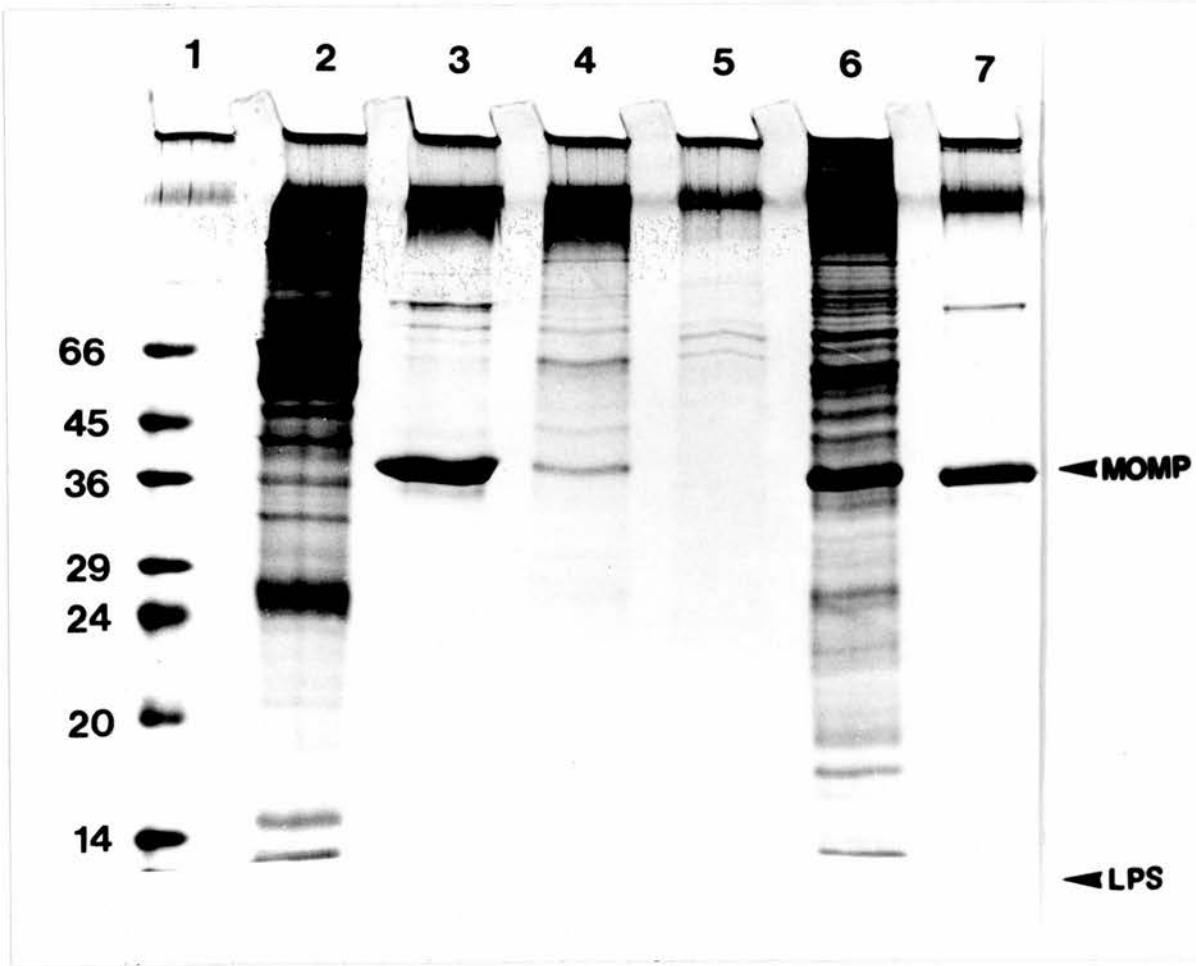


Figure 5.1. SDS-PAGE profiles of the preparations used in the vaccination-challenge experiment to test the efficacy of a purified EB and a MOMP-subcellular vaccine. The 12.5% gel was stained with silver. Key: M_r standards labelled in kDa (lane 1). BHK-21 cell preparation used in Group A ewes as a placebo vaccine (lane 2). Purified EB preparation used to vaccinate Group B ewes (lane 6). Outer membrane preparation used to vaccinate Group C ewes (lane 3 - 3μg and lane 7 - 1μg). PBES-soluble components from the first detergent extraction of EBs (lane 5). PBESD-soluble components from the second extraction (lane 4).

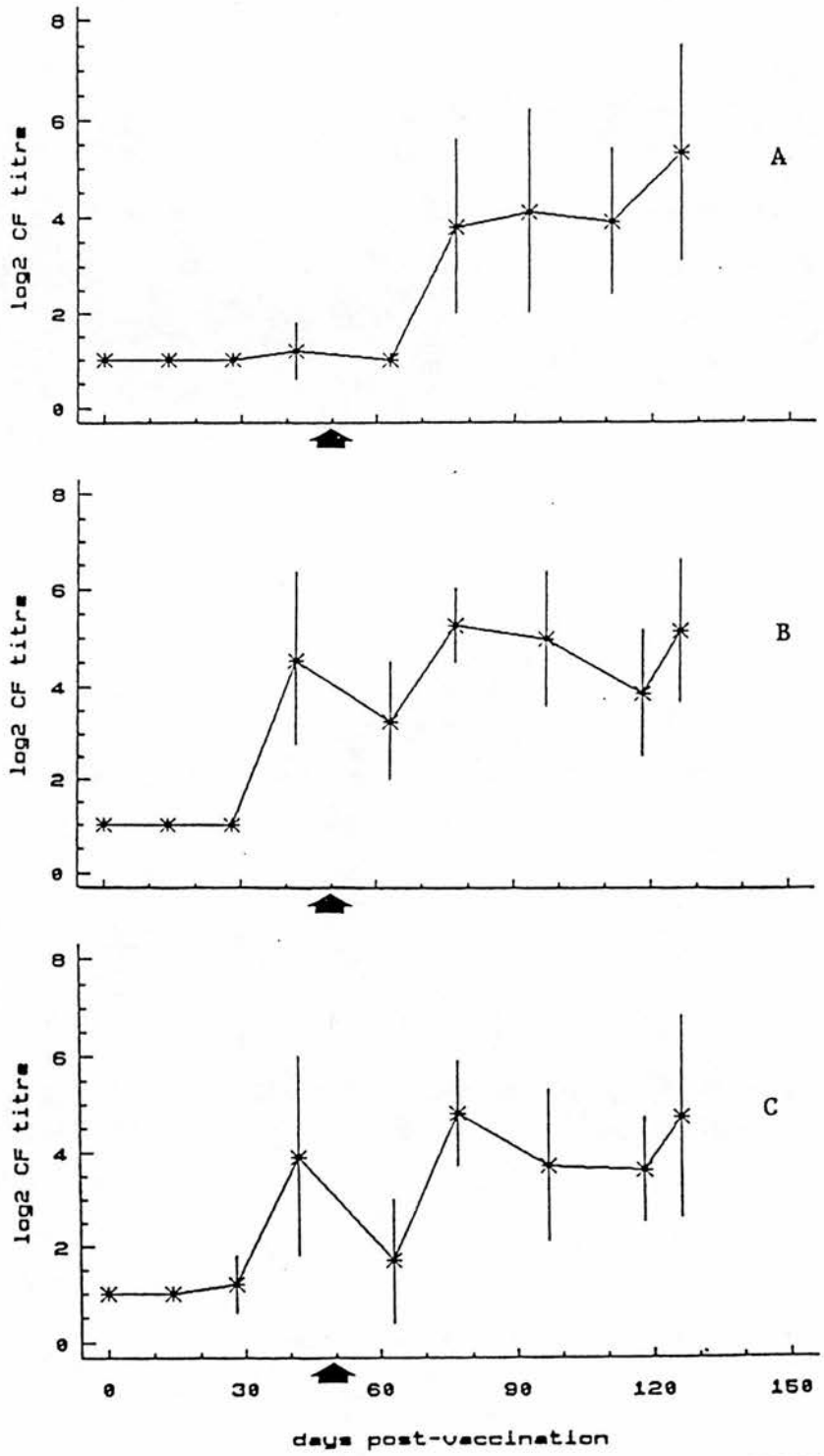


Figure 5.2. Kinetics of the complement fixing (CF) antibody respor of each vaccine group: A. placebo vaccine group (n=12). B. purifi vaccine group (n=7). C. MOMP-enriched subcellular group (n=11). T day of subcutaneous challenge with live chlamydiae is indicated with arrow on each abscissa. Mean $\log_2$ CF titres of all ewes in each gr are displayed with error bars (±one standard deviation).

Vaccination with purified EBs or the outer membrane vaccines induced a primary response in most sheep within 42 days (Figure 5.2B and 5.2C). Titres generally declined by the time of challenge whereupon a secondary response was subsequently induced in all vaccinated sheep. One ewe from group B and three from group C did not produce a detectable CF titre before challenge (Table 5.1A) but none of them became infected or aborted. No significant correlation was found between the presence of pre-challenge CF titres and immunity.

An immunoblot of the serum samples at week 4 post-vaccination is shown in Figure 5.3. Nearly all vaccinated ewes had by then produced a detectable antibody response particularly to MOMP. No chlamydia-specific antibodies were detected in the negative control animals.

Lambing results

Table 5.1C shows the protection results of the vaccines. 10 out of 11 animals in group C were protected against abortion. No chlamydiae could be isolated from the vaginal swabs or fetal membranes taken at parturition from these 11 ewes. Only one lamb from the single ewe died. In group B, 6 out of 7 were protected and a similar low ratio of lamb mortality was recorded. In contrast, 7 out of 11 control animals were infected of which 5 subsequently aborted. The lamb mortality was significantly higher ($P < 0.5\%$).

Table 5.1. Results of OEA vaccination-challenge experiment testing a subcellular outer membrane preparation highly enriched in MOMP.

	Panel 5.1A				*Panel 5.1B*				*Panel 5.1C*						*Panel 5.1D*		
Group	Number of Aborted and/or infected ewes with Pre-Challenge Sera showing CF titre		Number of Resistant ewes with		Number of Aborted and/or infected ewes with Post-Parturition Sera showing CF titre		Number of Resistant ewes with		Number of ewes Infection only		Number of ewes infection and abortion		Number of lambs		Significance level of vaccine vs placebo (P value <) by criterion:		
Serological status	*-ve*	*+ve*	*-ve*	*+ve*	*-ve*	*+ve*	*-ve*	*+ve*	*-ve*	*+ve*	*-ve*	*+ve*	live	dead	Infected or Uninfected	Aborted or Unaborted	Live Lamb: Dead Lamb
A. Placebo control n= 12	7	0	5	0	0	7	1[+]	4	7	5	5	7	8	9			
B. Purified EB Vaccine n=7	0	1	1	5	0	1	0	6	1	6	1	6	15	1	0.080	0.238	0.004
C. Subcellular MOMP vaccine n=11	0	1	3	7	0	1	1[~]	9	1	10	0	11	16	1	0.019	0.024	0.003

Panel 5.1A divides the ewes of each group firstly into two classes, resistant or affected, and divides each class into two subclasses according to the CF titre of the pre-challenge sera (log2 dilution <4 is negative, or else positive). The table shows the number of ewes in each category (no significant correlation P>0.05). Similarly Panel 5.1B tabulates the outcome of the post-parturition sera. Panel 5.1C shows the efficacy of the vaccines by three criteria; the significance levels calculated by the Fisher's exact test (one-tailed) of the test vaccine against the placebo is given in Panel 5.1D.
(+) one ewe with weak CF response; (~) sera showed rapid decline in titre from 5.

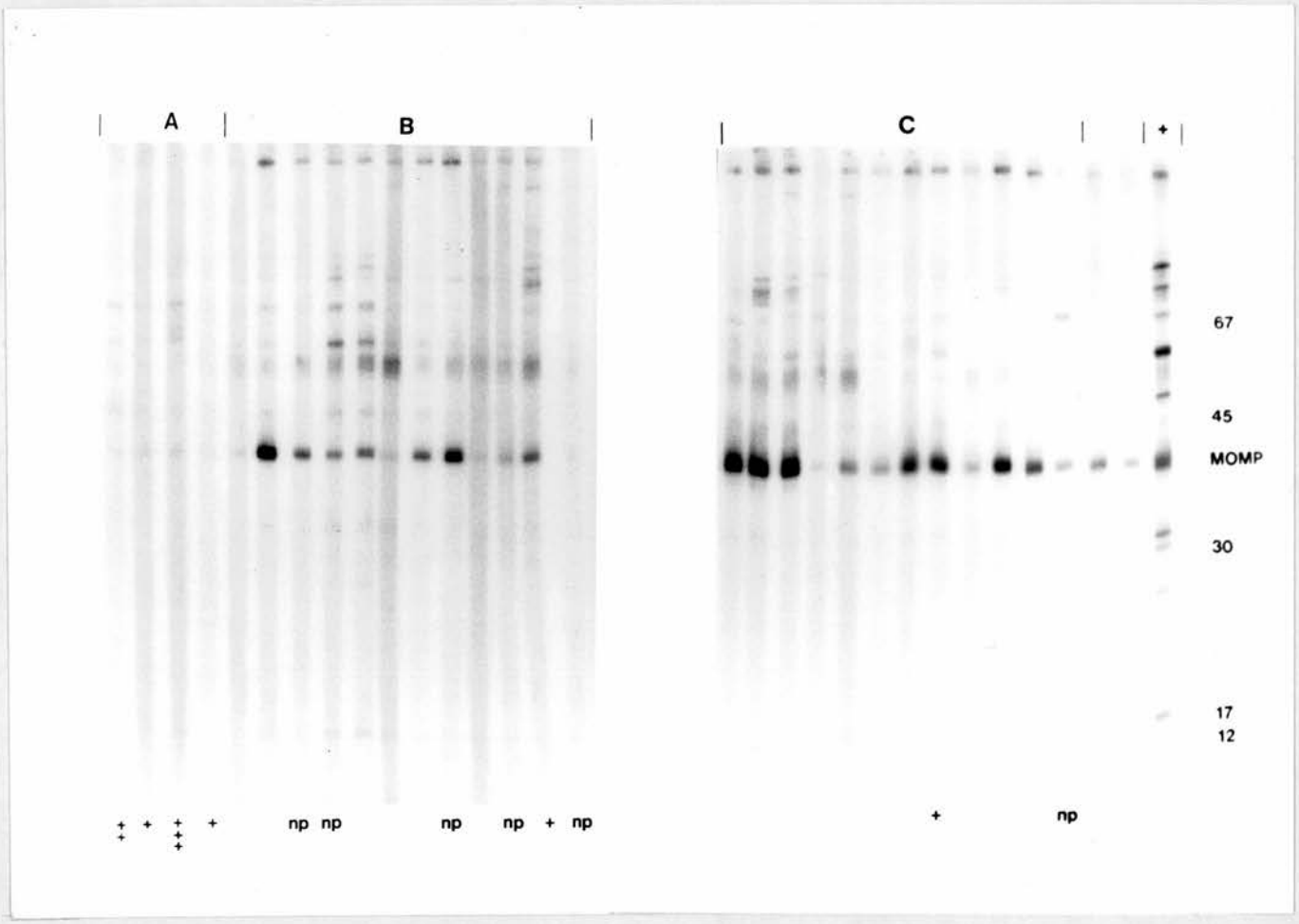


Figure 5.3. Immunoblot of sera from ewes of all groups, four weeks post-vaccination. Panel A shows the immunoblot profiles of four sets of pooled sera from three controls animals each. Each symbol (+) at the bottom indicates an ewe which eventually became infected. Panel B shows the profiles for the purified EB vaccine group. The letters (np) stand for non-pregnant ewes which were not challenged and subsequently not used in the experiment. Panel C shows the profiles for the subcellular MOMP vaccine group. Panel + contains the profile of a post-abortion sheep serum. The unmarked panel contains the profiles of two ewes vaccinated with the PBESD supernatant as part of another experiment outside the scope of this thesis.

DISCUSSION

Experimental design

The widespread nature of chlamydial infection in sheep flocks whether from abortigenic or non-abortigenic strains of *C.psittaci* such as enteric strains necessitated stringency in the selection of experimental animals for this study. In addition to ensuring that animals were obtained from a source with no known history of OEA, the CF test, immunodot-blot and immunoblot analyses were carried out on the serum samples. Although these criteria do not completely eliminate all possibility of chlamydia-infected animals, it was thought necessary at least to exclude from the study animals with specific antibodies.

The purified EB vaccine described in Chapter 3 has been incorporated in this study firstly as positive control and secondly, to test its efficacy in a single dose. This vaccination regime induced protection in 6 out of 7 animals and a lamb survival count which was significantly better than the placebo group thereby confirming and extending the previous result. The use of a single dose in this study rather than the two-dose regime of the previous experiment may have caused the slightly reduced vaccine efficacy.

Previous vaccination-challenge experiments suggested that a 55% to 70% rate of abortion could be expected from the negative control ewes using the subcutaneous challenge procedure described in this study (I.E.Anderson and G.E.Jones, personal communication). Although the abortion rate of the control group was not significantly different from previous results, a more effective challenge would be desirable. It appears that in future experiments a stronger challenge may be required, possibly via a more natural route such as intra-tonsillar instillation (Jones and Anderson, 1988). The lamb survival rate in the control group was 47%, comparable to the 50% rate obtained in a previous vaccination experiment.

Vaccination in chlamydial infections

In contrast to attempts at vaccination against *C.trachomatis* infections in humans (Grayston *et al*, 1963; Woolridge *et al* 1967) and in animal models (Bell *et al*, 1959; Collier and Blyth; 1966; Wang *et al*, 1967; Orenstein *et al*, 1973; Cui *et al*, 1989) using crude whole organisms as immunogens, vaccination against chlamydial abortion in ewes has had a long and successful history (reviewed by Foggie, 1973). It was only in the past decade that signs of vaccine breakdown emerged (Linklater and Dyson, 1979) which led to renewed investigations. These included studies on cross-protection between strains (Aitken *et al*, 1981; Aitken *et al*, 1986), strain variation (McClenaghan *et al*, 1984; Anderson 1986a and 1986b; Anderson and Baxter, 1986; Johnson and Clarkson, 1986), antigenic structure of *C.psittaci* (McClenaghan *et al*, 1986), pathogenesis (Blewett *et al*, 1982; Jones and Anderson, 1988; Buxton *et al*, in press) and the immune response (Dawson *et al*, 1986a and 1986b; Buzoni-Gatel *et al*, 1987; Huang, M.Phil. thesis, 1988; Huang *et al*, in press).

The immunoprotective antigens in OEA, however, have not been well characterised. In Chapter 3, a number of *C.psittaci* antigens have been identified of which MOMP was implicated as a promising candidate antigen for OEA vaccines. In this chapter it has been demonstrated that a vaccine based on a subcellular fraction can protect pregnant ewes against infection and abortion from experimental subcutaneous challenge with a virulent ovine abortion strain of *C.psittaci* - S26/3. The efficacy of this subcellular vaccine compares favourably with other experimental OEA vaccines and the commercial product (G.E.Jones, personal communication). The subcellular outer membrane fraction contained largely MOMP and LPS with trace amounts of other chlamydial antigens. Being the main components of the vaccine, MOMP and/or LPS are suspected to possess the immunoprotective epitopes. Their relative importance in immunoprotection are discussed in a following section.

Outer membranes prepared from detergent extractions are known to lose varying amounts of lipids, LPS and other components, resulting in varying degrees of structural reorganisation (Lugtenberg and van Alphen, 1983). However, the detergent-extracted subcellular preparations appeared as fine particles and rosettes under the electron microscope. These structures corresponded to those in preparations not treated with detergent as shown here and in the literature (reviewed by Matsumoto, 1988). This suggested that, if reorganisation of the outer membrane structure did occur during detergent extraction, it was not sufficient to alter the general morphology of the outer membrane preparations. The retention of the three-dimensional structure and the membrane context of MOMP in both vaccine preparations may have been important for eliciting immunity. The loss of such structure may be a possible explanation as to why a purified subunit MOMP vaccine failed to induce an adequate antibody response and elicited poor protection against eye infection in cynomolgus monkeys (Taylor *et al*, 1988).

Immune response and immune mechanisms

The exact nature of the immune mechanisms which are elicited by chlamydial infections is presently unclear. The most detailed studies have been made with chlamydial infections of small rodents, for example, with the *C.trachomatis* mouse pneumonitis agent (MoPn) in a murine model for chlamydial genital infection and with the *C.psittaci* guinea pig inclusion conjunctivitis agent (GPIC) in guinea pigs (reviewed by Williams, 1988 and Rank, 1988; Ramsey *et al*, 1988; Ramsey *et al*, 1989; Rank and Batteiger, 1989; Rank *et al*, 1989).

In the mouse-MoPn model, infection can be resolved in the absence of an antibody response (Ramsey *et al*, 1988) suggesting that cell mediated immunity plays the dominant role in resolution of infection and resistance to reinfection. Although *in vitro* studies have established that antibodies do neutralise *C.trachomatis* infectivity, for example with plasma (Barenfonger

and MacDonald, 1974), with antiserum (Howard, 1975; Williams *et al*, 1982), with anti-MOMP immunoglobulin (Caldwell and Perry, 1982) and with monoclonal antibodies to MOMP (Peeling *et al*, 1984; Lucero and Kuo, 1985), recent data question the value of the humoral response in MoPn genital infections (Ramsey *et al*, 1989).

A recent study of *C.psittaci* in a mouse model of systemic infection has shown that T-cell mediated immunity was more effectively transferred than humoral immunity (Buzoni-Gatel *et al*, 1987). However, both humoral and cell mediated immune responses have been shown to be essential in the guinea-pig-GPIC model (Rank *et al*, 1979; Rank and Barron, 1983a and 1983b; Rank and Batteiger, 1989; Rank *et al*, 1989). Any extrapolation to the situation in sheep is presently premature and should take into account that the immune mechanisms responsible for resolution of the infection and resistance to reinfection may differ between types of chlamydiae or between host species.

Although studies of immune responses have been reported for ovine abortion strains in sheep (Dawson *et al*, 1986a and 1986b; Huang, M.Phil. thesis, 1988; Huang *et al*, in press), the immunological mechanisms for protection by vaccination remain undefined. The results in this chapter has confirmed the finding described in Chapter 3 that anti-MOMP antibodies feature prominently in immunoblot profiles of vaccinated sheep. Whether such antibodies are mediators of immunity or are merely indicators of immunity needs to be resolved. Recently, these sera were analysed by a newly developed microplate serum neutralisation test and results showed that all animals which possessed a neutralising titre were protected (I.E.Anderson, personal communication). In contrast, the CF test did not show such a correlation, confirming the findings of other investigators (Dawson *et al*, 1986a and 1986b; Buzoni-Gatel *et al*, 1987).

The role of the antibody response in immunity to OEA was recently assessed in a passive protection experiment. However, initial findings did not show any evidence that post-abortion serum antibodies could protect pregnant ewes against experimental challenge with live organisms (G.E.Jones, personal communication). Since *C.psittaci* is an intracellular parasite, cellular immunity is probably involved. Some evidence for this has been reported (Dawson *et al*, 1986a and 1986b; Huang, M.Phil. thesis, 1988; Huang *et al*, in press). Studies are in progress to characterise the cell mediated responses that may be involved in immunity (C.M. McCafferty, D.Buxton and D.M.Haig, personal communication).

The role of MOMP in immunoprotection

Having demonstrated though not exclusively that the immunoprotective element of previous OEA vaccines resides on the outer membrane preparation, the role of each chlamydial constituent of the subcellular vaccine now remains to be assessed. The chlamydial LPS carries genus-specific epitope(s) (Caldwell and Hitchcock, 1984) and is not thought to be an important antigen for protection in OEA or in other chlamydial infections for the following reasons. Polyclonal antibodies directed against LPS could not neutralise chlamydial infectivity *in vitro* (Caldwell and Perry, 1982). Serum prepared against heat-killed organisms, which is likely to contain antibodies against the thermostable genus-specific epitope of LPS, did not confer protection in passive transfer experiments using a mouse model of systemic infection (Buzoni-Gatel *et al*, 1987). A monoclonal antibody which recognises a genus-specific epitope located on chlamydial LPS could not protect mice from toxic death or neutralise infectivity for the monkey eye by challenge with a trachoma serovar (Zhang *et al*, 1987a; Zhang *et al*, 1989b). Oral immunisation with a recombinant lipopolysaccharide bearing this genus-specific epitope of chlamydial LPS could not protect monkeys against ocular infection (Taylor and Prendergast, 1987).

In the case of sheep, the CF test which is thought to detect LPS-genus-specific epitope(s) did not correlate with protection (Dawson *et al*, 1986a and 1986b; Buzoni-Gatel *et al*, 1987). The immunoblotting technique used in this study or in previous studies on OEA (McClenaghan *et al*, 1986) did not detect a strong antibody reaction against LPS. Although chlamydial LPS is unlikely to carry protective epitopes, it may play a role in enhancing the immunogenicity of OEA vaccines in view of the well-known properties of LPS as an adjuvant (Johnson *et al*, 1956).

SDS-PAGE analysis showed the presence of 10 to 15 minor proteins in the subcellular outer membrane vaccine. Only one or two of these contaminants were weakly reactive by immunoblotting (Figure 4.5B and Figure 5.3). Since the total administered dose was estimated at 20 μg of protein, each component was present in submicrogram quantities. For large animals such as sheep, such amounts are unlikely to constitute sufficient antigenic mass to contribute a significant effect in protection. MOMP, in contrast, was the major protein component. Several lines of evidence suggested that it was the immunoprotective element of the subcellular vaccine used in this study.

Functionally, MOMP has been shown to be involved in structural rigidity of the EB (Caldwell *et al*, 1981), in the developmental cycle and in the permeability of the outer membrane (Newhall and Jones, 1983; Hatch *et al*, 1984; Bavoil *et al*, 1984) and in infectivity (Su *et al*, 1988). A complex hierarchy of antigenic epitopes has been shown to reside on MOMP (Stephens *et al*, 1982; Ma *et al*, 1987). Polyclonal and serovar-specific monoclonal antibodies raised against MOMP have been demonstrated to neutralise chlamydial infectivity *in vitro* (Caldwell and Perry, 1982; Peeling *et al*, 1984; Lucero and Kuo, 1985) and *in vivo* (Zhang *et al*, 1987a; Zhang *et al*, 1989b). Sheep lymph node cannulation experiments described in Chapter 3 (reported in Huang *et al*, in press) have indicated that MOMP is an immunodominant antigen against which the lymph

node response is initially directed. The vaccination-challenge experiment also described in Chapter 3 showed that all protected animals directed a strong antibody response almost only to MOMP (Anderson *et al*, submitted). Although a recent experiment testing purified MOMP as a vaccine demonstrated only a partial effect against ocular *C.trachomatis* infection in monkeys (Taylor *et al*, 1988), it is possible that the use of strong detergent in their preparation of MOMP may have destroyed some protective epitopes.

Taken as a whole, a body of data has accumulated in the literature and in this thesis that strongly implicates MOMP as a useful immunogen. A definitive experiment to determine if MOMP alone is sufficient for protection would require a stringent purification procedure to eliminate all contaminating antigens. This has so far been difficult to achieve in quantity without denaturing conditions. Consequently, a recombinant DNA approach to produce a MOMP subunit vaccine was undertaken. This entailed the characterisation of the structure of the MOMP gene and its subsequent manipulation into vectors for expression, the results of which are described and discussed in the following chapters.

CHAPTER 6

ANALYSIS OF THE MAJOR OUTER MEMBRANE PROTEIN GENE

INTRODUCTION

The data presented in the previous chapters have shown that the ovine immune system recognises the major outer membrane protein of OEA strains of *C.psittaci* as a dominant antigen. A further experiment has provided evidence that MOMP is immunogenic and is likely to contain protective epitopes. It was at this point in the project that the characterisation of the MOMP gene was made an important priority for the research group as a whole. The author of this thesis became involved in the sequencing of the MOMP gene already cloned by the other members of this group (Herring *et al*, 1989), specifically in mapping the restriction sites of the gene, sequencing the 5′ end and other segments of the MOMP gene, as well as reading and checking the complete DNA sequence as presented in this chapter and in Herring *et al* (1989; see Appendix).

In order to obtain a better understanding of the structure of the gene and its encoded product, the sequence data thus obtained was analysed by using various computer programs for DNA sequence analysis, and was compared with other published sequences of chlamydial MOMPs and with outer membrane proteins of other bacteria. Such a knowledge is essential for the design of strategies to express the *C.psittaci* S26/3 MOMP gene. The derivation of a viral vector containing the MOMP gene is briefly described below.

A λEMBL3 library was made from a partial *Mbo*I digest of the S26/3 genome. A positive clone carrying the S26/3 MOMP gene was selected using gene probes (gift of Dr R.Stephens, University of California, San Francisco) made from two pUC9 plasmid clones containing the 5′ and 3′ ends of the MOMP gene from *C.trachomatis* serovar L2 (A.J.Herring and N.F.Inglis, personal communication). Apparently, there is DNA homology

within the MOMP gene family (Stephens *et al*, 1985) even though the two chlamydial species share very little DNA homology at the genomic level (Kingsbury and Weiss, 1968). The positive clone thus identified had hybridised with both probes and further analysis showed that this hybridisation was restricted to a 2.6kb *Sst*I fragment. To avoid potential problems of plasmid instability when cloning outer membrane proteins (Clarke and Lambden, 1988), this *Sst*I fragment was subcloned into the viral vector, M13 mp19 to produce clone A1/4.

RESULTS

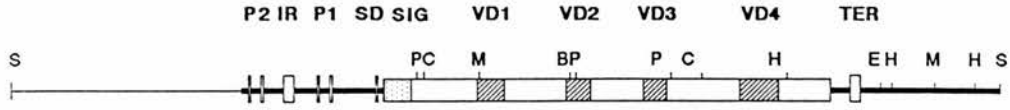
Restriction Endonuclease Analysis

The 2.6 kb *Sst*I fragment was subjected to digestion with a panel of restriction endonucleases (REs) namely, *Eco*RI, *Hin*dIII, *Cla*I, *Pvu*II, *Msp*I, *Alu*I, *Mbo*I and *Acc*I. Restriction sites were identified and mapped on the *Sst*I fragment by double RE digests. Some of these restriction sites and other important sites deduced subsequently from the DNA sequence are shown in Figure 6.1A. The sizes of the restriction fragments were estimated by interpolation from a log-linear calibration curve derived from DNA standards.

DNA sequencing

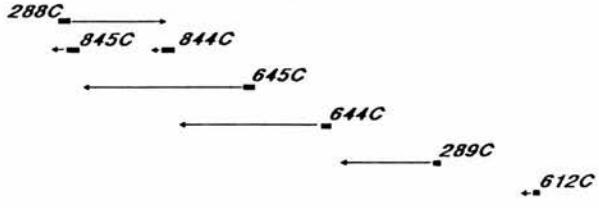
The M13 mp19-derived clone carrying the *Sst*I insert (A1/4) was sequenced using the M13 universal primer in a chain termination reaction (see Chapter 2). Initial sequence data from the 3′ end of the insert matched the 3′ end of the MOMP genes from *C.trachomatis* L2 (Stephens *et al*, 1986) and the *C.psittaci* A22/M (Pickett *et al*, 1988a). Thus the S26/3 MOMP gene is located on the 3′ end of the *Sst*I insert. A method for making a library of nested deletions from the 3′ end of the MOMP gene was attempted but without success. Consequently, *Eco*RI, *Hin*dIII, *Cla*I and *Msp*I RE fragments derived from the 2.6 kb *Sst*I fragment were subcloned into M13 mp18 and mp19 vectors for sequencing. The sequencing strategy is illustrated in Figure 6.1B. Confirmation of the DNA sequence was obtained by using a

A.



B.

C.



D.

Primers	Sequence 5' to 3'	Direction
288C	TTG CCT GTA GGG AAC CCA GCT	→ VD1
289C	ACC AAC AGC TAC ACC ACA AGC T	VD4 ←
612C	TTT CAC TGC AGG TTG TCA ATC TCT AGG TTT CAA GC	TER ←
644C	CCT GAT CAG TAC CAG C	VD3 ←
645C	TGC ACC TAC ACT CCA AG	VD2 ←
844C	TGG TGA ACC ATT CGG CG	VD1 ←
845C	CCC ACA TAG TGC CAT CG	SIG ←

Figure 6.1 The 2.6 kb SstI-SstI fragment containing the MOMP gene of C.psittaci S26/3. A. Gene Structure and. Restriction Map; B. Sequencing Strategy; C. Positions of oligonucleotide primers; D. Sequences of the primers. Abbreviations: Promoters - P1 and P2; inverted repeat - IR; Shine-Dalgarno translation recognition site - S-D; putative signal peptide - SIG; variable domains - VD1 to VD4; putative transcription terminator - TER; restriction endonuclease sites - BamHI, ClaI, EcoRI, HindIII, MspI, PvuII, SstI.

set of synthetic oligonucleotides including 288C, 289C, 612C, 644C and 645C for priming the sequencing reaction (Figure 6.1C). The sequences of the synthetic oligonucleotides are listed in Figure 6.1D. In total, approximately 2 kb from the 3' end of the 2.6 kb fragment were sequenced (Figure 6.2).

Gene structure

The DNA sequence revealed one long open reading frame (ORF) which consisted of 1170 bp beginning with a codon for methionine (ATG) and ending with a stop codon TAA. The ORF encoded 389 amino acids and was preceded by promoter-like sequences and a ribosome-binding site AGAGG (Shine and Dalgarno (1974). Further upstream of these regulatory sequences, more promoter-like sequences and a perfect 9 bp inverted repeat (IR) were detected (Figure 6.2).

Downstream of the ORF, another IR followed by 8 thymine residues was also found by comparison with the L2 MOMP sequence and by using the TERMINATOR program (Brendel and Trifonov, 1984) of UWGCG sequence analysis package (Devereux *et al*, 1984). The RNA transcript of this IR may form a stable RNA stem-loop structure followed by seven uridine residues (Figure 6.3; Table 6.1). The free energy of formation of this 13 bp stem and 4-base hairpin loop structure was estimated to be -14.5 kcal/mol using the FOLD program (UWGCG) which is based on the method of Zuker and Stiegler (1981). This stable stem-loop structure followed by a poly-U tail is characteristic of prokaryotic rho-independent terminators (Platt and Bear, 1983).

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-381                                                                                    C AATATAATAT AAGAAGCCTG

-360  TACACTCTTC TATGAGGTAA TTCCAACTTA TTCTAAGTGC ATAAGAAAAT AAAAATGTGT ACAAAAATCT AATAGCTCTC TTATTAGCAA
                                                                                      P2  -35

-270  GTATAAGGAG TTATTGCTTG AAATCTGTGC CTGAAACAGT CTTTTTTCTT ATCGTCTTTA CTATAATAAG AAAAGTTTGT TATGTTTTCG
       P2 -10                                          --------->InvertedRepeat<---------

-180  AATAATGAAC TGTATGTTCA TGCTTAAGGC TGTTTTCACT TGCAAGACAC TCCTCAAAGC CATTAATTGC CTACAGGATA TCTTGTCTGG


-90   CTTTAACTTG GACGTGGTGC CGCCAGAAGA GCAAATTAGA ATAGCGAGCA CAAAAAGAAA AGATACTAAG CATAATCTTT AGAGGTGAGT
                      P1  -35                             P1  -10                              -S-D-

-22   Met Lys Lys Leu Leu Lys Ser ALA Leu Leu Phe Ala Ala Thr Gly Ser Ala Leu Ser Leu Gln Ala Leu Pro Val
  1   ATG AAA AAA CTC TTG AAA TCG GCA TTA TTG TTT GCC GCT ACG GGT TCC GCT CTC TCC TTA CAA GCC TTG CCT GTA
                                                                                          288Cprimer___

  4   Gly Asn Pro Ala Glu Pro Ser Leu Leu Ile Asp Gly Thr Met Trp Glu Gly Ala Ser Gly Asp Pro Cys Asp Pro
 76   GGG AAC CCA GCT GAA CCA AGT TTA TTA ATC GAT GGC ACT ATG TGG GAA GGT GCT TCA GGT GAT CCT TGC GAT CCT
      __________PvuII\                  ClaI/_________845Cprimer


 29   Cys Ser Thr Trp Cys Asp Ala Ile Ser Ile Arg Ala Gly Tyr Tyr Gly Asp Tyr Val Phe Asp Arg Val Leu Lys
151   TGC TCT ACT TGG TGT GAT GCT ATC AGC ATC CGC GCA GGA TAC TAC GGA GAT TAT GTT TTC GAT CGT GTA TTA AAA


 54   Val Asp Val Asn Lys Thr Ile Thr Gly Met Gly Ala Val Pro Thr Gly Thr Ala Ala Ala Asn Tyr Lys Thr Pro
226   GTT GAT GTG AAT AAA ACT ATC ACC GGC ATG GGT GCA GTT CCT ACA GGA ACC GCA GCA GCT AAT TAC AAA ACT CCT
                        >>>VD1    MspI

 79   Thr Asp Arg Pro Asn Ile Ala Tyr Gly Lys His Leu Gln Asp Ala Glu Trp Phe Thr Asn Ala Ala Phe Leu Ala
301   ACG GAT AGA CCC AAC ATC GCT TAC GGC AAA CAC TTA CAA GAC GCC GAA TGG TTC ACC AAT GCA GCT TTC CTC GCA
           VD1<<<                                        /__________844Cprimer

104   Leu Asn Ile Trp Asp Arg Phe Asp Ile Phe Cys Thr Leu Gly Ala Ser Asn Gly Tyr Phe Lys Ala Ser Ser Ala
376   TTG AAT ATC TGG GAT CGC TTT GAT ATT TTC TGC ACA TTA GGC GCT TCT AAT GGG TAC TTC AAA GCT AGT TCT GCG


129   Ala Phe Asn Leu Val Gly Leu Ile Gly Val Lys Gly Ser Ser Ile Ala Ala Asp Gln Leu Pro Asn Val Gly Ile
451   GCA TTC AAC CTC GTT GGT TTG ATT GGT GTT AAA GGA TCC TCC ATA GCA GCT GAT CAG CTT CCC AAT GTA GGC ATC
                               >>>VD2          BamHI            PvuII

154   Thr Gln Gly Ile Val Glu Phe Tyr Thr Asp Thr The Phe Ser Trp Ser Val Gly Ala Arg Gly Ala Leu Trp Glu
526   ACT CAA GGA ATC GTT GAA TTT TAT ACA GAT ACA ACA TTC TCT TGG AGT GTA GGT GCA CGC GGA GCT TTA TGG GAG
           VD2<<<                                        /__________645Cprimer

179   Cys Gly Cys Ala Thr Leu Gly Ala Glu Phe Gln Tyr Ala Gln Ser Asn Pro Lys Ile Glu Met Leu Asn Val Val
601   TGT GGT TGT GCG ACT TTA GGA GCA GAG TTC CAA TAC GCT CAG TCT AAT CCT AAA ATT GAA ATG TTG AAT GTA GTC
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204   Ser Ser Pro Ala Gln Phe Val Val His Lys Pro Arg Gly Tyr Lys Gly Thr Ala Phe Pro Leu Pro Leu Thr Ala
676   TCC AGC CCA GCA CAA TTT GTG GTT CAC AAG CCT AGA GGA TAC AAG GGA ACA GCA TTT CCT TTA CCT CTA ACA GCT
                                                                  >>>VD3                    PvuII/__

229   Gly Thr Asp Gln Ala Thr Asp Thr Lys Ser Ala Thr Ile Lys Tyr His Glu Trp Gln Val Gly Leu Ala Leu Ser
751   GGT ACT GAT CAG GCA ACT GAC ACT AAG TCG GCT ACA ATT AAA TAC CAC GAA TGG CAA GTT GGT TTA GCG CTC TCT
      644CprimerVD3<<<

254   Tyr Arg Leu Asn Met Leu Val Pro Tyr Ile Ser Val Asn Trp Ser Arg Ala Thr Phe Asp Ala Asp Ala Ile Arg
826   TAT CGA TTG AAC ATG CTT GTT CCT TAC ATT AGC GTA AAC TGG TCA CGA GCA ACT TTT GAT GCT GAC GCT ATC CGC
          ClaI

279   Ile Ala Gln Pro Lys Leu Ala Ala Ala Val Leu Asn Leu Thr Thr Trp Asn Pro Thr Leu Leu Gly Glu Ala Thr
901   ATC GCT CAA CCT AAA TTA GCT GCT GCT GTG TTA AAC TTG ACC ACA TGG AAC CCA ACC CTT TTA GGA GAA GCT ACA
                        >>>VD4

304   Ala Leu Asp Thr Ser Asn Lys Phe Ala Asp Phe Leu Gln Ile Ala Ser Ile Gln Ile Asn Lys Met Lys Ser Arg
976   GCT TTA GAT ACT AGC AAC AAA TTC GCT GAC TTC TTG CAA ATT GCT TCG ATT CAG ATC AAC AAA ATG AAG TCT AGA
      AluI                                    VD4<<<

329   Lys Ala Cys Gly Val Ala Val Gly Ala Thr Leu Ile Asp Ala Asp Lys Trp Ser Ile Thr Gly Glu Ala Arg Leu
1051  AAA GCT TGT GGT GTA GCT GTT GGT GCA ACG TTA ATC GAC GCT GAC AAA TGG TCA ATC ACT GGT GAA GCA CGC TTA
        /HindIII_____primer289C

354   Ile Asn Glu Arg Ala Ala His Met Asn Ala Gln Phe Arg Phe 367
1126  ATC AAT GAA AGA GCC GCT CAC ATG AAT GCT CAA TTC AGA TTC TAA GGATTTA GTTTATACTA TCCTAACTTT TTGTCCCGCT
                                                        1167
         rho-independent terminator
+41    ATCAGAACCT AGGAATCTCT GGGTTCTGAT TTTTTTTGCT CTCATCCTTT TGTAGAGCTT CAAATCTCTT TTCTAAAATC CATTCGCATA
       --------> ->      <- <--------                          AluI/_____________________612Cprimer

+131   AGAATTCACT GATTATCTAA AATTTTCTAG AAGCTTGAAA CCTAGAGATT ACAACCTTGC GTAAAAAGCA TTATTAAATT AACATCTCTA
         EcoRI                          HindIII/AluI

+221   TTCTTAGCAC GCGCCCGTAG CTCAATGGTA GAGCTGTAGC CTTCCAAGCT ACCGGTGTCA GTTCGATTCT GATCGGGCGC TTTCTGAACA
                       AluI                            AluI  MspI

+311   CCACCAAGAC TGAAATCTTG ACATCGGTTC AAGATGCCAT TGTTAACATA TTCAAGAAAT AGAGGTACAA AGCTTGGAAG AACAACCACT

+401   ATGCAATCTT TCAGTTAAAG ACTTAATGGA AGCGGGAGCT +440
                                       SstI
```

Figure 6.2. DNA sequence and deduced amino acid sequence of the major outer membrane protein gene from Chlamydia psittaci (ovine abortion strain S26/3). Putative promoters (P1 and P2). Shine-Dalgarno ribosome recognition box (S-D). Location of oligonucleotide primers are marked by arrows.

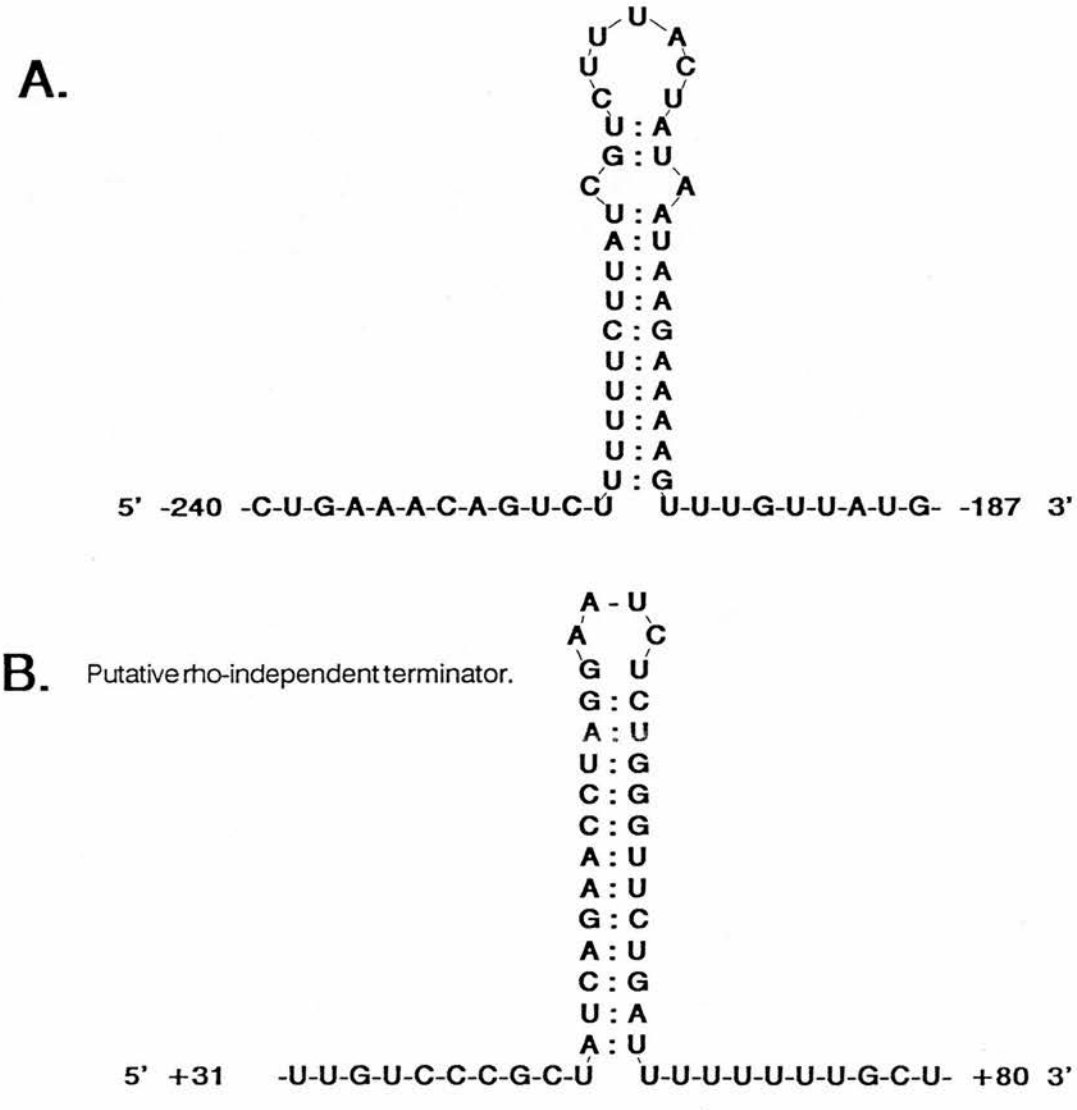


Figure 6.3 Potential secondary structure of the inverted repeats located 5' (A) and 3'(B) of the S26/3 MOMP gene. The stem-loop sturctures shown begin and end at the nucleotides corresponding to the DNA sequence. The RNA sequence is shown here in the based paired conformation with the minimum free energy as deduced by the computer program of Zuker and Stiegler. The free energies of formation are -5.9 kcal/mol and -14.5 kcal/mol respectively.

Table 6.1. Calculated free energies of formation (kcal/mol)*. Comparison of the IR and the putative terminators of S26/3, A22/M and L2 MOMP genes.

	S26/3	A22/M	L2
3′ IR**	-14.5	-16.5	-12.9
5′ IR	-9.5	-	-7.2

* Note that these values were calculated only for the underlined regions of the IR and terminator of S26/3 MOMP gene (Figure 6.2) and the corresponding sequences of A22/M (Pickett *et al*, 1988a) and L2 (Stephens *et al*, 1986). More stabler conformations or alternative folding patterns may be formed if more bases are taken into account.

** putative rho-independent terminators.

MOMP 5′ and 3′ regulatory sequences

The 5′ flanking regions of the MOMP genes of the following chlamydial strains have been aligned and displayed in Figure 6.4: *C.psittaci* strains S26/3 (OEA) (this thesis; Herring *et al*, 1989), A22/M (putative OEA) (Pickett *et al*, 1988a), Cal10 (meningopneumonitis Mn), GPIC strain 1 (guinea pig inclusion conjunctivitis) (Zhang *et al*, 1989a) *C.trachomatis* serovars L2, B and C (Stephens *et al*, 1987). The sequence of the *C.psittaci* strains from -1 to -227 were identical except for two A→T base substitutions in the GPIC sequence (-56T and -179T) and an extra base (-186T) in the S26/3 sequence. Comparison of the S26/3 sequence farther upstream with that of *C.trachomatis* serovars B, C and L2 showed that while the *C.trachomatis* sequences were virtually identical, only specific boxes of conservation were present across species. Thus there were several more genus-conserved regions than the twelve control regions, CR1 to CR12, previously designated by Pickett *et al* (1988a) for a putative ewe abortion agent, A22/M.

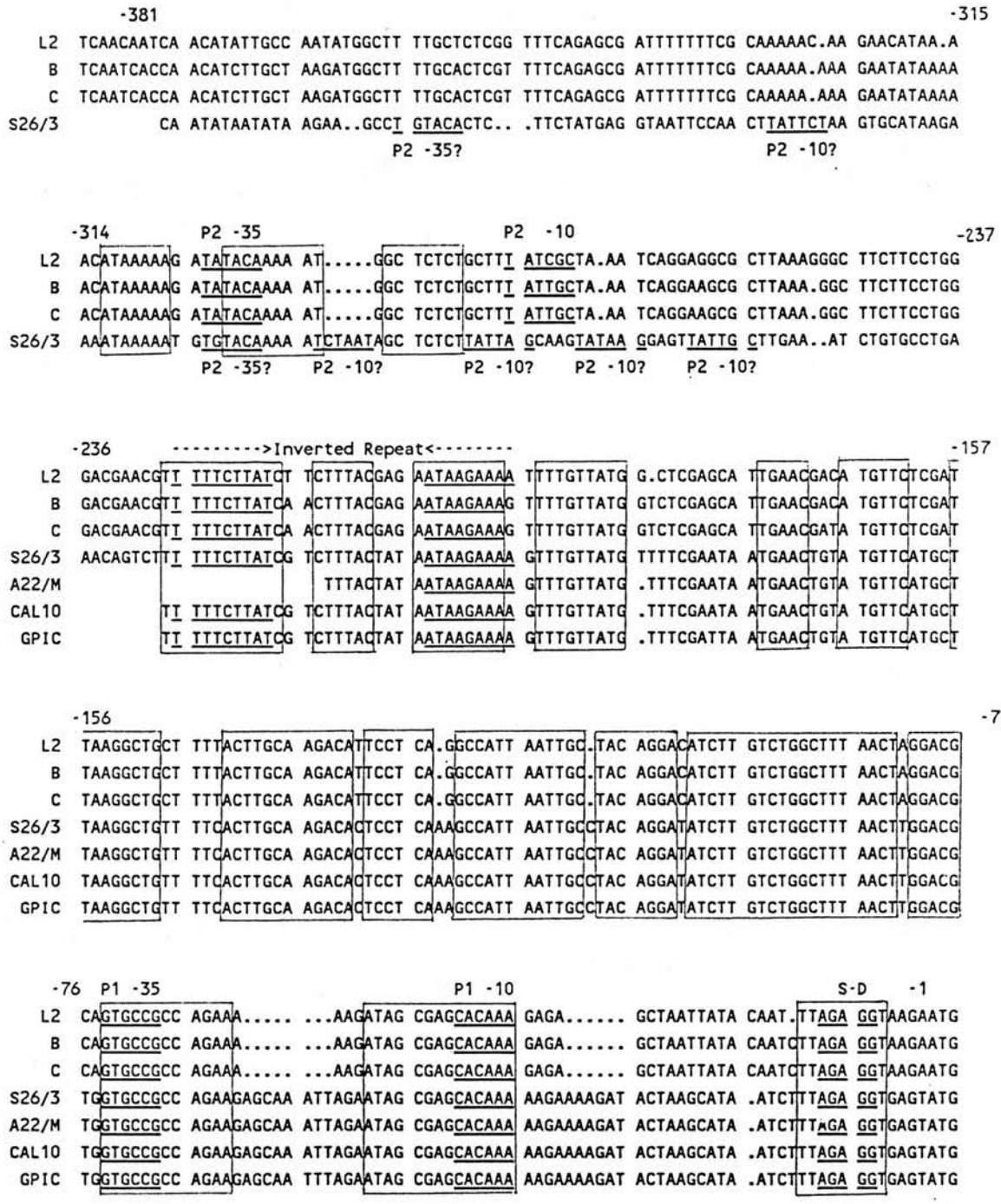


Figure 6.4. Comparison of the 5' regulatory sequences of C.psittaci strains S26/3 (OEA) A22/M (putative OEA), GPIC (strain 1), Cal10 (meningopneumonitis) and C.trachomatis serotypes L2, B, C. To optimise alignment, gaps have been inserted as indicated by dots. The numbering begins backwards from the start codon of the ORF of the S26/3 sequence. Putative promoter sequences (P1 and P2 - underlined). Shine-Dalgarno box (S-D). Sequences of complete identity analogous to CR1 to CR12 of Pickett et al (1988a) are boxed.

Potential promoter elements of the S26/3 MOMP gene were located by correspondence of position and sequence similarity to that of published chlamydial promoters (Engel and Ganem, 1987; Stephens *et al*, 1988a), assuming that chlamydial promoters follow the basic motif of *E.coli* promoters (Hawley and McClure, 1983). The putative -10 and -35 hexamers (CACAAA and GTGCCG) of the first promoter (P1) were found in conserved regions, identical to those proposed by Stephens *et al* (1988a) for *C.trachomatis* MOMP genes. The spacing between these elements, however, was 26 bases, 8 more than that of the *C.trachomatis* P1 promoter. The P1 promoters for *C.psittaci* MOMPs were 5 - 6 bases farther from the initiation codon than that of *C.trachomatis* MOMPs.

About 120 bp upstream of all the putative chlamydial P1 promoter elements was an inverted repeat (IR) theoretically capable of coding for an RNA stem loop and previously described only for *C.trachomatis* MOMPs (Stephens *et al*, 1988a) (Figures 6.3A and 6.4). Sequences identical to this dyad symmetry were present in GPIC and Call0 MOMP sequences and were followed by a T-rich region as shown, but not described, in the data of Zhang *et al* (1989a). Only half the structure was provided by Pickett *et al* (1988a) who sequenced the A22/M MOMP gene.

Several regions resembling chlamydial promoter sequences for the S26/3 sequence were present upstream of this IR and are underlined and designated as the putative second promoter (P2) in Figure 6.4. Except for one base, the putative P2 -35 hexamer is identical to the analogous structure in the P2 promoter of *C.trachomatis* MOMP gene. These sequences matched the *E.coli* -35 consensus sequence (TTGACA) in 4 out of 6 bases. Several hexamers similar to the *E.coli* -10 consensus sequence (TATAAT) were present 4 bp (TCTAAT), 18 bp (TATTAG), 28bp (TATAAG) and 38 bp (TATTGC) downstream. An alternative pair of -35 and -10 hexamers are also shown.

Comparison of the 3' flanking region showed that the putative rho-independent terminators for *C.psittaci* MOMPs were 38 bp downstream of the termination codon whilst those of *C.trachomatis* were an additional 34 bp downstream (Figure 6.5 - see next section for details of the sequence alignment). The terminator IRs followed by 8Ts (Figure 6.3B) were conserved in *C.psittaci* MOMP sequences but were fairly divergent from that of *C.trachomatis*. Within the *C.psittaci* terminator IR, part of the downstream arm TCYGGGTTC was very similar to the upstream arm of the *C.trachomatis* L2 MOMP IR, TCTGGGCTC, as shown aligned in Figure 6.5 (see Appendix for ambiguity code).

MOMP ORFs

A comparison of the S26/3 MOMP ORF sequence with the corresponding sequences from three other *C.psittaci* strains (Pickett *et al*, 1988a - putative ovine abortion isolate, A22/M; Zhang *et al*, 1989a - Call0 and GPIC) and a representative *C.trachomatis* serovar (Stephens *et al*, 1986 - L2) was made. The sequences were optimally aligned to S26/3 by the GAP program (UWGCG package) with gap weight of 1.5 and gap length weight 0.15. These gapped sequences were then realigned with each other by inspection and by correlation with conservation in the amino acid sequence (Figure 6.5). Differences in nucleotides at each position in Figure 6.5 were counted for each pair of ORFs and displayed in Table 6.2.

Pickett *et al* (1988a) reported A22/M as an ovine abortion strain, thought to be derived from the Moredun Research Institute (personal communication to Dr A.J.Herring). However, the reported A22/M MOMP sequence was quite different from the S26/3 MOMP sequence (Table 6.2). Instead, the A22/M sequence was strikingly similar to the Call0 (meningopneumonitis) sequence, differing only at 12 nucleotides. Differences between other pairs of MOMP sequences were more than an order of magnitude greater than that between A22/M and Call0. The next most similar pair of sequences were from S26/3 and GPIC, both of mammalian origin.

```
      1
S26/3 ATGAAAAAAC TCTTGAAATC GGCATTATTG TTTGCCGCTA CGGGTTCCGC TCTCTCCTTA 60
 GPIC .......... .......... .......... ......A... .......... .......... 60
A22/M .......... .......... .......... .......... .......... .......... 60
CAL10 .......... .......... .......... .......... .......... .......... 60
   L2 .......... .......... ..T....G.. .........T T.A....T.. .TC......G 60

          288C_____________PvuII\                ClaI/_____845C
S26/3 CAAGCCTTGC CTGTAGGGAA CCCAGCTGAA CCAAGTTTAT TAATCGATGG CACTATGTGG 120
 GPIC .......... .......... T......... .......... ....T..... .......... 120
A22/M .......... .......... .......... .......... .......... .......... 120
CAL10 .......... .......... .......... .......... .......... .......... 120
   L2 .....TC... ....G..... T..T...... .....CC.TA .G.....C.. A.T.C.A... 120

S26/3 GAAGGTGCTT CAGGTGATCC TTGCGATCCT TGCTCTACTT GGTGTGATGC TATCAGCATC 180
 GPIC .....C.... ....C..... ...T...... .......... .......... .......... 180
A22/M .......... ....A..... .......... ...G...... .......C.. C..T...... 180
CAL10 .......... ....A..... .......... ...G...... .......C.. C..T...... 180
   L2 ......TTCG GC..A..... .......... ...A.C.... .......C.. .........G 180

S26/3 CGCGCAGGAT ACTACGGAGA TTATGTTTTC GATCGTGTAT TAAAAGTTGA TGTGAATAAA 240
 GPIC ........G. .......... .......... .....CA.C. .......... ...T...... 240
A22/M .......... .......... .......... .......... .......... .......... 240
CAL10 .......... .......... .......... .......... .......... .......... 240
   L2 ..TATG..T. ....T..T.. C.T....... ..C.....T. .GC..ACA.. .......... 240

      >>> VS1 MspI
S26/3 ACTATCACCG GCATGGGTGC AGTTCCT--- ------ACAG GAACCGCA-- -GCAGCTAAT 288
 GPIC .......G.- --......GA. ..C...A--- -------..T. .T.AT...-- -..T...G.C 285
A22/M ...T.T.G.. ......C... .AC....ACG CAGGCT.... .T.A....AG TAATA..... 300
CAL10 ...T.T.G.. ......C... .AC....ACG CAGGCT.... .T.A....AG TAATA..... 300
   L2 GAAT..CAA- --........ CAAG...ACA ACTGCT.... .C.AT..T-- -......CCA 294

                            <<<                              /_____
S26/3 TACAAAACTC CTACGGATAG ACCCAACATC GCTTACGGCA AACACTTACA AGACGCCGAA 348
 GPIC .TT.....CG T.G.A..C.. GAAT.....A ..C....... ....TA.G.. ...T..A... 345
A22/M C.GCC.GAAG .A.AT.GC.. ...G...... ........A. GG..TA.G.. ...T..A..G 360
CAL10 C.GCC.GAAG .A.AT.GC.. ...G...... ........A. GG..TA.GG. ...T..A..G 360
   L2 .C..CTTG.- --..A.CA.. .GAG..TCCT .........C G...TA.G.. G..T..T..G 351

      844C
S26/3 TGGTTCACCA ATGCAGCTTT CCTCGCATTG AATATCTGGG ATCGCTTTGA TATTTTCTGC 408
 GPIC ....C...A. .C..G..... .T.A.....A ..C..T.... ....T..... .G.C...... 405
A22/M .....TT.A. .......C.. ...A..C..A ..C..T.... .......C.. C......... 420
CAL10 .....TT.A. .......C.. ...A..C..A ..C..T.... .......C.. C......... 420
   L2 AT...T..A. ....T....A .A.G...... .....T.... ....T..... .G.A.....T 411

S26/3 ACATTAGGCG CTTCTAATGG GTACTTCAAA GCTAGTTCTG CGGCATTCAA CCTCGTTGGT 468
 GPIC ........G. .A.....C.. C..TC..... ..A.A.G... .A..T..... T..A..C..C 465
A22/M ..C.....G. .A..C..... A......... T.A.....G. .T........ .T.G.....G 480
CAL10 ..C.....G. .A..C..... A......... ..A.....G. .T........ .T.G.....G 480
   L2 ........A. .CA.C.G... A..TC.T... .GA.A...A. .AT.T..... .T.A.....C 471
```

```
          >>> VS2   BamHI                PvuII
S26/3  TTGATTGGTG TTAAAGGATC C---TCCATA GCAGCTGAT- --------CA GCTTCCCAAT 516
 GPIC  ..AC....G. .A.C....-- -------.C. ------...C TTCAAGGC.. ATA...A..C 510
A22/M  ..A..A..GT ..TC..CTA. .AGC..A.CC T.TA.C..GC TTCCAATG.. AC....T..C 540
CAL10  ..A..A..GT ..TC..CTG. AAGC..A..C T.TA.C...C TTCCAACG.. AC....T..C 540
   L2  ..AT.C..A. A...T.AGAA .---CATGCT A...T.TCAG ATAGTAAG.T TG.A..... 528

                          <<<                          /
S26/3  GTAGGCATCA CTCAAGGAAT CGTTGAATTT TATACAGATA CAACATTCTC TTGGAGTGTA 576
 GPIC  ....C...CT .......CC. T..A..GC.. .....T..C. ....C..... ......C..T 570
A22/M  ........T. .C.....TG. T..G...... ........C. ..T....T.. ......C... 600
CAL10  ........T. .C.....TG. T..G...... ........C. ..T....T.. ......C... 600
   L2  A.GA..T.AG A....TCTG. T.....G..G .......... .T..T..TG. ........CT 588

       645C
S26/3  GGTGCACGCG GAGCTTTATG GGAGTGTGGT TGTGCGACTT TAGGAGCAGA GTTCCAATAC 636
 GPIC  .....G..T. .......... ...A...... ..C..A.... .......... .........T 630
A22/M  ........T. .......... ...A...... .....A.... .......T.. .......... 660
CAL10  ........T. .......... ...A...... .....A.... .......T.. .......... 660
   L2  ..A..T..T. C......G.. ...A.....A ..C....... ....C..TTC T......... 648

S26/3  GCTCAGTCTA ATCCTAAAAT TGAAATGTTG AATGTAGTCT CCAGCCCAGC ACAATTTGTG 696
 GPIC  ..G....... .......G.. C......C.T ......A.T. .T......A. .......... 690
A22/M  .....A.... .......G.. ....G..C.C ..C..CACT. .A........ .......... 720
CAL10  .....A.... .......G.. .......C.C ..C..CACT. .A........ .......... 720
   L2  .....A..C. .G......G. C...GAA..A ..C..TC... GT.A.G.... TG.G...ACT 708

                                   >>> VS3                      PvuII
S26/3  GTTCACAAGC CTAGAGGATA CAAGGGAACA GCA------T TTCCTTTACC TCTAACAGCT 750
 GPIC  A....T.... .......... T..A..G... ..GGCCAAC. .C...C.G.. .T....C... 750
A22/M  A.......A. .A.....C.. T..A...G.T AGCTCGAAT. .......... .A....G... 780
CAL10  A.......A. .A.....C.. T..A...G.T AGCTCGAAT. .......... .A....G... 780
   L2  A.CA.T.... ...A...... TGTA..GCA. .A.-------. .C...C.TGA ...T.A...A 762

       /     644C<<<
S26/3  GGTACTGATC AGGCAACTGA CACTAAGTCG GCTACAATTA AATACCACGA ATGGCAAGTT 810
 GPIC  ..A..A..GA GC..T..... T.....A..A .......... .G..T..T.. .......A.. 810
A22/M  ..A..AACAG .A..T..A.. ...C..A..A .......... .......T.. .........A 840
CAL10  ..A..AACAG .A..T..A.. ...C..A..A .......... .......T.. .........A 840
   L2  ..A..A...G GT.TG..A.G A......GAT ..CT.T...G .T.....T.. ........C. 822

                       ClaI
S26/3  GGTTTAGCGC TCTCTTATCG ATTGAACATG CTTGTTCCTT ACATTAGCGT AAACTGGTCA 870
 GPIC  ........T. .T......A. .......... ..C.....A. .T...G.A.. .........C 870
A22/M  ..CC.C..C. .G.....CA. ......T... ........A. .T...G.... .......... 900
CAL10  ..CC.C..C. .G.....CA. ......T... ........A. .T...G.... .......... 900
   L2  A.......T. .......... .C....T... T.CAC...CT .......A.. T..A.....T 882

                                                    >>> VS4
S26/3  CGAGCAACTT TTGATGCTGA CGCTATCCGC ATCGCTCAAC CTAAATTAGC TGCTGCTGTG 930
 GPIC  A....T..A. .......... .T........ ..T.....G. ........C. .A.G..CA.T 930
A22/M  A.......... .......... TA........ ..T....... ........AA AT.G.AGA.T 960
CAL10  A.......... .......... TA........ ..T....... ........AA AT.G.AGA.T 960
   L2  .......G.. .......A.. ...G..T..T ..T.....G. .G..G.C... .A.AA....C 942
```

```
S26/3  TTAAACTTGA CCACATGGAA CCCAACCCTT TTAGGAGAAG CTACAGCTTT A------GAT  984
 GPIC  ......C.A. .T........ ...T..TT.A .....G..G. ....TA..A. .------A.C  984
A22/M  C.T...A.T. .T........ .....G.... C.....TC.A .C..TA.... GCCCAATA..  1020
CAL10  C.T...A.T. .T........ .....G.... A.....TC.A .C..TG.... GCCCAATA..  1020
   L2  ..TG.TG.T. ....TCT... ......TA.. GCT....CT. ---GC.A.G. G---AAA.C.  996

                                      <<<
S26/3  ACTAGC---A ACAAATTCGC TGACTTCTTG CAAATTGCTT CGATTCAGAT CAACAAAATG 1041
 GPIC  ...G.A---G CA....AT.. ....CAG..A .......... ..C....A.. .......... 1041
A22/M  GG.G.TAAGG .TGTTC.AT. ...TG..... .......... .......... .......... 1080
CAL10  .G.G.TAAGG .TGTTC.AT. ...TG..... .......... .......... .......... 1080
   L2  .GCGCAGAGG GTC.GC...G A..TAC.A.. .....C.T.. .CT.G..ATT G.....G... 1056

                  /HindIII_____________289C
S26/3  AAGTCTAGAA AAGCTTGTGG TGTAGCTGTT GGTGCAACGT TAATCGACGC TGACAAATGG 1101
 GPIC  .......... .......... .A.T...... ........C. ....T..T.. .......... 1101
A22/M  .......... .......... .......... .......... .......... .......... 1140
CAL10  .......... .......... .......... .......... .......... .......... 1140
   L2  ..A....... ..T....C.. .A.T..A..A ..AA....TA .TG.G..T.. A.......AC 1116

S26/3  TCAATCACTG GTGAAGCACG CTTAATCAAT GAAAGAGCCG CTCACATGAA TGCTCAATTC 1161
 GPIC  ..G....... .......T.. .........C ........T. .....G.A.. C......... 1161
A22/M  .......... .......... .......... ........T. .......... .......... 1200
CAL10  .......... .......... .......... ........T. .......... .......... 1200
   L2  G..G.T..A. T...GA.T.. ...G...G.. ..G.....T. .....G.A.. ...A...... 1176

        STP
S26/3  AGATTCTAAG GATTTAGTTT ATACTATCCT AACTTT---- ---------- ---------- 1197
 GPIC  .......... .......... .......... ......---- ---------- ---------- 1197
A22/M  .......... .......... .......... ......---- ---------- ---------- 1236
CAL10  .......... .......... .......... ......---- ---------- ---------- 1236
   L2  C.C......T T.A..GTA.A ..TT.G.TA- ......GGCA AGTTTATCTT TGTTAATAAC 1235

                              ------------>    <------------
S26/3  ---------- TTGTCCCGCT ATCAGAACCT AGGAATCTCT GGGTTCTGAT TTTTTTTGCT 1247
 GPIC  ---------- .......... .......... ....GCG... .......... .....A.TTA 1247
A22/M  ---------- .......... .......... G...G....C .......... .......... 1286
CAL10  ---------- .......... .......... G...G....C .......... .......... 1286
   L2  GTTAATAACA C.A...GTG. T..T.GG.TC GACTTCGGTC ....C.A.T. .....TGCAA 1295
                               ----------->    <-----------
                        /_____________________________612C   EcoRI
S26/3  CTCATCCTTT TGTAGAGCTT CAAATCTCTT TTCTAAAATC CATTCGCATA AGAATTCACT 1307
 GPIC  AAGCC..                                                          1254
A22/M  AC..C..... .C-....T.. ....                                       1309
CAL10  AC..C..                                                          1293
   L2  AAAT.TT... .C.TACTT.C G.TCT.C..C C.A.CTCTCT                       1335
```

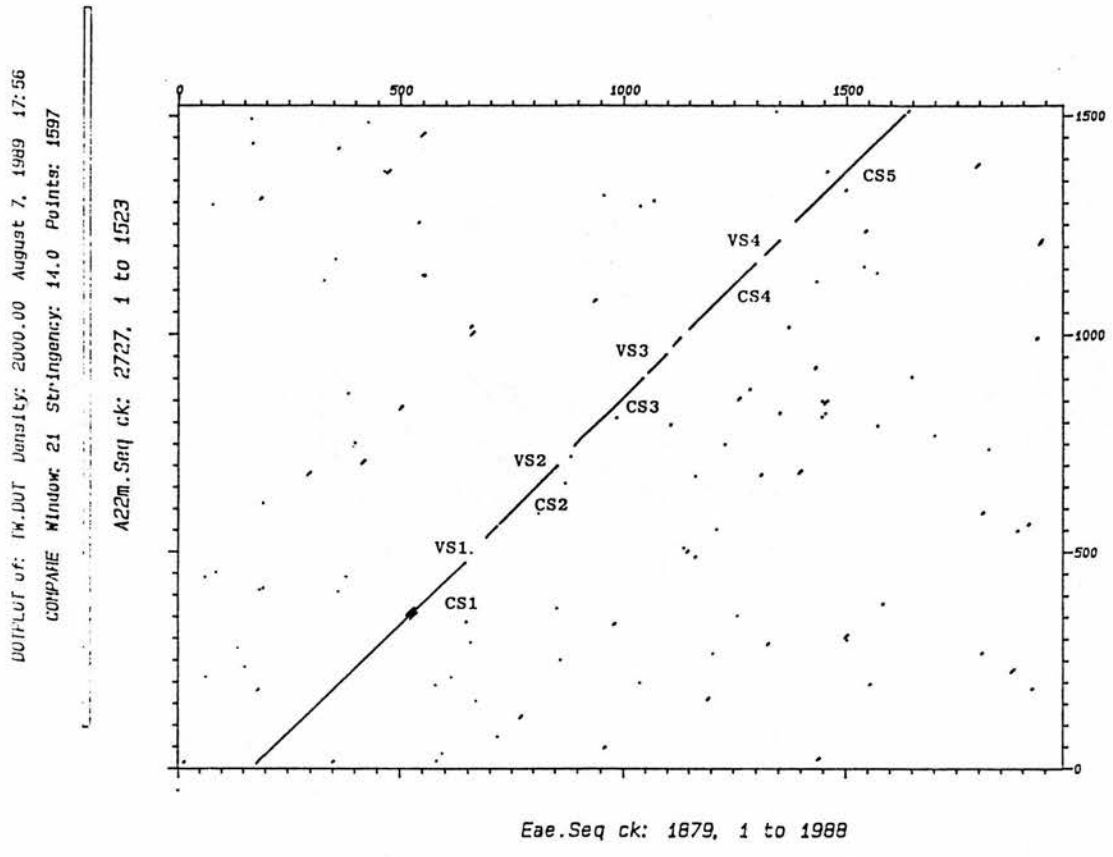
Figure 6.5. Comparison of coding and 3' flanking regions of MOMP genes of C.psittaci strains S26/3, GPIC, A22/M and Cal10 and C.trachomatis serotype L2. The four variable sequences (VSs) corresponding to variable domains (VDs) in the polypeptide chain are marked by triple arrows. Gaps are indicated by dashes (-); identical bases by dots (.); putative terminator by broken arrows. Restriction sites are underlined; primer sequences are overlined with an arrow.

Within the *C.psittaci* sequences, many base changes were third base "wobbles" and did not result in changes in the amino acid sequence. Across species, there were more differences but it was interesting to note that the S26/3 MOMP gene was the least different from L2 MOMP gene among the *C.psittaci* MOMP genes.

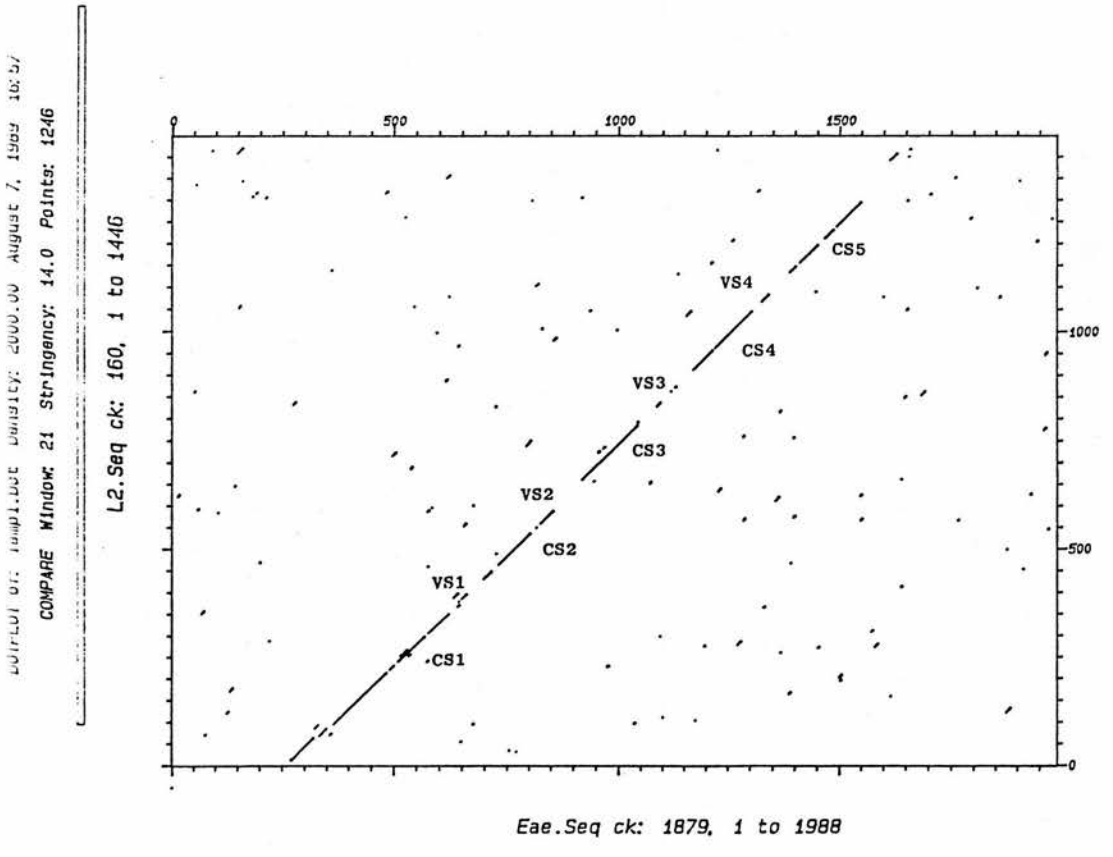
TABLE 6.2. Differences in nucleotides within the ORF as a percentage of the mean of N for the two ORFs. The number of amino acids in the ORF and the number of differences are given within brackets. Gaps were also counted as differences.

	S26/3	GPIC	A22/M	CAL10	L2
N=	1167 (389)	1167 (389)	1206 (402)	1206 (402)	1182 (372)
S26/3	0	17.9%(56)	12.9%(66)	12.6%(62)	33.0%(139)
GPIC		0	22.5%(78)	22.8%(80)	35.4%(140)
A22/M			0	1.0%(10)	35.8%(146)
CAL10				0	36.0%(146)
L2					0

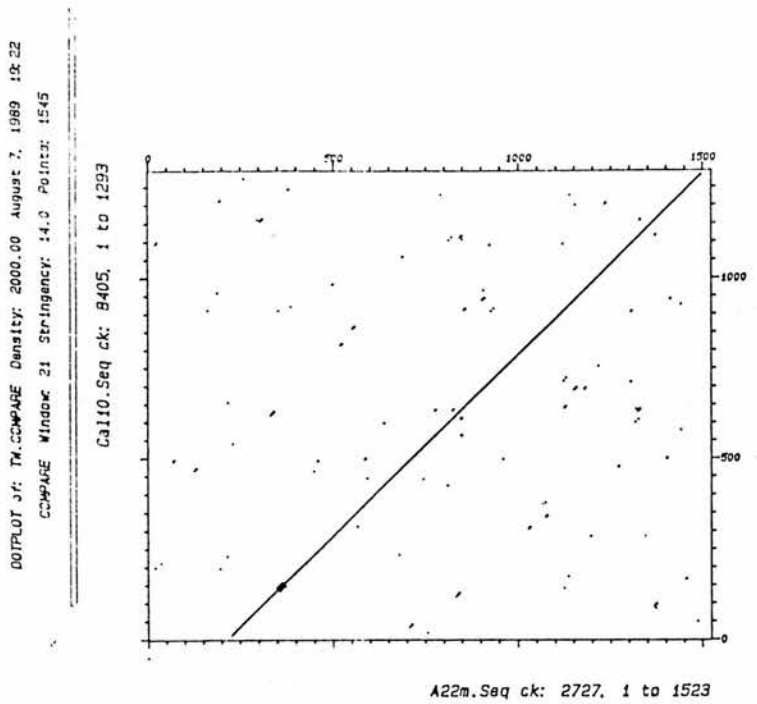
To visualise the distribution of these differences, several ungapped sequences were compared pairwise using the COMPARE program (window = 30 and stringency = 9) and displayed by the DOTPLOT program to highlight the main regions of similarity and differences (Figure 6.6). (As a control, the S26/3 MOMP sequence was compared with the *E.coli* PhoE outer membrane porin sequence and did not show any homology.) The plots revealed a localisation of consecutive base changes in four regions, first identified for *C.trachomatis* MOMPs and termed variable sequences VS1 to VS4 by Stephens *et al* (1987), and subsequently for *C.psittaci* MOMPs by Pickett *et al* (1988a) and Zhang *et al* (1989a) while this project was in progress.


DOTPLOT of: fw.DOT Density: 2000.00 August 7, 1989 17:56
COMPARE Window: 21 Stringency: 14.0 Points: 1597
A22m.Seq ck: 2727, 1 to 1523
Eae.Seq ck: 1879, 1 to 1988
CS5
VS4
CS4
VS3
CS3
VS2
CS2
VS1
CS1
0
500
1000
1500

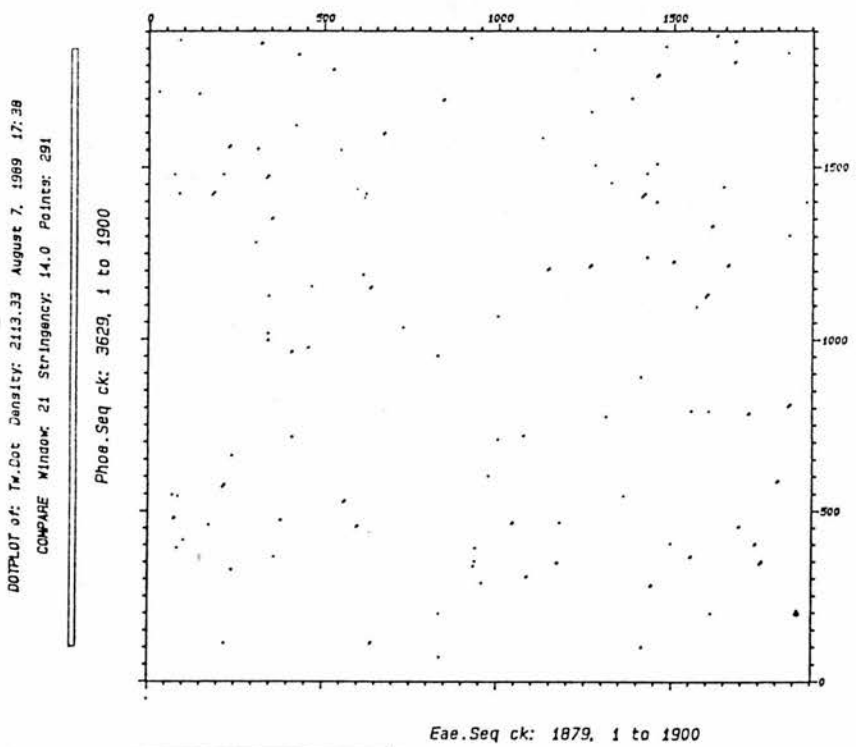
A


COMPARE Window: 21 Stringency: 14.0 Points: 1246
L2.Seq ck: 160, 1 to 1446
Eae.Seq ck: 1879, 1 to 1988
CS5
VS4
CS4
VS3
CS3
VS2
CS2
VS1
CS1
0
500
1000
1500

B



C



D

Figure 6.6. DOTPLOTS of comparisons between MOMP nucleotide sequences of A. S26/3 and A22/M; B. S26/3 and L2; C. A22/M and Cal10. Panel D is a comparison between the *E.coli phoE* gene and S26/3 MOMP gene. The variable sequences are marked VS1 to VS4 and the conserved sequences CS1 to CS5.

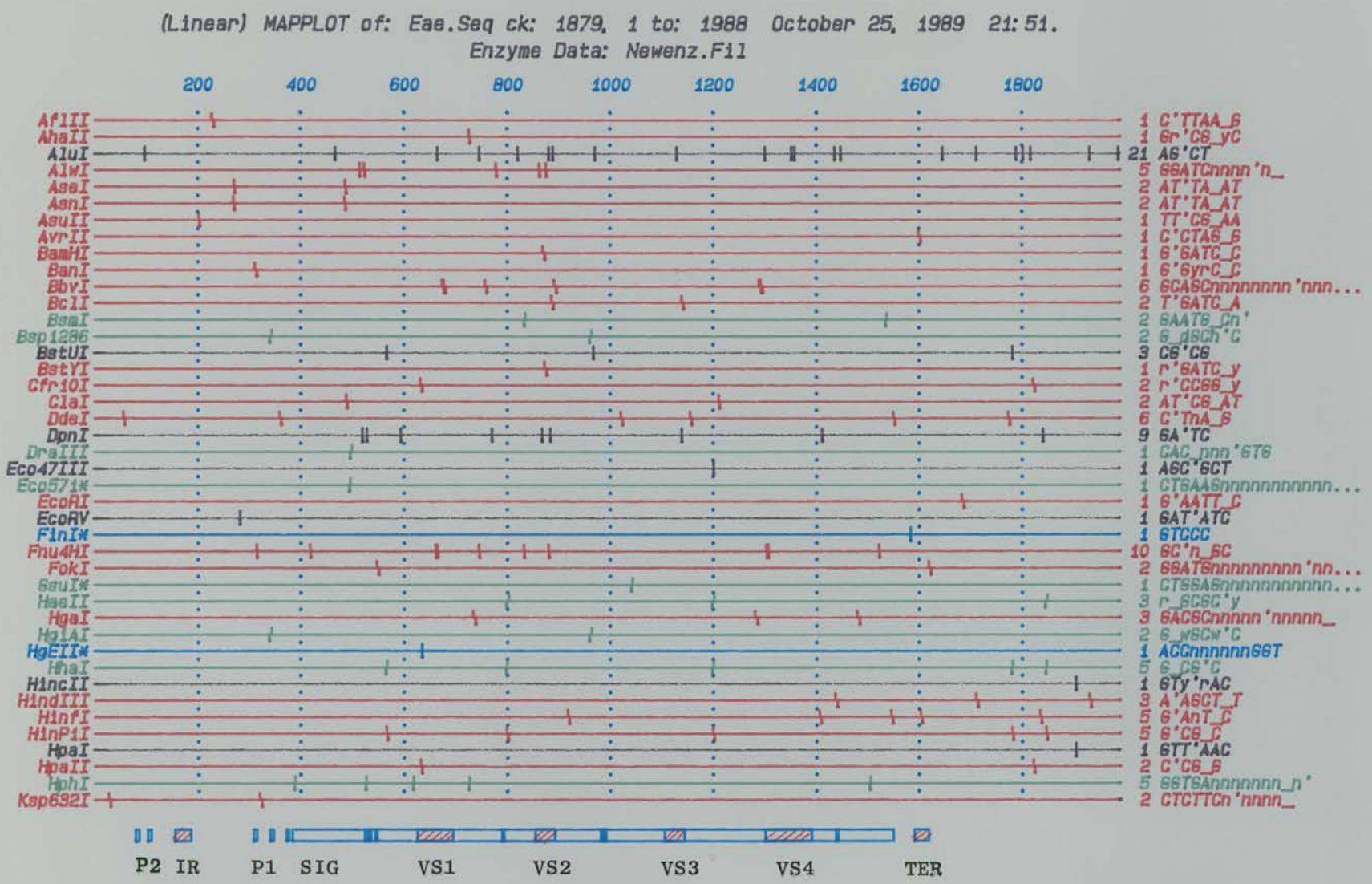
These variable sequences (VSs) are marked in Figure 6.5. Within these sequences, gaps have been introduced in the sequences to optimise the alignments. Equally conspicuous were large stretches of sequence identity in the 5' and 3' ends of the *C.psittaci* MOMP ORFs, about 240 and 160 bp in length respectively. In between the VS1 to VS4 were regions of intermediate diversity in sequence. For convenience, these regions of conservation interdigitating with the variable sequences are termed conserved sequences CS1 to CS5.

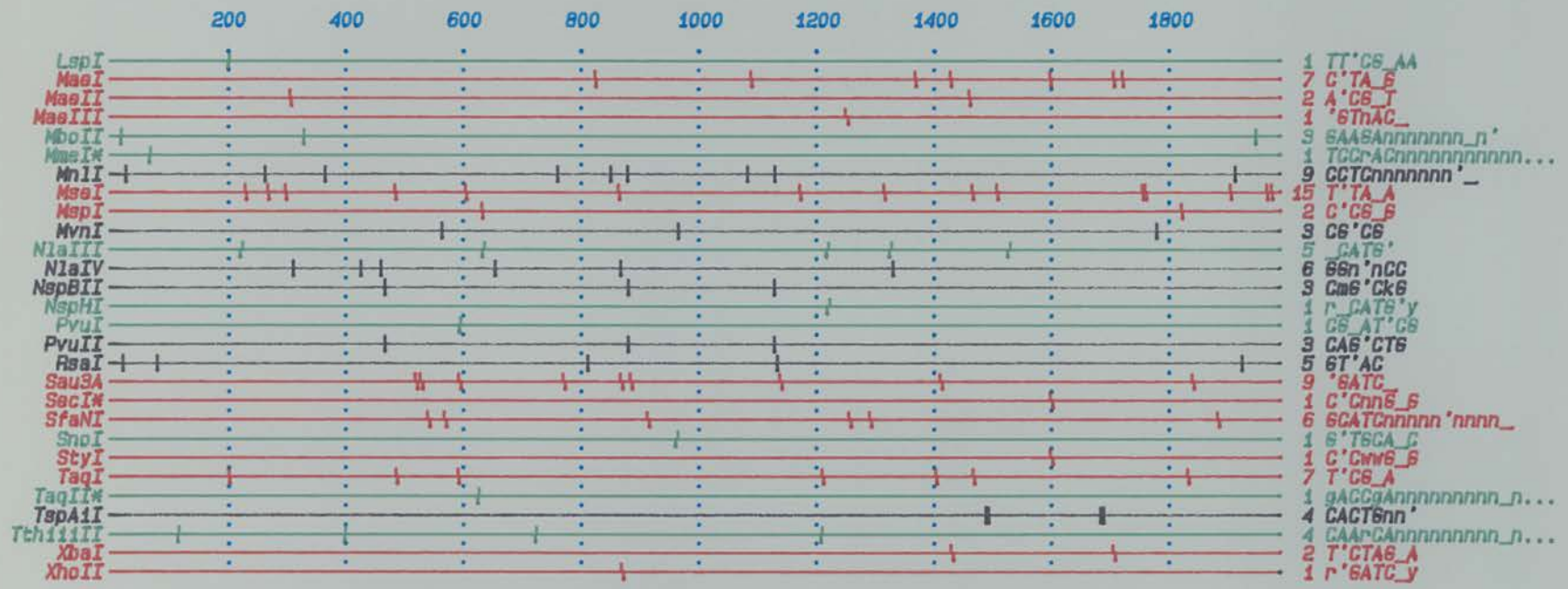
Several of the more common restriction endonuclease sites have been underlined. Some sites are unique to the strain but others are conserved within *C.psittaci* strains. An exhaustive restriction map of the S26/4 MOMP sequence is shown in Figure 6.7 (of restriction endonucleases commercially available up to January 1989).

Deduced amino acid sequence

The translation of the *C.psittaci* S26/3 MOMP gene is displayed in Figure 6.2 corresponding to the nucleotide sequence. It encoded 389 amino acids with a calculated M_r of 41883 Da. Since the N-terminus of the mature MOMP began with L(D)P(G)VGNPAEPS (Chapter 4), which corresponded to the 23rd to the 32nd residue of the *C.psittaci* pre-MOMP, the initiation codon was designated -22 and the N-terminus of the mature protein as +1. The calculated M_r of the mature protein was 39.639 kDa, which compared favourably with the estimated 39 to 40 kDa by SDS-PAGE analysis (Chapter 3 and 4). The length of the signal sequence and the cleavage site is identical to that of MOMP from *C.trachomatis* serovar L2 (Nano *et al*, 1985). The signal sequence cleavage site in the MOMPs of other strains were assumed by their investigators to be the same as that of serovar L2.

Alignment of the amino acid sequence of S26/3 MOMP and those from other sources (Stephens *et al*, 1986; 1987; Pickett *et al*, 1987 and 1988a; Zhang *et al*, 1989a) was carried out using


(Linear) MAPPLOT of: Eae.Seq ck: 1879, 1 to: 1988 October 25, 1989 21:51.
Enzyme Data: Newenz.Fil
200
400
600
800
1000
1200
1400
1600
1800
AflII 1 C'TTAA_G
AhaII 1 Gr'CG_yC
AluI 21 AG'CT
AlwI 5 GGATCnnnn'n_
AseI 2 AT'TA_AT
AsnI 2 AT'TA_AT
AsuII 1 TT'CG_AA
AvrII 1 C'CTAG_G
BamHI 1 G'GATC_C
BanI 1 G'GyrC_C
BbvI 6 GCAGCnnnnnnnn'nnn...
BclI 2 T'GATC_A
BsaI 2 GAATG_Cn'
Bsp1286 2 G_dGCh'C
BstUI 3 CG'CG
BstYI 1 r'GATC_y
Cfr10I 2 r'CCGG_y
ClaI 2 AT'CG_AT
DdeI 6 C'TnA_G
DpnI 9 GA'TC
DraIII 1 CAC_nnn'GTG
Eco47III 1 AGC'GCT
Eco571* 1 CTGAAGnnnnnnnnnnn...
EcoRI 1 G'AATT_C
EcoRV 1 GAT'ATC
FinI* 1 GTCCC
Fnu4HI 10 GC'n_GC
FokI 2 GGATGnnnnnnnnn'nn...
GsuI* 1 CTGGAGnnnnnnnnnnn...
HaeII 3 r_GCGC'y
HgaI 3 GACGCnnnnn'nnnnn_
HglAI 2 G_yGCw'C
HgiEII* 1 ACCnnnnnnGGT
HhaI 5 G_CG'C
HincII 1 GTy'rAC
HindIII 3 A'AGCT_T
HinfI 5 G'AnT_C
HinP1I 5 G'CG_C
HpaI 1 GTT'AAC
HpaII 2 C'CG_G
HphI 5 GGTGAnnnnnnn_n'
Ksp632I 2 CTCTTCn'nnnn_
P2
IR
P1
SIG
VS1
VS2
VS3
VS4
TER



Enzymes that do not cut:

AatII	AccI	AflIII	AlwNI	ApaI	ApyI	Asp700	Asp718	AvaI	AvaII	AviII	AxyI
BalI	BanII	BbeI	BbvII*	BglI	BglII	BspHI	BspMI	BssHII	BstEII	BstNI	BstXI
CfrI	Cfr13I	DraI	DsaI*	EcoK	EcoB	Eco311*	EaeI	EagI	Eco81I	Eco0109	EcoNI
EcoRII	EcoT22I	EspI	FspI	GdiII*	HaeI*	HaeIII	KpnI	MluI	MroI	NaeI	NarI
NciI	NcoI	NdeI	NheI	NotI	NruI	NsiI	PflMI	PleI	PmaCI*	PpuMI	PssI
PstI	RspXI	RsrII	SacI	SacII	SalI	Sau96I	ScaI	ScrFI	SfiI	SmaI	SnaBI
SpeI	SphI	SplI	SspI	SsoII*	StuI	Tth111I	XcaI	XhoI	XmaI	XmaIII	

Figure 6.7. Restriction endonuclease map of S26/3 MOMP gene. Key: Putative promoters (P1 and P2), inverted repeat (IR), signal peptide (SIG), variable sequences (VS1 to VS4), terminator (TER).

the CLUSTAL program (Chapter 2). For optimal alignment, further adjustments were made to the CLUSTAL output by inspection (Figure 6.8). Corresponding closely but not exactly to the four VSs in Figure 6.5 were four variable peptide domains (VD1 to VD4 - boxed); similarly the conserved domains (CD1 to CD5) corresponded to the five conserved sequences. The VD assignments were similar but not identical to those reported for *C.trachomatis* (Stephens *et al*, 1987) and *C.psittaci* (Zhang *et al*, 1989a) due to the larger number of strains compared here. It was noted that intraspecies VD boxes tended to be smaller than those interspecies and this is particularly obvious in VD3. General data and amino acid composition of S26/3 MOMP are displayed with that of other chlamydial MOMPs in Table 6.3.

From Figure 6.8, it was observed that the A22/M MOMP was identical to the Cal10 MOMP in polypeptide length and identical in sequence except for changes in 10 residues which were located in CD2 (2), VD2 (4), CD3 (1) and VD4 (3). These changes were the result of 10 nucleotide differences out of the 12 for the whole ORF. S26/3 MOMP on the other hand was a shorter polypeptide with the least M_r of the MOMPs cited and was different at 67 residue positions compared with A22/M, 55 of which occurred in the VDs.

The M_r of the MOMPs without the signal sequences agreed closely with SDS-PAGE estimations (Chapter 3). The signal sequences were nearly identical within each species but differed in 5 to 6 out of the 22 residues between the two species. These were mainly conservative changes and did not alter the basic structure of positively charged residues followed by hydrophobic residues.

Although the total number of positively charged residues in each MOMP was fairly constant, there were 10 more Asp and Glu residues in the *C.trachomatis* MOMPs than those of *C.psittaci* (Table 6.3). In general, the negatively charged residues were more numerous in *C.trachomatis* MOMPs. The computer-predicted isoelectric points of these MOMPs were lower than their reported

```
       -22 +                +1      -       -       -   - -       -     +     -
S26/3  MKKLLKSALL FAATGSALSL QALPVGNPAE PSLLIDGTMW EGASGDPCDP CSTWCDAISI RAGYYGDYVF 48
 GPIC  .......... ..T....... .......... .......... .......... .......... .......... 48
 A22M  .......... .......... .......... .......... .......... .A........ .......... 48
CAL10  .......... .......... .......... .......... .......... .A........ .......... 48
    A  .......V.V ...LS..S.. .......... ...M...IL. ..FG...... .T.......M .M.....F.. 48
    C  .......V.V ...LS..S.. .......... ...M...IL. ..FG...... .T.......M .V.....F.. 48
    B  .......V.V ...LS..S.. .......... ...M...IL. ..FG...... .T.......M .M.....F.. 48
   L2  .......V.V ...LS..S.. .......... ...M...IL. ..FG...... .T.......M .M.....F.. 48
   L1  .......V.V ...LS..S.. .......... ...M...IL. ..FG...... .T.......M .M.....F.. 48

       -+  + -  +                                +   -+      ++  - -           -+ -
S26/3  DRVLKVDVNK TITGMGAVPT ---GTAAA-N YKTPTDRPNI AYGKHLQDAE WFTNAAFLAL NIWDRFDIFC 114
 GPIC  ..I.......  ..S-..TA.. ---.N...-D F..VA..N.. .....M.... .S........ .......V.. 113
 A22M  ..........  .FS..A.T.. QAT.N.SNT. QPEANG.... ...R.M.... ..S....... .......... 118
CAL10  ..........  .FS..A.T.. QAT.N.SNT. QPEANG.... ...R.ME... ..S....... .......... 118
    A  .....T....  EF-Q...A.. TSDVAGLEKD PVANVA...P .....M.... M.....YM.. .......V.. 117
    C  .....T....  EF-Q...A.. TSDVAGLQND PT.NVA...P .....M.... M.....YM.. .......V.. 117
    B  .....T....  EF-Q...K.. TTT.N.V.-- PS.L.A.E.P ...R.M.... M.....CM.. .......V.. 115
   L2  ....QT....  EF-Q...K.. TAT.N...-- PS.C.A.E.P ...R.M.... M.....YM.. .......V.. 115
   L1  ....QT....  EF-Q...K.. ATT.N...-- PS.C.A.E.P ...R.M.... M.....YM.. .......V.. 115

              +                  +       -                    -   -       +   -
S26/3  TLGASNGYFK ASSAAFNLVG LIGVKG-SSI AAD---QLPN VGITQGIVEF YTDTTFSWSV GARGALWECG 180
 GPIC  .........L. .NA.....  ...T.---- -T.LQG.Y.. .A.S..L..L .......... .......... 178
 A22M  .......... S.........  ...FSAT..T STELPM.... ......V... ....S..... .......... 188
CAL10  .......... ..........  ...FSAA... ST.LPT.... ......V... ....S..... .......... 188
    A  ....TT..L. GN..S.....  .F.T.TQ..- GFDTANIV.. TALN.AV..L ......A... ...A...... 186
    C  ....TT..L. GN..S.....  .F.T.TQ..- SFNTAKLI.. TALNEAV..L .IN...A... ...A...... 186
    B  .....S..L. GN..S.....  .F.NNENQTK VSNGAF-V.. MSLD.SV..L ....A.A... ...A...... 184
   L2  ....TS..L. GN..S.....  .F.DNENHAT VS.S-KLV.. MSLD.SV..L ......A..A ...A...... 184
   L1  ....TS..L. GN..S.....  .F.DNENQ.T VKKDA--V.. MSFD.SV..L ......A... ...A...... 183

             -         + -                ++ +  +                -   - +     + +-
S26/3  CATLGAEFQY AQSNPKIEML NVVSSPAQFV VHKPRGYKGT A--FPLPLTA GTDGATDTKS ATIKYHEWQV 248
 GPIC  .......... .......... ..I...T... I.........  .AN....... ..ES...... .........I 248
 A22M  .......... ........V. ..T....... I.........A SSN....I.. ..TE...... .......... 258
CAL10  .......... .......... ..T....... I.........A SSN....I.. ..TE...... .......... 258
    A  ......S... ...K..V.E. ..LCNASE.T IN..K..V.A E--...DI.. ..EA..G..D .S.D.....A 254
    C  ......S... ...K..V.E. ..LCNASE.T IN..K..V.A E--...NI.. ..EA..G..D .S.D.....A 254
    B  ......S... ...K..V.E. ..LCNA.E.T IN..K..V.K E--L..D... ...A..G..D .S.D.....A 252
   L2  ......S... ...K..V.E. ..LCNA.E.T IN..K..V.Q E--...D.K. ...GV.G..D .S.D.....A 252
   L1  ......S... ...K..V.E. ..LCNA.E.T IN..K..V.K E--...D... ...A..G..D .S.D.....A 251
```

```
              +               +   - -  +     +                   -     -    +    -
S26/3   GLALSYRLNM LVPYISVNWS RATFDADAIR IAQPKLAAAV LNLTTWNPTL LGEATAL-DT SNK--FADFL 315
GPIC    .......... .....G.... .......S.. ......PT.I .......... .....TI-N. GA.--Y..Q. 315
A22M    .......... .....G.... .......T.. ......KSEI ..I.....S. ..ST.T.PNN GG.DVLS.V. 328
CAL10   .......... .....G.... .......T.. ......KSEI ..I.....S. I.ST...PNN .G.DVLS.V. 328
A       S...F..... FT...G.K.. .VS....T.. .......KP. .DT..L...I A.KG.VV-S- .AENEL..TM 322
C       S......... FT...G.K.. .VS....T.. .......E.I .DV..L...I A.KGSVV-SA GTDNEL..TM 323
B       S......... FT...G.K.. ..S....T.. .....S.ETI FDV..L...I A.AGDV--K. .AEGQLG.TM 320
L2      S......... FT...G.K.. ..S....T.. .....S.TT. FDV..L...I A.AGDV--KA .AEGQLG.TM 320
L1      S......... FT...G.K.. ..S....T.. .......T.I FDT..L...I A.AGEV--KA NAEGQLG.TM 319

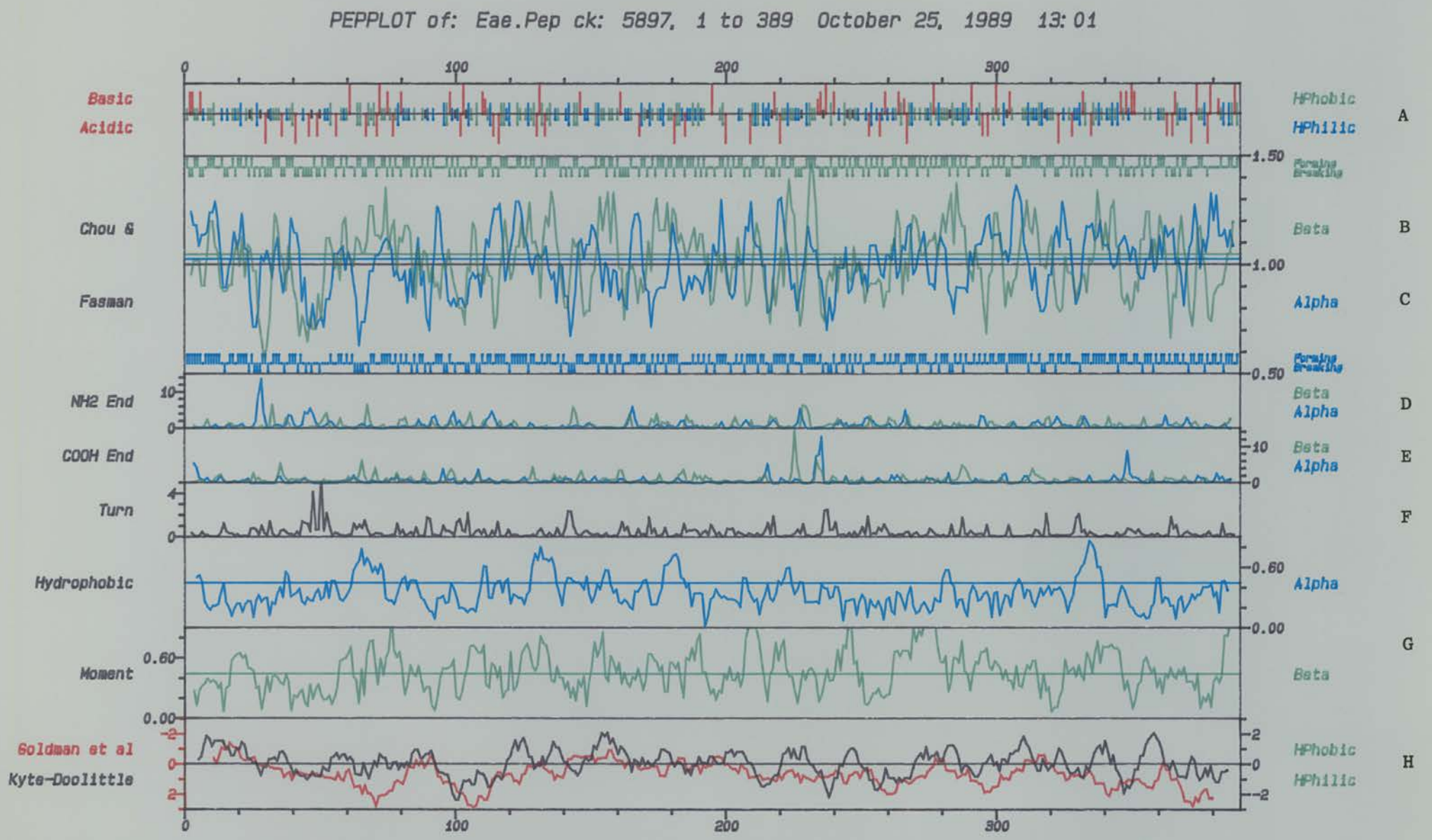
                +  + ++            - -+      - +    -+  +      +
S26/3   QIASIQINKM KSRKACGVAV GATLIDADKW SITGEARLIN ERAAHMNAQF RF 367
GPIC    ....L..... .......I.. .......... .......... .....V.... .. 367
A22M    .......... .......... .......... .......... .......... .. 380
CAL10   .......... .......... .......... .......... .......... .. 380
A       ..V.L.L... ....S..I.. .T.VV....Y AV.I.T...D .....V.... .. 374
C       ..V.L.L... ....S..I.. .T.IV....Y AV.V.....D .....V.... .. 375
B       ..V.L.L... ....S..I.. .T.IV....Y AV.V.T...D .....V.... .. 372
L2      ..V.L.L... ....S..I.. .T.IV....Y AV.V.T...D .....V.... .. 372
L1      ..V.L.L... ....S..I.. .T.IV....Y AV.V.T...D .....V.... .. 371
```

Figure 6.8. Comparison of the amino acid sequences of chlamydial MOMPs. The C.psittaci S26/3 MOMP sequence is shown, aligned with those of C.psittaci strains GPIC, A22/M and Cal10 (Mn) and those of C.trachomatis serotypes A,B, C, L1 and L2. Residues identical to the S26/3 MOMP sequence are represented by dots. To optimise alignment, gaps have been inserted as indicated by dashes. The signal peptide is assumed to be 22 residues for MOMPs from strains other than L2 and S26/3. The numbering begins from the first residue of the mature MOMP. The variable domains VD1 to VD4 are marked by boxes slightly modified from the assignment given by Zhang et al (1989a). Charged residues are marked by (+) and (-). Cysteine residues are underlined; conserved ones are marked. L1 and A MOMP sequence were from Pickett et al (1987) and Zhang et al (1989a). Other sequences are as cited in the text. Note that there are several apparent discrepancies between the published sequences and the sequences deposited in the databases. MOMP serovar B: Cys-33 (database) but Val-33 (Stephens et al, 1987); Ser-149 (database) but Gly-149 (Stephens et al, 1987). MOMP serovar C: Ile-80 (database) but Thr-80 (Stephens et al, 1987).

---

pI of 5.3-5.5 for LGV strains and 6.9 for trachoma strains (Batteiger *et al*, 1985). For *C.psittaci* MOMPs, the positively charged residues outnumbered the negatively charged residues taking into account histidine residues as positive unlike Zhang *et al* (1989a) who did not. The pIs of *C.psittaci* MOMP have not been reported although the pI of whole *C.psittaci* EBs is about 5 (Vance and Hatch, 1980). These differences between species were mainly accounted for by variation in the VDs (VD3 and VD4 in particular as arrowed in Table 6.3).

S26/3 MOMP possessed 18% charged residues which were evenly distributed throughout the sequence, similar to other MOMPs (16% to 20%). However, charges of one type to the exclusion of the other were found localised in several interesting loci. Six negatively charged residues spaced between 2 to 5 residues apart were located in a positive-charge-free region between residues +8 to +34 (Figure 6.9 panel A - note that 22 should be added to the amino acid number to account for the signal sequence). Five out of the 17 Pro residues were located within or near this region. While intervening residues diverged across species, these Asp, Glu and Pro residues were completely conserved. The carboxyl end of this region coincided with a triplet of conserved Cys residues (26, 29 and 33) and was predicted to possess a high antigenic index using the method of Jameson and Wolf (1988) and a high β-turn potential by both the methods of Chou and Fasman (1978) and Garnier *et al* (1978)(Figure 6.9 panels F,L,M,P). This region was termed a cysteine- and proline-rich acidic (CAPRA) sequence. Charged sequences exclusively rich in Asp and Glu can also be found in the hydrophilic β-sheet porins of *E.coli*, OmpF, OmpC and PhoE (data not shown) but not in the hydrophobic α-helical bacteriorhodopsin of Halobacterium halobium (sequence data cited from GENBANK and EMBL databases using the UWGCG package). These membrane proteins do not contain any cysteines.


PEPPLOT of: Eae.Pep ck: 5897, 1 to 389 October 25, 1989 13:01
0
100
200
300
Basic
Acidic
HPhobic
HPhilic
A
1.50
Turns
Breaks
Chou &
Fasman
Beta
B
1.00
Alpha
C
0.50
NH2 End
10
0
Beta
Alpha
D
COOH End
10
Beta
Alpha
E
0
Turn
4
0
F
Hydrophobic
0.60
Alpha
0.00
G
Moment
0.60
0.00
Beta
Goldman et al
Kyte-Doolittle
2
0
2
HPhobic
HPhilic
H
0
100
200
300

PLOTSTRUCTURE of: EAE.PLS October 25, 1989 13:07

PEPTIDESTRUCTURE of: eae.pep Ck: 5897, 1 to: 389

TRANSLATE of: eae.seq check: 9925 from: 282 to: 1448

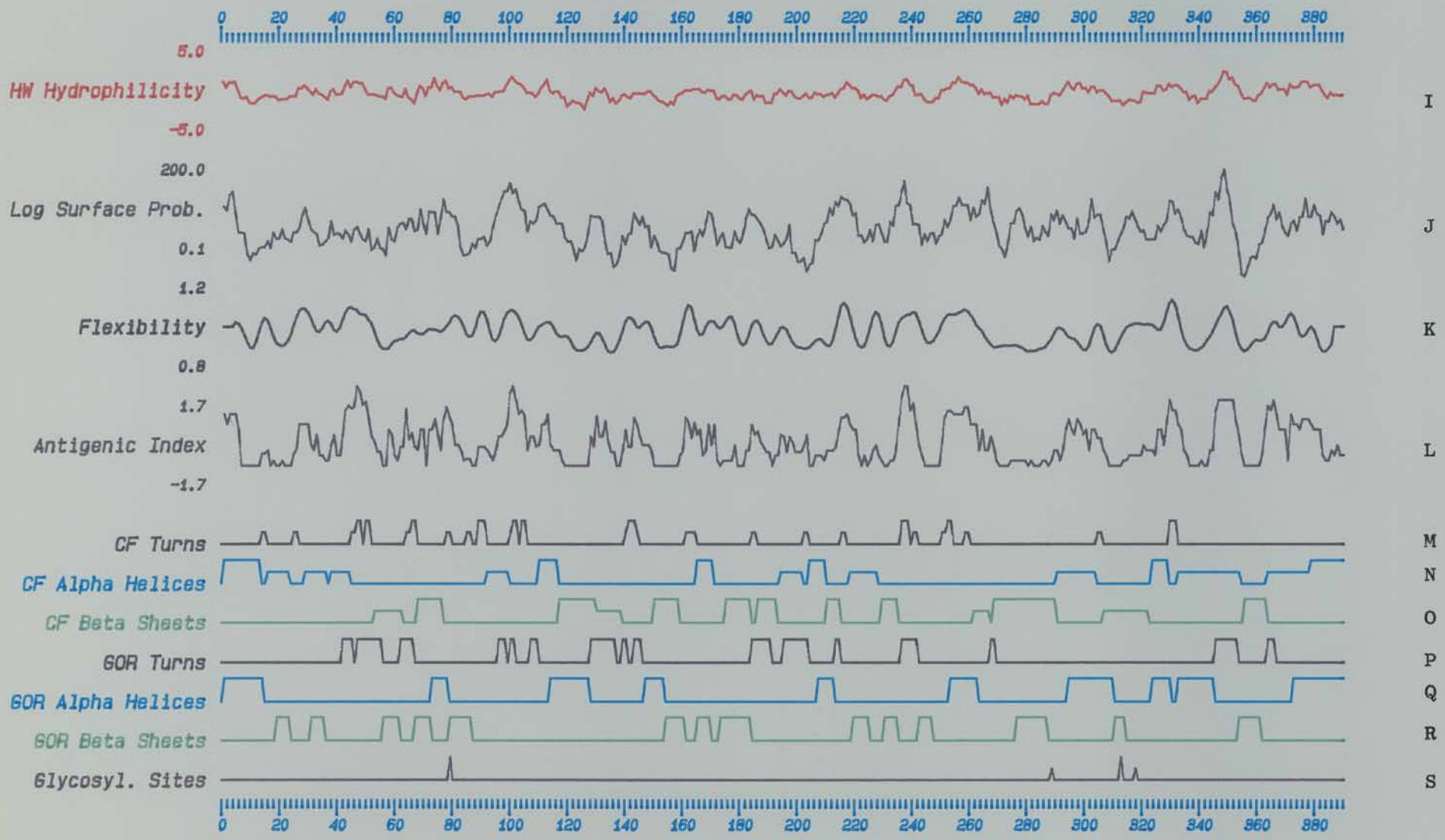


Figure 6.9. Outputs from the PEPPLOT, PEPTIDESTRUCTURE and PLOTSTRUCTURE programs of the UWGCG sequence analysis package. See text and Chapter 2 for a detailed description of the plots.

A series of four closely spaced, positively charged residues was found preceding VD3 in a predicted region of high turn potential, hydrophilicity, surface probability, flexibility and antigenic indices (residues +212 to +218) (Figure 6.9 panels A,F,I,J,K,L,M,P - add 22 to residue number to obtain corresponding values on the plots). (For details of these indices, see below or Chapter 2.) This cationic sequence was found in other *C.psittaci* MOMP sequences but in the *C.trachomatis* MOMP, there were only two positively charged residues (Figure 6.8). A similar positively charged region ending with a cysteine residue, 323-NKMKSRKAC-331 was located in the N-terminus of CD5, near the genus-specific epitope described by Conlan *et al* (1988). This cysteine, lysine and arginine-containing (CLAC) sequence was highly conserved across species. Clusters of basic residues resembling these sequences were also present in *E.coli* porins but not in bacteriorhodopsin.

Unlike bacteriorhodopsin which possesses seven hydrophobic α-helices 20 to 25 residues long that can span the membrane, no hydrophobic stretches of similar lengths except the putative signal sequence could be found in the MOMP sequence despite 47% hydrophobic residues. This type of primary sequence resembled the *E.coli* porins which possess numerous hydrophilic residues evenly distributed in the sequence without any long stretches of hydrophobicity.

All MOMPs possessed 7 Cys residues that were conserved in location and context throughout the genus. In addition, *C.trachomatis* MOMPs had 1 or 2 more. Although not all of these residues may be involved in disulphide cross-linking, some may participate in the intra- and inter-molecular linkages that have been suggested previously (Hatch *et al*, 1984; Bavoil *et al*, 1984), possibly those located in the CAPRA and CLAC sequences. An exhaustive search (PROSRCH and PATSRCH) of the current major databases of protein sequences for CAPRA and CLAC-like sequences did not pick out any sequences of significant homology other

than those of other MOMPs. Local regions of the CAPRA sequence, for example, at the cysteine residues, produced homology with human, murine and *Drosophila* laminin, *E.coli* alanyl-tRNA synthetase, sheep microfibrillar keratin, yeast methionyl-tRNA synthetase but the significance of these similarities was low.

Table 6.3 shows that the composition of MOMPs with respect to certain amino acids were similar whereas some displayed interspecies differences (marked with x) or intraspecies differences (marked with square brackets). In every VD of all the MOMPs examined, there was at least one conserved Pro residue despite nearly complete variation in other surrounding residues.

Table 6.3. Molecular mass of MOMPs, amino acid composition.

		C.psittaci MOMPs			*C.trachomatis* MOMPs			
	S26/3	GPIC	A22/M	CAL10	C	B	L2	
PreMOMP	41883	41946	43277	43232	42833	42532	42550	
LENGTH	389	389	402	402	397	394	394	
CHARGE	+5	+4	+4	+3	-3	-4	-5	
AVE	107.7	107.8	107.7	107.5	107.9	107.9	108.0	
SIG	2263	2293	2263	2263	2293	2293	2293	
CHARGE	+3	+3	+3	+3	+3	+3	+3	
MOMP	39639	39672	41032	40987	40559	40257	40275	
LENGTH	367	367	380	380	375	372	372	
CHARGE	+2	+1	+1	0	-6	-7	-8	
pI	6.50	6.14	6.15	5.78	4.82	4.76	4.69	
CHARGED RESIDUES								
SIG	3-0	3-0	3-0	3-0	3-0	3-0	3-0	
CD1	4-9	4-9	4-9	4-9	4-9	4-9	3-9	
CD2	4-4	4-4	4-4	4-5	4-4	4-4	4-4	
CD3	6-5	6-5	6-5	6-5	5-5	5-6	5-6	
CD4	7-4	7-4	7-4	7-4	7-5	7-5	7-5	
CD5	9-4	9-4	9-4	9-4	9-5	9-5	9-5	
CHARGE	33-26	33-26	33-26	33-27	32-30	32-31	31-31	
	+7	+7	+7	+6	+2	+1	0	
VD1	2-1	2-2	1-1	1-1	1-3	2-2	2-2	
VD2	1-1	0-1	0-1	0-1	2-1	1-2	2-4	
VD3	0-1	0-1	0-1	0-1	0-2	1-3	1-3	<-
VD4	1-3	1-2	2-3	2-3	1-5	1-5	1-4	<-
CHARGE	-2	-3	-3	-3	-5	-5	-5	

		C.psittaci MOMPs				*C.trachomatis* MOMPs		
	S26/3	GPIC	A22/M	CAL10		C	B	L2
pre-MOMP								
A Ala	54	51	47	50		49	47	48
G Gly	30	31	32	31		28	30	30
I Ile	22	24	23	25	x	18	15	14
L Leu	35	38	34	33		33	32	31
V Val	21	17	20	19	x	28	26	26
M Met	7	7	8	8	x	10	12	12
P Pro	17	16	[19	19]		16	15	15
F Phe	18	[14]	18	18		19	19	19
W Trp	10	10	10	10	x	7	7	7
N Asn	19	22	[24	24]		22	20	18
Q Gln	13	14	14	13		12	13	14
C Cys	7	7	7	7	x	8	9	9
D Asp	21	20	[18	19]		22	24	26
E Glu	11	12	14	14	x	17	17	16
H His	4	4	4	4		3	3	4
K Lys	20	19	18	18		21	21	20
R Arg	13	13	14	14		12	13	13
S Ser	25	24	[35	35]		27	28	28
T Thr	30	33	32	30		33	32	32
Y Tyr	12	13	11	11		12	11	12
AG	84	82	79	81		77	77	78
ST	55	57	67	65		60	60	60
DE	32	32	32	33		39	41	42
HKR	37	36	36	36		36	37	37
%charged	(17.7%)	(17.5%)	(16.9%)	(17.2%)		(18.9%)	(19.8%)	(20.0%)
NQ	32	36	38	37		34	33	32
DEHKR	69	68	68	69		75	78	79
ILMV	85	86	85	85		89	85	83
FWY	28	24	28	28		26	26	26

Note:
- AVE = average M_r of each residue
- SIG = putative signal peptide of 22 amino acid long
- LENGTH = number of amino acids
- preMOMP= putative precursor of MOMP before cleavage of SIG.
- CHARGE = summation of the total number of residues with positively charged side chains (H,K and R) and negative charged side chains (D and E).
- pI = predicted isoelectric point

Although there is no evidence of host glycosylation of chlamydial proteins, several potential eukaryotic glycosylation sites having the sequence Asn-X-Thr or Asn-X-Ser were present in the MOMP sequences. A weak glycosylation site (Asn-Pro-Thr or Ser) was present in MOMPs of both species; three strong sites were conserved in the *C.psittaci* MOMPs whereas only one was conserved in *C.trachomatis* MOMPs. These are displayed in Figure 6.9 panel S for S26/3 MOMP.

The codon usage of S26/3 MOMP was compared with that of other MOMPs (Zhang *et al*, 1989a; Pickett *et al*, 1988a; Stephens *et al*, 1986) and with other outer membrane proteins of *E.coli*, OmpF, OmpC and PhoE porins (EMBL database) and displayed in Table 6.4. The codon usage for S26/M MOMP was not random but did not display preferences as distinctly as those of *E.coli* OMPs. Several codons corresponding to the major isoaccepting species of tRNA in *E.coli* were not used in the gene eg. CTG for Leu, CCG for Pro.

Secondary structure prediction of S26/3 MOMP and other analyses based on primary structure

The MOMP amino acid sequences were analysed by a variety of tests for secondary structure and the results are displayed in Figure 6.9. As a guide to interpretation and to assess its predictive value, the secondary structure prediction was carried out firstly for the the matrix protein of *E.coli*, OmpF, a major outer membrane porin with two-thirds β-sheet structure (Inokuchi *et al*, 1982; Kleffel *et al*, 1985), some of which form intermolecular aqueous channels (Schindler and Rosenbusch, 1984; Engel *et al*, 1985) and secondly, for bacteriorhodopsin from *Halobacterium halobium*, a hydrophobic transmembrane protein known to contain seven transmembrane helices (Henderson and Unwin, 1975). The two conventional algorithms (Chou and Fasman, 1978; Garnier *et al*, 1978) predicted a high degree of β-structure in OmpF porin as expected. Several helical regions were also predicted. However, the prediction for bacteriorhodopsin was generally ambiguous. (Sequence data were obtained directly from the EMBL database.)

For example, bacteriorhodopsin sequences (data not shown) known to be transmembrane helices were not predicted accurately if at all by the C-F (Chou and Fasman, 1978) or GOR (Garnier *et al*, 1978) algorithms. They were identified by the GES (Engelman *et al*, 1986) and hydropathy (Kyte and Doolittle, 1982) plots strikingly well, which confirmed a previous report (Engelman *et al*, 1980). The surface probability (Emini *et al*, 1985), flexibility (Karplus and Schulz, 1985) and hydrophilicity (Hopp and Woods, 1981) plots accurately predicted those regions which projected into either the internal or external surface of the membrane. The correspondence of the antigenic index to such regions was striking, but did not distinguish between the internal or external surface. In the C-F and GOR predictions the helices showed up as random coil, probably caused by collisions of simultaneously predicted α-helix and β-sheet.

Consequently, these data for MOMP were interpreted with caution bearing in mind the predictive limitations and were used in combination with other analyses also displayed in the figure. The signal peptide was predicted to be hydrophobic and helical, carrying negatively charged lysines (-21, -20 and -17) at the N-terminus. Twenty residues at the C-terminus were also predicted by both C-F and GOR algorithms to be helical. No other continuous stretches of hydrophobic residues long enough to span a membrane in an helical structure (20-25 residues) could be detected in the various hydropathy plots of the MOMP sequence in contrast to those detected for bacteriorhodopsin. Instead short stretches of α-helices generally less than 20 residues were predicted throughout the sequence. These helices tended to be spaced about 40 to 60 residues apart. Overall, more β-sheet than α-helix structure was predicted as has been previously reported (Baehr *et al*, 1988).

Surface probability, flexibility and hydrophilicity plots tended to fall into a trough over VD1 and VD3 whereas flanking sequences were sharp peaks. Most residues in these VDs were

hydrophobic and comparison with other MOMPs showed that despite substantial sequence variation, the hydrophobic character of these sites was retained. Both VDs possessed β-turn potential.

In contrast, VD2 and VD4 contained relatively more hydrophilic residues; the surface probability, flexibility and antigenicity indices were high in these regions. Several peaks of β-turn potential occurred in these segments. This pattern was present in L2 MOMP which agreed well with epitope mapping data identifying these regions as highly antigenic sites (Stephens *et al*, 1988b; Baehr *et al*, 1988; Conlan *et al*, 1988).

The CAPRA and CLAC sequences previously mentioned were predicted to be regions of β-turns sandwiched by α-helix or β-sheet structure. These two regions possessed very low hydrophobic moment as were the corresponding sequences in A22/M and L2 MOMP. (VD1 was also another region of strikingly low hydrophobic moment.) CAPRA sequences possessed the strongest β-turn potential in S26/3 MOMP as well as A22/M and L2 MOMPs.

A program for joint prediction of secondary structure (Sawyer *et al*, 1986) was recently made available (courtesy of Dr Lindsay Sawyer) and the S26/3 peptide sequence was analysed. Preliminary results were not in conflict with any of the interpretations made (data not shown). Conflicting structural predictions produced by different programs made any useful deductions about the secondary structure of MOMP difficult.

T-cell epitope prediction

A computer program written by D.Thomson (University of Edinburgh - unpublished) was used to detect motifs commonly found in known T-cell epitopes (Rothbard and Taylor, 1988). Several such motifs were clustered in the following regions.

a. 29-CSTWCDIASIRAGYYGDYVFDRVLKVDVNK-58 which contains segments with high antigenic index and high β-turn potential;

b. 122-YFKASSAAFNLVGLIGVKGSSIAADQLPNV-151 which possesses a high antigenic index at the N-terminus and included the whole of VD2 at the C-terminus; and

c. 292-TTWNPTLLGEATALDTSNKFADFLQIASIQ-321 which is part of VD4.

Other tetrameric or pentameric 'Rothbardian' motifs were found localised in other segments of the sequence. An independent computer-assisted search for T-cell epitopes was carried out using the method of DeLisi and Berzofsky (1985) as adapted by D.Thomson (unpublished data). Amphipathic sequences longer than four residues are listed, several of which also contained 'Rothbardian' tetramers or pentamers.

3-VGNPAEP-9	
15-GTMWEGASGDPCDPCS-30	in the CAPRA sequence
43-YGDYVFDRV-51	in **a**.
93-AEWFTN-98	
154-TQGIV-159	in **C**. (see below)
295-NPTLLGE-301	in **c**.
306-DTSNKFADF-319	in **D** and in **c**.
353-LINER-357	

The hydrophobic moment mentioned in the preceding section is a marker of periodicity in hydrophobicity of a protein sequence (Eisenberg *et al*, 1984). Amphipathic α-helices or β-sheets possess a periodicity in their hydrophobicity of about 3.6 or 2.3 residues respectively. The hydrophobic moment plots displayed in Figure 6.9 are profiles of the moment at 100° for α-helices and 160° to 180° for β-sheets. T-cell epitopes tend to be amphipathic helices (DeLisi and Berzofsky, 1985); a scan of the helix hydrophobic moment profile revealed large peaks at

A. 41-GYYGDYVFDRV-51	in **a**. (see above)
B. 105-NIWDRFDIFC-114	
C. 156-GIVEFYT-162	
D. 308-SNKFADFLQ-316	in **c**.

The first two are in highly conserved regions CD1 and CD2 while the second two are located at the C-termini of the putative antigenic sites, VD2 and VD4. These are also present

in the corresponding sites in L2 MOMP despite differences in sequence. The first and the fourth are contained in the sites predicted above as containing many 'Rothbardian' motifs. Both the second and the third contain several such motifs. All four corresponding sites were present in A22/M MOMP. L2 MOMP possessed several more regions with large hydrophobic moment peaks.

DISCUSSION

Serology, peptide mapping and sequence data of MOMP has shown it to be the site of antigenic variation for *C.trachomatis* (see discussion of previous chapters). Data in preceding chapters showed that MOMP is likely to carry immunoprotective epitopes. This dual involvement of MOMP in antigenic variation and immunity has prompted the cloning and sequencing of its encoding gene from the OEA strain.

In this chapter, the structure of the MOMP gene has been elucidated as a prelude to gene expression. The sequence data of OEA *C.psittaci* MOMP has been presented together with that of other chlamydial MOMP to demonstrate inter-species and intra-species variation. Comparison was also made with well-studied outer membrane proteins of *E.coli* and with the paradigmatic bacteriorhodopsin of *Halobacterium*. Techniques in computer-assisted analysis of DNA and protein sequence data were used to make observations and inferences about higher level structure in relation to known functions and immunological effects of MOMP.

Resolving the identity of OEA MOMPs

The species *C.psittaci* is a diverse grouping within which strains have been defined by their origin of isolation and biological properties only until recently. Techniques such as restriction endonuclease analysis (REA)(McClenaghan *et al*, 1984; Timms *et al*, 1988), micro-immunofluorescence (MIF) (Perez-Martinez and Storz, 1985), monoclonal antibodies

(DeLong and Magee, 1986; Fukushi *et al*, 1987) and immunoblotting (Fukushi and Hirai, 1988) have been used to show variation at specific loci. Despite demonstrably large differences among strains of *C.psittaci*, strains that cause OEA when analysed by REA (McClenaghan *et al* 1984) and SDS-PAGE profiling (A.J.Herring, in preparation) are virtually identical. Some evidence for biological variation has been adduced (Russo *et al*, 1979; Aitken *et al*, 1981; Johnson and Clarkson, 1986) but SDS-PAGE and peptide mapping (Chapter 4) analyses on MOMP from several OEA strains do not show clear evidence for the loci of variation (if any) to reside within MOMP.

Given that A22/M as reported by Pickett *et al* (1988a) is purportedly an ovine abortion isolate, the degree of variation found between S26/3 and A22/M MOMPs was surprising. From the sequence data and DOTPLOT comparisons, A22/M MOMP resembles that of the meningopneumonitis strain Call0 far more closely than it does S26/3 MOMP. S26/3 MOMP in turn resembles that of the other strain of mammalian origin, GPIC. Since the Call0 strain is avian in type as judged by its origin from a case of presumptive ornithosis (Francis and Magill, 1938), by its REA profile (McClenaghan *et al*, 1984) and its DNA reassociation properties (Cox et al, 1988), the A22/M strain could be of avian origin. Moreover, genomic REA profiles and Southern blotting with a MOMP gene probe showed A22/M to be virtually identical to Call0 and avian strains; similarly, S26/3 was identical to other OEA strains (A.J.Herring and S.Baxter, personal communication; Herring et al, 1989). The comparison of A22/M and S26/3 MOMP sequences is thus an inter-type comparison rather than an inter-strain comparison.

The history of the A22/M strain is not clear but it should have been identical to A22, which is the original ovine abortion strain isolated at the Moredun Institute (Stamp *et al*, 1950); the "M" stands for Moredun (I.Clarke and A.J.Herring, personal communication). In contrast, the S26/3 MOMP gene was isolated from the same preparation of chlamydial organisms as that used in a vaccination-challenge experiment (Anderson *et al*,

submitted). Since the A22 isolate has had a very long passage history, one obvious explanation is that at some point an accidental substitution with an avian isolate has occurred, probably after it had left the institute.

As this thesis was being prepared, further evidence indicated that A22/M is unlikely to be the A22 OEA strain and that the S26/3 sequence is unequivocally from an OEA strain. Using primers based on the DNA sequence of S26/3 MOMP CDs (Figure 6.1D), amplification of MOMP genes by the polymerase chain reaction (Saiki *et al*, 1985), particularly from field isolates of OEA strains, followed by DNA sequencing and/or REA has shown that the S26/3 sequence is identical to that of other strains isolated from cases of OEA, including the A22 strain (S.Baxter, PhD thesis in preparation). Thus, while MOMP may be the loci of inter-type variation in *C.psittaci* as shown by the comparisons with Cal10, A22/M and GPIC, it does not appear to be a loci of primary structure variation in the OEA isolates tested so far. This conclusion agrees with the data on limited proteolysis of MOMP described in Chapter 4 although the question of post-translational modification remains to be resolved.

Promoter Region

Based on sequence data and Northern blot analysis, it has been shown that the *C.trachomatis* MOMP gene is monocistronic, possessing promoter sequences upstream of the ORF and termination signals immediately downstream (Stephens *et al*, 1986). It was subsequently demonstrated that the L2 MOMP gene (omp1L2) is differentially transcribed from two tandemly arranged promoters, a shorter transcript (P1) being constitutively synthesised and the longer (P2) under regulatory control (Stephens *et al*, 1988a). Since the regulatory sequences of S26/3 MOMP are highly conserved with *C.trachomatis* sequences and possess identical P1 -35 and -10 regions and IR sequences, with very similar P2 and terminator sequences, it is likely that S26/3 MOMP gene is also monocistronic and differentially

transcribed from two tandem promoters. However, whether this is the case needs to be formally demonstrated with *C.psittaci* MOMP.

Although the *C.psittaci* MOMPs presented in Figure 6.4 and 6.5 are from diverse sources, they were virtually identical as much as 200 bp upstream of the start codon despite clear differences in the ORF, particularly in the VSs. Inter-species comparison in this region showed 75% identical residues after alignment, much higher than the corresponding degree of conservation within the coding region between the two species. Such a striking degree of conservation suggests that either these strains are more related than previously thought (Kingsbury and Weiss, 1968), or that there is an extremely strong selection pressure on this non-coding region as a result of its putative promoter and other regulatory functions, or both. An example where highly conserved upstream sequences has been linked to transcriptional regulation is the *pho*E promoter of *Enterobacteriaceae* (van der Ley *et al*, 1987a) but the degree of conservation is not as high as that in the MOMP promoters.

Mechanisms of transcriptional regulation in prokaryotes involve RNA polymerase modification, its association with sigma factors, repressors and activators, and the association of the transcription apparatus with specific nucleotide sequences (von Hippel *et al*, 1984). For instance, the *omp*R product is an osmolarity-dependent activator of both OmpF and OmpC porin expression in *E.coli* (Hall and Silhavy, 1981 - review). Both the genes encoding these major outer membrane porin possess a highly conserved sequence in the promoter that has been subsequently shown to be within the binding site of OmpR (Mizuno *et al*, 1983; Mizuno and Mizushima, 1986). Similarly the *pho*E, *pho*A, *pho*B, and *pst* genes of *Enterobacteriaceae* are thought to be regulated by the products of the *pho* regulon via upstream elements called *pho* boxes, originally deduced by sequence comparison of their respective promoters (Tommassen *et al*, 1987).

Comparison of the S26/3 MOMP promoter with *C.trachomatis* MOMP promoters revealed that while sequences upstream of the promoter-IR motif had diverged, there were many identical sequences in the motif, including the perfect IR, the cognate elements of P1 and the ribosome-binding site. In extending the suggestion of Pickett *et al* (1988a) that these conserved regions might function in regulation and expression of the gene, it is proposed that a search for chlamydial proteins that bind at these sites might uncover trans-acting regulatory factors of MOMP gene transcription. It is also interesting to note that another highly transcribed gene, the rRNA cistron of *C.trachomatis* also possesses tandem promoters (Engel and Ganem, 1987) but they bear little homology with either *C.trachomatis* or *C.psittaci* MOMP promoters. The suggestion by Engel and Ganem (1987) that chlamydial transcription signals are significantly different from all previously described prokaryotic promoters, if true, also requires the proposition that chlamydial RNA polymerases and accessory factors differ, at least in the critical DNA binding sites.

The sequence and the spacing of the -10 and -35 promoter elements are critically important for optimal binding of RNA polymerase holoenzyme (Esigma^{70}) of *E.coli* (Hawley and McClure, 1983), particularly where highly transcribed genes are involved. Generally, conformity to the -10 (TATAAT) and -35 (TTGACA) consensus sequences and their spacing (17$\pm$1) results in optimal expression in *E.coli*. *C.trachomatis* MOMP promoters appear to function poorly in E.coli and this has been attributed to the lack of sequence homology between the promoters of *C.trachomatis* MOMP and the *E.coli* consensus (Stephens *et al*, 1988a). The putative *C.psittaci* promoters particularly P1 possess a longer spacing between the promoter elements in addition to the lack of homology in sequence to that of *E.coli*. The obvious implication for the development of recombinant S26/3 MOMP is that the coding gene should be attached to a strong *E.coli* promoter rather than retain the natural promoter.

It has been shown for *Bacillus subtilis* (Rather *et al*, 1986) and for *E.coli* (Cowing *et al*, 1985) that the utilisation of different sigma factors by the RNA polymerase core enzyme (E) occurs with differences in promoter sequence specificity. McClure (1985) proposed that if "consensus is best" for transcription initiation in prokaryotes, then the data in the literature suggest that poor homologies at the -35 region might indicate control by dissociable activators (or repressors) while those at the -10 region might suggest the presence of additional sigma subunits. The possibility that *C.trachomatis* might use different sigma subunit(s) from *E.coli* has been explored by Stephens *et al* (1988a). The longer spacing between the -35 and -10 of the *C.psittaci* promoters compared to that of *C.trachomatis* suggests that if sigma subunits are involved, they are likely to be different between the two chlamydial species. Efforts to test these speculations will be hampered by the absence of a transformation system for *Chlamydia* spp. despite many studies of the chlamydial plasmid (Joseph *et al*, 1986; Sriprakash and MacAvoy, 1987; McClenaghan *et al*, 1988; Hatt *et al*, 1988). In addition, there have been reports of chlamydia-specific phage (Harshbarger *et al*, 1977; Richmond *et al*, 1982; Bevan and Labram, 1983) but its development as an agent of transfection has yet to be demonstrated.

Coding Region

Prokaryotic outer membrane proteins have signal sequences at their N-termini which are cleaved off as they mediate transport to the surface location (reviewed by Perlman and Halvorson, 1983). Such sequences of outer membrane porins of *E.coli* are very similar in length (20 residues) and structure: positively charged residues followed by a hydrophobic core and a post-translational cleavage recognition site, Leu-X-Ala (Oliver, 1985 - review). In addition to the positively charged N-terminus and the hydrophobic core (n-region and h-region respectively), von Heijne (1985) also describes the c-region of the signal sequence, a region of more polar character which is at the C-terminus.

The N-terminus of the *C.trachomatis* L2 pre-MOMP conforms to these requirements and the N-terminal residue Leu of the mature MOMP begins immediately after the consensus site, Leu-Gln-Ala (Nano *et al*, 1985; Stephens *et al*, 1986). Protein microsequencing of the mature *C.psittaci* S26/3 MOMP showed that the N-terminus is also identical to that of *C.trachomatis* L2 (Chapter 4). Although MOMPs from other strains have not been sequenced, it is likely that the processing site is identical to that of *C.trachomatis* L2 or *C.psittaci* S26/3 because of the high degree of conservation, particular at the proposed cleavage site. The three Lys residues and the consensus site were identical in all these chlamydial MOMPs. Substitutions were conservative and maintained the overall hydrophobic character of the h-region.

It is an established observation that heterogeneity in MOMP sizes exists (see Chapter 1). The sequence comparisons made here and that of others (Stephens *et al*, 1987; Pickett *et al*, 1987 and 1988a; Yuan *et al*, 1989; Zhang *et al*, 1989a) have shown that such size differences are due to differences in the length of the peptide sequence at these variable domains. However, estimations of M_r from SDS-PAGE do not always agree closely with calculated values prompting speculations of post-translational modification.

There are eukaryotic signals for glycosylation within the MOMP sequence but the evidence for glycosylation of MOMP has so far been ambiguous (Hatch *et al*, 1981; Caldwell *et al*, 1981; Carlson *et al*, 1986). Hatch *et al* (1981) demonstrated the presence of glycosyl moieties on MOMP using the sensitive periodic acid-dansyl hydrazine (PAD) stain where Caldwell *et al* (1981) failed with the periodic acid-Schiff (PAS) stain. Carlson *et al* (1986) have shown that MOMP and other chlamydial proteins bound specific lectins in a ligand blotting study. The lectin specificities of these proteins were consistent with O-linked oligosaccharide-containing glycoproteins and these investigators suggested that chlamydial glycosylation is not derived from host cell N-linked lipid pathways.

The composition of the VDs from the sequence analyses appeared to vary significantly thus influencing hydrophilicity and antigenicity . Within these VDs are often found regions of high turn potential and high hydrophilicity indices suggesting that these are regions which are exposed on the surface of the outer membrane. In studies with *C.trachomatis* MOMP using monoclonal antibodies and recombinant proteins (Stephens *et al*, 1988b; Baehr *et al*, 1988) or solid-phase peptides (Conlan *et al*, 1988), VD2 and VD4 have been found to be the loci of a complex hierarchy of serovar-, subspecies-, species- and genus-specific epitopes. Su *et al* (1988) have also found trypsin cleavage sites located in two VDs showing the accessibility of such domains. For trachoma serovar B, they have correlated loss of infectivity to trypsin cleavage at VD2. Visual inspection of the S26/3 sequence showed potential sites for trypsin or chymotrypsin cleavage in VD2. Further investigations on the effect of protease treatment of S26/3 EBs may reveal whether an analogous situation occurs for *C.psittaci* infectivity.

Chlamydial MOMP is a structural component and functions as a porin (Bavoil *et al*, 1984). It is covalently linked through disulphide bridging to itself and to other outer membrane proteins (Bavoil *et al*, 1984; Hatch *et al*, 1984; Newhall and Jones, 1983) and that redox control of these bonds affect differentiation of EB to RB (Hackstadt *et al*, 1985) and regulate porin function (Bavoil *et al*, 1984). Comparison of the *C.psittaci* with the *C.trachomatis* MOMP sequences in this chapter and by Zhang *et al* (1989a) revealed five highly conserved portions of the MOMP sequence designated constant domains 1 to 5 (CD1 to CD5). It is likely that the biological functions common to all chlamydial MOMPs may be located in these regions. The 7 conserved cysteine residues in the CDs of *C.psittaci* and *C.trachomatis* MOMPs occur at the same location in a virtually identical context between species. Thus while the variable regions, thought to be the surface-exposed stretches, diverged at the primary sequence to give rise to the observed antigenic

variation, the constant regions may have been preserved to maintain the secondary, tertiary or quaternary structures necessary for the retention of these functions.

The data in the literature and in Chapter 4 suggests that MOMP possesses a hierarchy of disulphide bridges that mediate inter-oligomeric, inter-monomeric and intra-molecular linkages. The presence of at least seven cysteine residues provides MOMP with the capacity to form such linkages: at least one thiol group to form links from one oligomer complex to another either directly or with via other CRPs, at least two to form intra-complex links between monomers with two pairs left for intra-molecular stabilisation. The highly conserved context in which the cysteine residues occur suggests that it may be possible to reconstitute hybrid oligomers made of MOMPs from different strains.

The aggregation of denatured and reduced MOMP to form putative dimers, trimers and higher order oligomers under partially renaturing and non-reducing conditions was described in Chapter 4. The striking features of CAPRA and CLAC sequences described in this chapter suggest a possible explanation and a testable hypothesis for this observation. These oppositely charged sequences may fold into stable secondary structures that facilitate close interaction of MOMP monomers, thereby bringing the thiol residues into close proximity for disulphide bonds to form.

For example, it is speculated that two cysteine residues at the end of the CAPRA sequence may cross-link with each other to stabilise a β-turn, leaving one free cysteine to project out from a negatively charged domain. Alternatively, if at least part of the CAPRA sequence can form a helix, it is possible to draw a model in which all three Cys residues occur on the same side and all the negatively charged Asp residues lie on another to form a negatively charged cysteine "barrel". The CLAC sequence, not necessarily from the same polypeptide chain, with its positively charged side chains, may form stable ion

pairs and a disulphide linkage with this moiety. Excess anionic groups on the CAPRA sequence may also be involved in the sites on the surface of EBs and RBs which bind polycationic ferritin molecules (Schiefer *et al*, 1982). Computer modelling of these sequences should reveal the thermodynamic and steric possibility of the speculated structures.

However, in the absence of experimental data, it would be premature to form firm inferences about the secondary structure of MOMP based on the computer predictions. It is generally accepted by workers in this field that VDs are surface-exposed, immunoaccessible and protease-accessible. The preliminary model of the way MOMP folds in the outer membrane as proposed by Baehr *et al* (1988) might be too simplistic in view of the complexity of the various parameter profiles. If the knob-like structures observed in the EM pictures of the outer membrane preparations (Chapter 4) are indeed the side elevations of MOMP aggregates, then these knobs may be globular MOMP domains on the exterior with contiguous segments of the polypeptide chain extending into the OM as anchor points and as channels. OmpF porin has already been shown to exist as a triple channel trimer at the external surface which merges into a single channel at the periplasmic face (Engel *et al*, 1985). Perhaps certain domains of MOMP may form a similar structure.

It is clear that MOMP does not possess the all helical structure of bacteriorhodopsin but it is not possible to rule out the presence of α-helices. The amphipathicity determinations and hydrophobic moments suggest that certain regions may fold into amphipathic helices at the surface of the polypeptide, either lining the putative channels or exposed to the exterior or the periplasmic space.

It is also clear that MOMP resembles *E.coli* porins in its parameter profiles as well as amino acid composition and charge distribution. This suggests that a large part of MOMP might be β-sheet structure. Circular dichroism analyses of outer membrane preparations such as those performed on OmpF by Kleffel

et al (1985) on reconstituted MOMP may provide some answers here. In the absence of transformation procedures for *Chlamydia* spp., a potential alternative for testing the porin function of MOMP may be to contrive porin-MOMP hybrids to determine how far MOMP domains can substitute for channel forming domains of porin. This has been successfully performed for OmpF, OmpC and PhoE (van der Ley *et al*, 1987b). In this respect, informed secondary structure predictions may assist in the selection of domains for testing. In the same manner, OmpA-MOMP hybrids may assist in assessing the basic molecular requirements for a structural function. Sites where the two cysteine residues of OmpA occur may be substituted for MOMP domains carrying cysteine residues.

Codon Usage

As proposed by Crick (1966) in his "wobble hypothesis", a single tRNA can usually respond to multiple codons. Despite this degeneracy in the genetic code, codon choices, particularly for some amino acids, are highly biased and the choice of synonymous codons varies with the type of organism (the "genome hypothesis" of Grantham, 1980). Such an organism-specific codon preference is related to the population profile of the isoaccepting tRNAs for each organism, as has been demonstrated for *E.coli* and yeast (reviewed by Ikemura, 1985)(isoaccepting tRNAs are tRNAs that are charged with the same amino acid but usually respond to different codons for that amino acid). The correlation between the usage frequency of a particular codon with the abundance of their respective tRNAs is especially evident for highly expressed genes, for example, *E.coli* RNA polymerase and major outer membrane proteins, yeast enolase and histone genes. Although the choice between synonymous codons does not affect the nature of the protein synthesised, it will influence the translation efficiency of mRNAs transcribed from these genes (Ikemura, 1981). As shown in Table 6.4, about half of the optimal codons of *E.coli* do not correspond to the frequently used codons of S26/3 MOMP. It is

possible that attempts at over-expression of MOMP in *E.coli* may be limited at the level of translation as specific tRNA populations become depleted.

TABLE 6.4. Codon usages.

AmAcid	Codon	S	G	A	Ca	L2	Ec	optima
Gly	GGG	2	7	4	4	2	0	
Gly	GGA	10	8	10	10	15	0	
Gly	GGT	13*	8	11	10	7	29	E
Gly	GGC	5	8	7	7	6	19	E H
Glu	GAG	2	4	4	3	8	0	H
Glu	GAA	9*	8	10	11	8	11	E
Asp	GAT	15*	14	12	13	20	9	
Asp	GAC	6	6	6	6	6	23	H
Val	GTG	3	1	4	3	6	2	E H
Val	GTA	7	7	7	7	6	9	E
Val	GTT	10*	7	7	7	12	12	E
Val	GTC	1	2	2	2	2	2	
Ala	GCG	3	4	0	0	1	3	E
Ala	GCA	17	13	15	17	15	8	E
Ala	GCT	30*	28	26	27	28	18	E
Ala	GCC	4	6	6	6	4	0	H
Arg	AGG	0	1	1	1	0	0	h
Arg	AGA	5*	6	7	7	4	0	H
Ser	AGT	3	1	3	4	6	1	
Ser	AGC	4	5	7	7	4	2	H
Lys	AAG	4	4	3	3	7	0	H
Lys	AAA	16*	15	15	15	13	17	E
Asn	AAT	10*	8	13	13	12	0	
Asn	AAC	9	14	11	11	6	32	E H
Met	ATG	7	7	8	8	12	4	
Ile	ATA	1	2	2	3	0	0	
Ile	ATT	7	12	13	13	9	0	
Ile	ATC	14*	10	8	9	5	10	E H
Thr	ACG	3	2	4	5	1	0	
Thr	ACA	10	12	8	8	13	0	
Thr	ACT	12*	15	13	12	14	12	E
Thr	ACC	5	4	7	5	4	12	E H
Trp	TGG	10	10	10	10	7	4	

AmAcid	Codon	S	G	A	Ca	L2	Ec	optima
End	TGA	0	0	0	0	0	0	
Cys	TGT	4*	4	4	4	5	0	
Cys	TGC	3	3	3	3	4	0	H
End	TAG	0	0	0	0	0	0	
End	TAA	1	1	1	1	1	1	
Tyr	TAT	3	10	4	4	4	5	
Tyr	TAC	9*	3	7	7	8	24	E H
Leu	TTG	9	4	7	7	10	0	
Leu	TTA	17*	20	14	14	8	1	
Phe	TTT	6	5	9	9	8	2	
Phe	TTC	12*	9	9	9	11	17	E H
Ser	TCG	3	3	5	5	1	0	
Ser	TCA	3	2	11	10	3	0	
Ser	TCT	7*	9	6	6	9	6	
Ser	TCC	5	4	3	3	5	8	h
Arg	CGG	0	0	0	0	0	0	
Arg	CGA	2	0	0	0	2	0	
Arg	CGT	1	2	2	2	5	12	E
Arg	CGC	5*	4	4	4	2	1	E
Gln	CAG	4	3	3	3	3	20	E H
Gln	CAA	9*	11	11	10	11	1	
His	CAT	0	3	2	2	3	0	
His	CAC	4*	1	2	2	1	1	H
Leu	CTG	0	1	1	1	3	24	E H
Leu	CTA	1	2	3	2	1	0	
Leu	CTT	3	7	5	5	5	1	
Leu	CTC	5	4	4	4	4	1	h
Pro	CCG	0	0	1	1	1	1	E
Pro	CCA	4	6	8	8	4	3	e
Pro	CCT	11*	10	9	9	9	0	h
Pro	CCC	2	0	1	1	1	0	H

Note:
S = S26/3 MOMP
G = GPIC MOMP (Zhang *et al*, 1989a)
A = A22/M MOMP (Pickett *et al*, 1988b)
Ca= Call0 MOMP (Zhang *et al*, 1989a)
L2= *C.trachomatis* L2 MOMP (Stephens *et al*, 1986)
Ec= OmpC major outer membrane porin of *E.coli* (Mizuno *et al*, 1983)
* = preferred codons in S26/3 MOMP
E = optimal codons and the major tRNA species for *E.coli* (Ikemura, 1985)
H/h = Most and second most preferred codon in human genes (Ikemura, 1985)

For highly expressed genes, similarities in codon usage in different unicellular organisms are thought to reflect similarities in the composition of the tRNA population and such similarities have been suggested as taxonomic markers (Ikemura, 1985). Table 6.4 shows that MOMP codon usages are similar within the genus but different from a typical *E.coli* major outer membrane porin gene, ompC. The degree of bias in the MOMP codon usage is not as pronounced as that of OmpC; less than 10 out of the 59 synonymous codons were used only once or not at all compared to more than half in OmpC. Since there is no evidence that chlamydial reticulate bodies utilise host tRNAs, correlation between the codon preferences in the chlamydial MOMPs and that of eukaryotes (as typified by human genes) was not expected. Indeed, Table 6.4 shows that there is little correlation in the preferences of MOMP genes from the representative avian, mammalian or human chlamydial strains with that of human genes.

Suitability of the MOMP gene for PCR

Although *C.psittaci* and *C.trachomatis* share some characteristics such as genus-specific epitopes (Dhir *et al*, 1972; Caldwell and Hitchcock, 1984), certain rRNA gene sequences (Palmer *et al*, 1986) and a unique developmental cycle (Higashi, 1965), they share only 10% or less DNA homology (Kingsbury and Weiss, 1968; Cox *et al*, 1988). Yet within the MOMP coding sequences, the overall inter-species homology is high. This accounts for the finding that L2 MOMP probes can hybridise to sequences from other chlamydial strains (Stephens *et al*, 1985) and was used in the cloning of the S26/3 MOMP gene (Herring *et al*, 1989). Within the VDs the homology declines to the overall percentage but in the conserved domains, it rises to greater than 90% between species, particularly at the termini of the gene.

The sequence conservation at the termini of the MOMP gene clearly makes it an ideal target for amplification by the polymerase chain reaction (PCR) (Saiki *et al*, 1985). PCR

primers (amplimers) 288C and 289C have been successfully used to amplify DNA of S26/3 and A22 MOMPs. Preliminary results have shown that it is possible to amplify the MOMP sequences from a wide range of *C.psittaci* strains using the appropriate amplimers (Figure 6.1) (S.Baxter and A.J.Herring, personal communication). Some of these PCR-amplified sequences have been easily cloned and sequenced (S. Baxter, personal communication). Restriction sites specific to each strain which are generated by differences within the VSs or by silent changes in the CDs imply that restriction endonuclease profiling may be used as a rapid method for typing strains. Thus the development of a generally applicable PCR-based test for use with clinical samples which can both detect and type *C.psittaci* infections seems imminent.

The next chapter describes how the PCR technique was used to amplify a precise MOMP gene fragment for insertion into an expression vector.

CHAPTER 7

EXPRESSION OF RECOMBINANT MOMP

INTRODUCTION

Allan *et al* (1984) were the first to report the expression of the complete MOMP gene of *C.trachomatis* (serovar L1) in *E.coli* using a bacteriophage vector, λ 1059. They showed that the recombinant produced a doublet of polypeptides (about 40 kDa) similar to purified MOMP by serological and by proteolytic analysis. Using a λ gt11 β-galactosidase fusion vector, Stephens *et al* (1985, 1988b), Baehr *et al* (1988) and Carlson *et al* (1989) have expressed fragments of the MOMP gene from serovars L2 and L3 of *C.trachomatis*. The N-terminus of β-galactosidase, however, constituted a major portion of the recombinant protein using this expression system. More recent attempts to express the MOMP gene from its own promoter either *in vivo* using another λ system (λ L47.1) or by *in vitro* transcription/translation have failed (M.A.Pickett, M.E.Ward and I.N.Clarke, unpublished data). Pickett *et al* (1988b) have subsequently succeeded by using plasmid vectors of the pUC series (Vieira and Messing, 1982; Yanisch-Perron *et al*, 1985). They reported high level expression of *C.trachomatis* L1 MOMP fragments of M_r 33, 26 and 13 kDa attached to short peptides from the N-terminus of the β-galactosidase. These fragments form an N-terminal deletion series progressively omitting MOMP antigenic sites. Expression is regulated by the *lac* promoter and is achieved via induction with isopropyl β-D-thiogalactoside (IPTG). As far as the author is aware at the time of the work described in this chapter, there have been no reports on the expression of MOMP genes from *C.psittaci* although there have been reports of sequence data on the MOMP gene from the putative abortion strain, A22/M (Pickett *et al*, 1988a), the GPIC strain 1 and the meningopneumonitis strain Cal10 (Zhang *et al*, 1989a).

From the analysis of the structure of the S26/3 MOMP gene in the preceding chapter, several important observations pertinent to the design of a MOMP expression construct were made. Firstly, the MOMP promoter is significantly different from the *E.coli* promoter consensus sequence and is unlikely to be a good substrate for *E.coli* RNA polymerase. This observation may explain why Pickett *et al* could not obtain expression using the L1 MOMP gene promoter, which is also quite different from the *E.coli* consensus. Thus expression vectors containing strong *E.coli* promoters were chosen. Several strong promoters are known to be used successfully for the expression of foreign genes, for example, the *lac*, *trp*, λ P_R and P_L promoters or the synthetic *tac* promoter (Marston, 1987 and references therein).

It has been suggested that chlamydial outer membrane protein (OMP) genes may be unstable in plasmid vectors and that cloning in M13 vectors may overcome instability (Clarke and Lambden, 1988). An M13 vector carrying a *tac* promoter (Larder *et al*, 1987) was readily available and this was used as the initial expression vector in this study. This vector, M13 mptac18, was derived from M13 mp18 (Yanisch-Perron *et al*, 1985) and carried in place of its *lac* promoter sequence, the *tac* promoter, a Shine-Dalgarno ribosome binding sequence and an ATG start codon followed by a multiple cloning site (MCS).

A second observation concerning the construction of a recombinant expressing MOMP was that there were no convenient restriction sites at the 5′ end for manipulating the MOMP gene into a vector. Several alternatives were available for circumventing this problem. One method would be to make a library of unidirectional nested deletions from the 5′ end of the MOMP *Sst*I insert. The option of creating a 5′ nested deletion was particularly attractive because it was envisaged that recombinants expressing progressively shorter polypeptides would be useful for analysing the relative importance of the variable domains. However, initial attempts to use this technique for creating a set of 3′ nested deletions to facilitate the sequencing of the MOMP gene failed (Chapter 6).

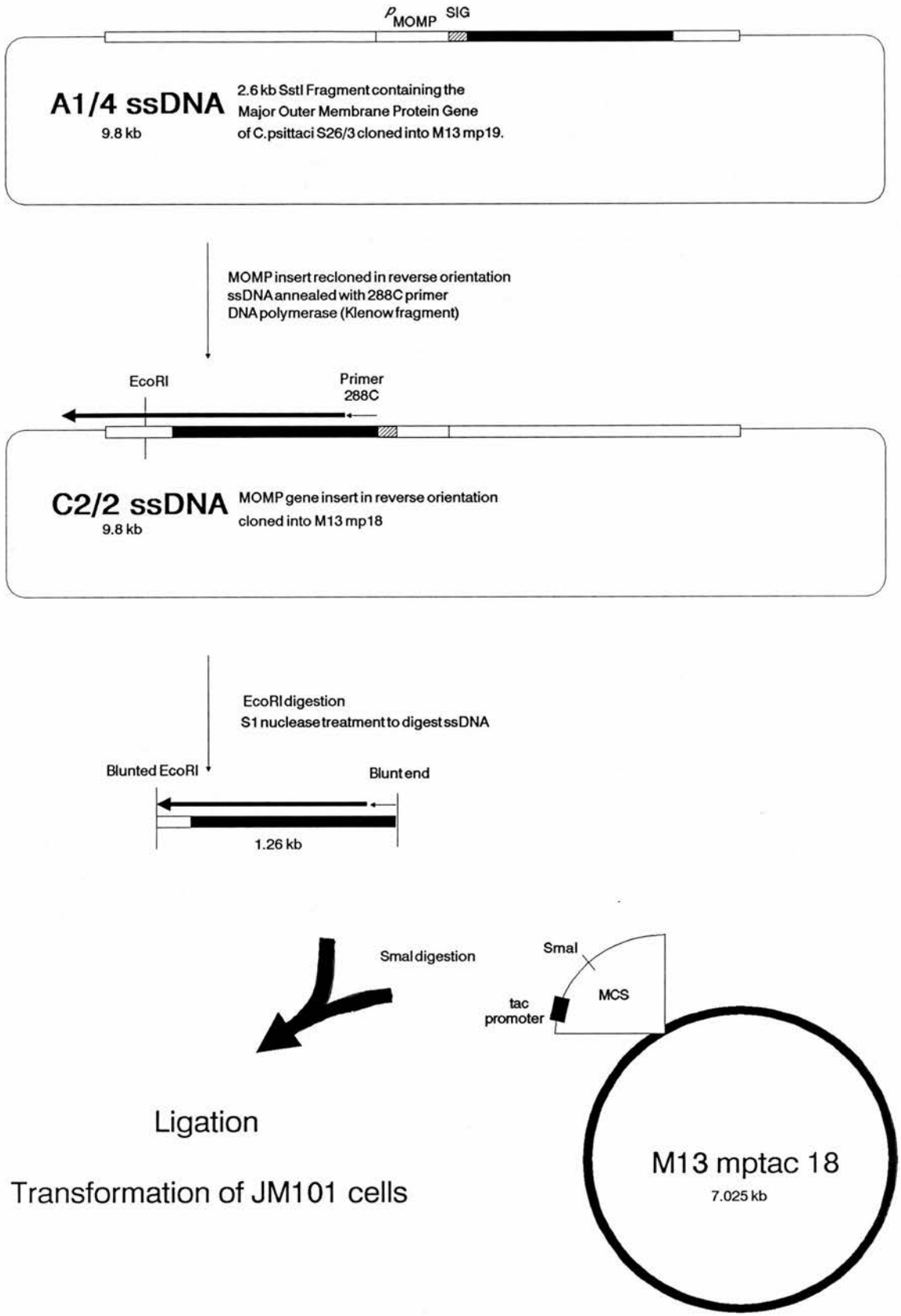


Figure 7.1 Attempt to construct an M13 mptac18 recombinant for the expression of mature MOMP using a primer extension method.

These attempts relied on reagents purchased from different manufacturers and the procedure required more experiments to optimise conditions. Alternatively, commercially available kits for this technique may be purchased.

Thirdly, a leader peptide sequence similar to that of *C.trachomatis* MOMP sequences was also present in S26/3 MOMP gene, as is expected for an OMP. This signal sequence is thought to direct the export of MOMP to the outer membrane. Since MOMP is a major structural protein with porin functions, expression and export to the *E.coli* outer membrane may have adverse effects on the growth of the host. Moreover, the signal peptide cleavage apparatus of *E.coli* may not recognise and cleave the MOMP leader sequence at the correct site. Based on these considerations, the cloning of the sequence coding for the processed MOMP was attempted instead.

EXPERIMENTAL STRATEGY

The strategy adopted was to design an oligonucleotide based on the elucidated S26/3 MOMP sequence (Chapter 6) for priming DNA polymerisation of the desired MOMP coding sequence. The oligonucleotide sequence chosen was 288C (Figure 6.1). It was used as a primer to initiate DNA polymerisation from the site corresponding to the N-terminus of the mature MOMP towards the 3′ end of the gene (Figure 7.1). The template (C2/2) used was the anti-sense strand of the MOMP gene cloned into the M13 ssDNA plus strand (courtesy of Dr A.J.Herring and Sue Dunbar). This clone, C2/2, was produced by recloning the *Sst*I dsDNA insert from A1/4, the original recombinant carrying the 2.6kb insert containing the MOMP gene (Chapter 6), into M13 mp18 and selecting for a recombinant containing the insert in the opposite orientation.

The experiment was carried out as outlined in Figure 7.1 but no positive recombinants could be isolated. One speculation was that the S1 nuclease digestion was not optimised for blunt end formation. Alternatively, the generation of many small blunt ended fragments may have blocked the desired ends.

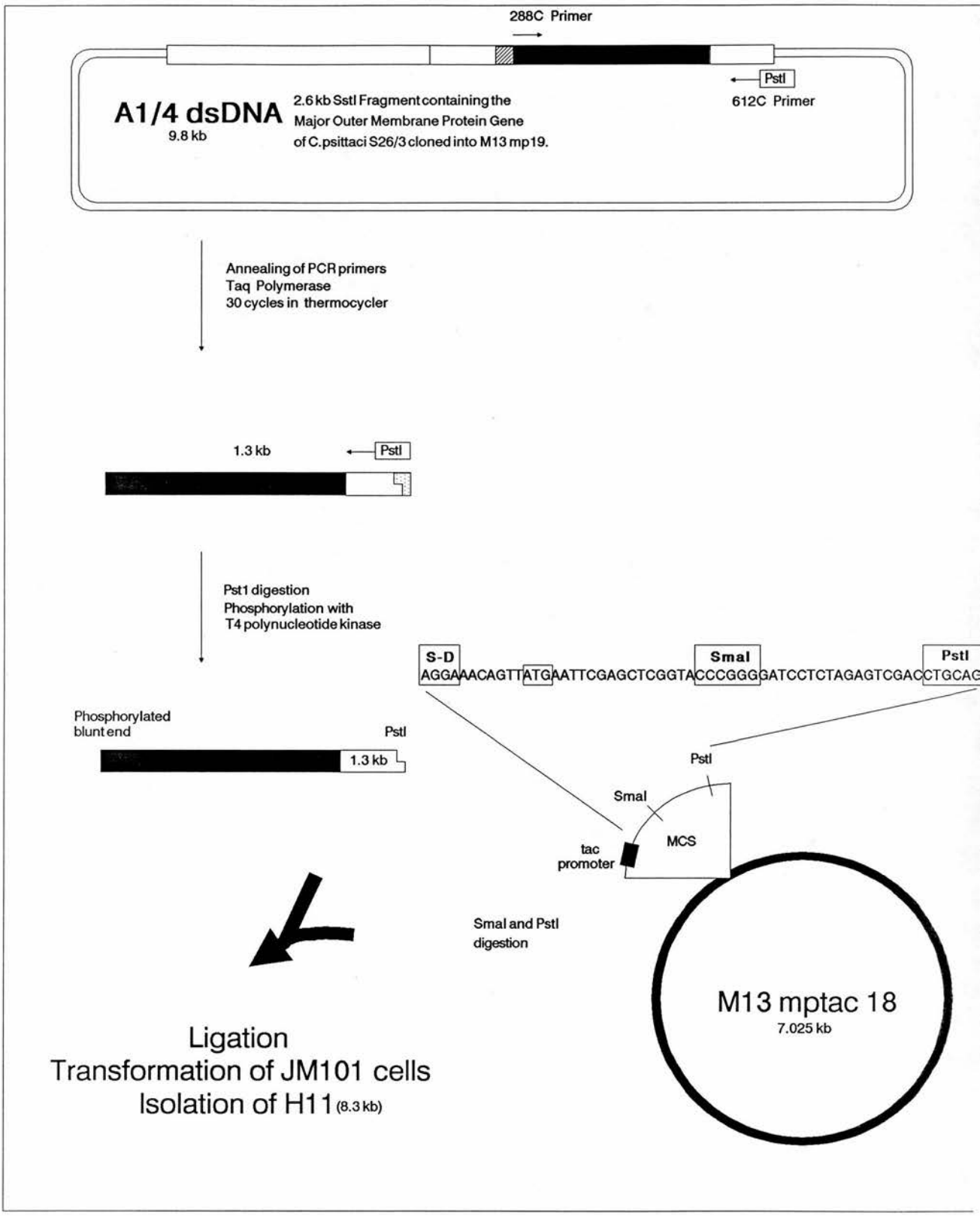


Figure 7.2 Construction of MOMP expression vector, H11, using the polymerase chain reaction (PCR).

The polymerase chain reaction (PCR) method of Saiki *et al* (1985) was tried next. Oligonucleotide 288C was used together with another oligonucleotide 612C to amplify the coding sequence plus the 3′ terminator from the replicative form of the original MOMP clone, A1/4. The location of primer 612C was selected such that it would prime into the MOMP gene terminator towards the coding region (Figure 6.1). The primer was designed to end at a well-characterised restriction site, *Hin*dIII, to ensure that it would act as a substrate for *Taq* polymerase, the thermostable DNA polymerase from the thermophile, *Thermus aquaticus*. To force cloning in the right orientation, a *Pst*I site was also designed into the primer 612C. The strategy as summarised in Figure 7.2 and described in the Results section, was carried out according to standard procedures as detailed in Maniatis *et al* (1982) and in Chapter 2. This strategy was successfully used to make an expression construct carrying the sequence information for directing the synthesis of the mature MOMP.

Once the desired MOMP sequence was stably cloned, the insert could be conveniently manipulated into other specialised expression vectors to optimise expression. Restriction sites of the multiple cloning site (MCS) of M13 mptac18 upstream from the 5′ end of the cloned MOMP gene facilitated this manipulation. Plasmid vector pUC8 (Yanisch-Perron *et al*, 1985) was used to express the complete processed MOMP and various truncated MOMP constructs. The N-terminus of these recombinant MOMPs (rMOMPs) were attached to several amino acid residues of the β-galactosidase N-terminus. Protein A-fusion vectors, pRIT5 and pRIT2T (purchased from Pharmacia) were also used to express these MOMP inserts. Details of the procedure will be described in the Results section (Figure 7.4 and 7.5).

The fusion vector pRIT5 (6.9kb) contains nucleotides 1 to 1103 of the staphylococcal Protein A gene which includes the Protein A promoter and terminator, the signal sequence and the coding sequence for a truncated form of the staphylococcal Protein A bearing the IgG-binding domains (Uhlen *et al*, 1984a;

Uhlen *et al*, 1984b; Nilsson *et al*, 1985). It is derived from the shuttle vector pSPA16 (Uhlen *et al*, 1984a), pEMBL9 (Dente *et al*, 1983) and the staphylococcal plasmid pC194 (Iordanescu, 1975). The *E.coli* and *Staphylococcus aureus* origins of replication together with the β-lactamase (ampicillin resistance) and chloramphenicol acetyltransferase (chloramphenicol resistance) genes are included for optimal replication and for selection in both *E.coli* and *S.aureus*. Five unique restriction sites, also present in pUC8, are located within the (MCS), downstream from the Protein A sequence: *Eco*RI, *Sma*I, *Bam*HI, *Sal*I and *Pst*I. Recombinant fusion polypeptides expressed from the Protein A promoter are directed to the periplasmic space in Gram-negative hosts or exported into the medium in Gram-positive hosts.

pRIT2T (4.25 kb) contains the thermoinducible λ P_R promoter and the first 12 codons of a λ *cro/lacZ* gene fused to a truncated form of the staphylococcal Protein A gene (nucleotides 385-1103) which codes for the IgG binding domains. Both the Protein A promoter and signal sequence have been removed. The 3' terminal region of the Protein A gene, nucleotides 1550-1920 and approximately 300 additional nucleotides from the non-coding region, are fused downstream of the MCS and provide transcription termination signals. The translation termination signals may be provided by the cloned DNA fragment but are also present in two reading frames immediately downstream of the MCS. The pBR322 origin of replication and the β-lactamase gene are present for replication and selection in *E.coli*. Strain N99cI$^+$ is used for the initial isolation of recombinant clones. It contains the wild type λ repressor which allows growth at 37°C. Suitable host *E.coli* strains for controlled expression include strain N4830-1 which has a temperature-sensitive λ cI857 repressor. When the temperature is shifted from 30°C to 42°C, the repressor is inactivated and the λ P_R promoter is derepressed, thus initiating expression of the fusion protein. Since no signal sequence is present, the fusion protein is not exported and remains in the cytoplasm.

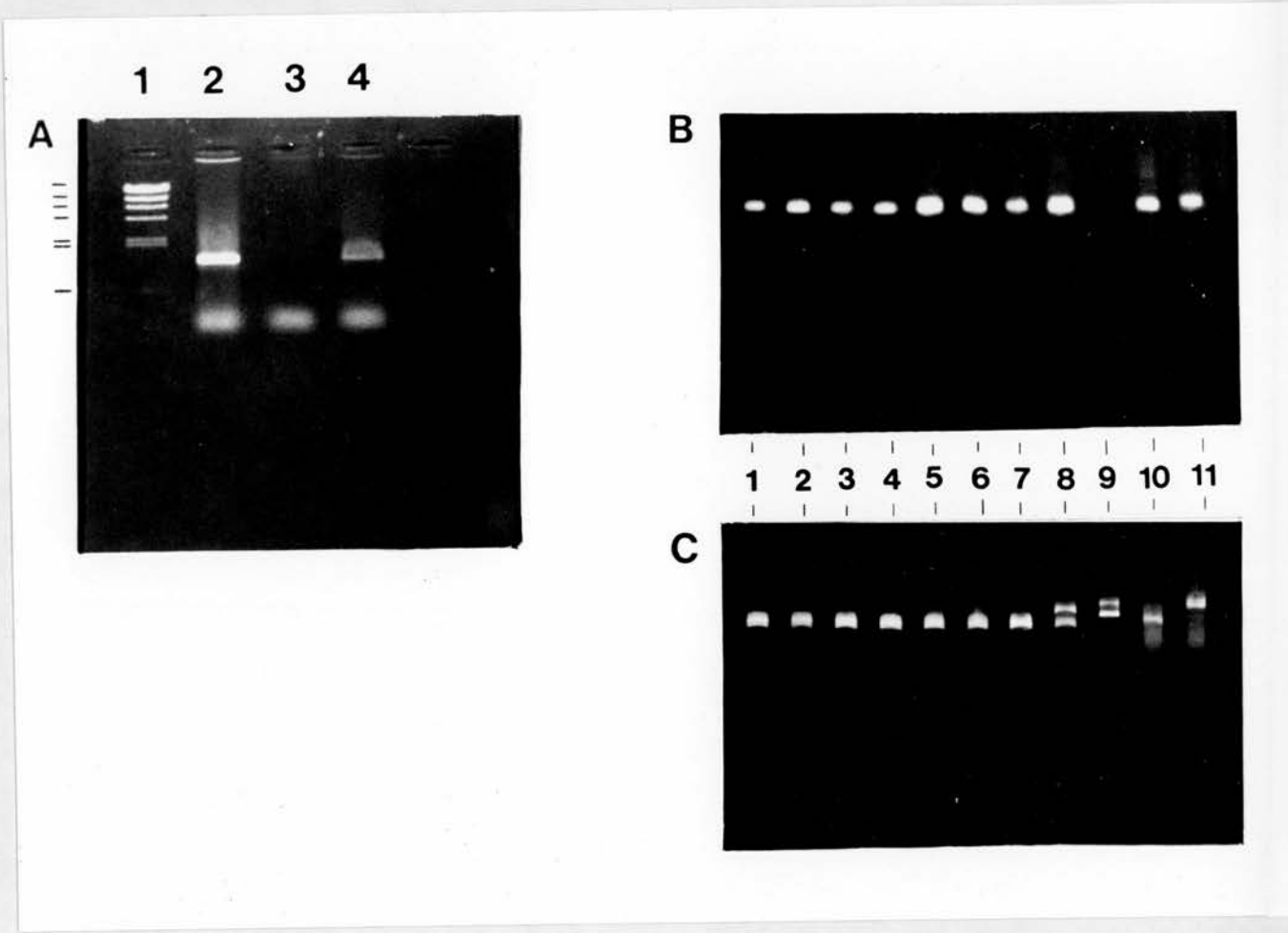


Figure 7.3. Cloning in M13 mptac18: analysis by agarose g electrophoresis. **Panel A.** PCR amplification of A1/4 (lane 2), H (lane 3) and H11 (lane 4). Nucleic acid standards (lane 1) are fron lambda *Hin*dIII digest: 23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kbp (fr the top). **Panel B.** Mobility of ssDNA from selected plaques. M mptac18 (lane 1). Clones H2 to H9 (lanes 2 to 9). H9 did not grow. H and H11 (lanes 10 and 11) possessed slightly lower mobilities. **Panel** Retardation assay of C2/2 ssDNA with H2 to H8, H10 and H11 ssDNA (lar 1 to 9). Retardation assay of A7/9 ssDNA with H11 ssDNA (lane 10) a with C2/2 ssDNA (lane 11).

Successfully isolated recombinants were cultured in larger quantities for SDS-PAGE and immunoblotting analyses of total cell lysate. Recombinants producing detectable amounts of fusion MOMPs were further characterised for optimal conditions of expression and the cellular location of accumulated rMOMPs. Procedures were developed to isolate rMOMPs in quantities sufficient for immunisation experiments in the future.

RESULTS

Cloning in M13 mptac18

The M13 recombinant carrying the *Sst*I MOMP insert, A1/4, was used as a template for the polymerase chain reaction (PCR). Typically, 10ng of the ssDNA vector was sufficient to generate 1000 ng or more of the insert material (1.3 kb) after 30 cycles of amplification using 50 pmol of each primer (Figure 7.3A - lane 2). To remove any potential 'ghost' bands (none detected by agarose gel electrophoresis (AGE) and ethidium bromide staining) and excess primers, preparative AGE was carried out and the 1.3 kb band excised. The insert was isolated by GENECLEAN (Bio101, USA), phosphorylated with T4 polynucleotide kinase and cleaved with *Pst*I to generate the downstream sticky end. The elaborated insert was ligated into M13 mptac18 vector cut with *Sma*I and *Pst*I. This construct was transformed into competent *E.coli* JM101 cells and plated out in top agar containing IPTG and Xgal.

Initial attempts to obtain an M13 mptac18 recombinant using IPTG and Xgal in the plating agar were not successful. Transfection efficiency of *E.coli* JM101 cells was low. Subsequently, IPTG and Xgal were omitted from the plating agar. Selected plaques were picked and cultured for the preparation of infectious phage, ssDNA and dsDNA. Single stranded DNA was analysed by AGE and gel retardation assay for the MOMP gene insert (Figure 7.3 panels B and C). Clone H11 was identified as a recombinant with a ssDNA electrophoretic mobility in agarose gel between that of A1/4 and the parent vector. In a gel retardation assay, H11 was retarded by C2/2 and not by A7/9 (similar to A1/4 but lacking the *Eco*RI-*Sst*I fragment at the 3' end of the *Sst*I MOMP insert - see Figure 6.1) indicating that it

was inserted in the correct orientation (Figure 7.3C lanes 9 and 10). Confirmation of the presence of the required insert was carried out by using primers 288C and 612C in the PCR amplification of H11 in comparison with A1/4 and a negative (H10) control (Figure 7.3A lanes 3 and 4). DNA sequencing using the 645C and 845C primers confirmed the orientation and showed that the ligation of the insert into the vector sequence carrying the promoter signal was in frame:

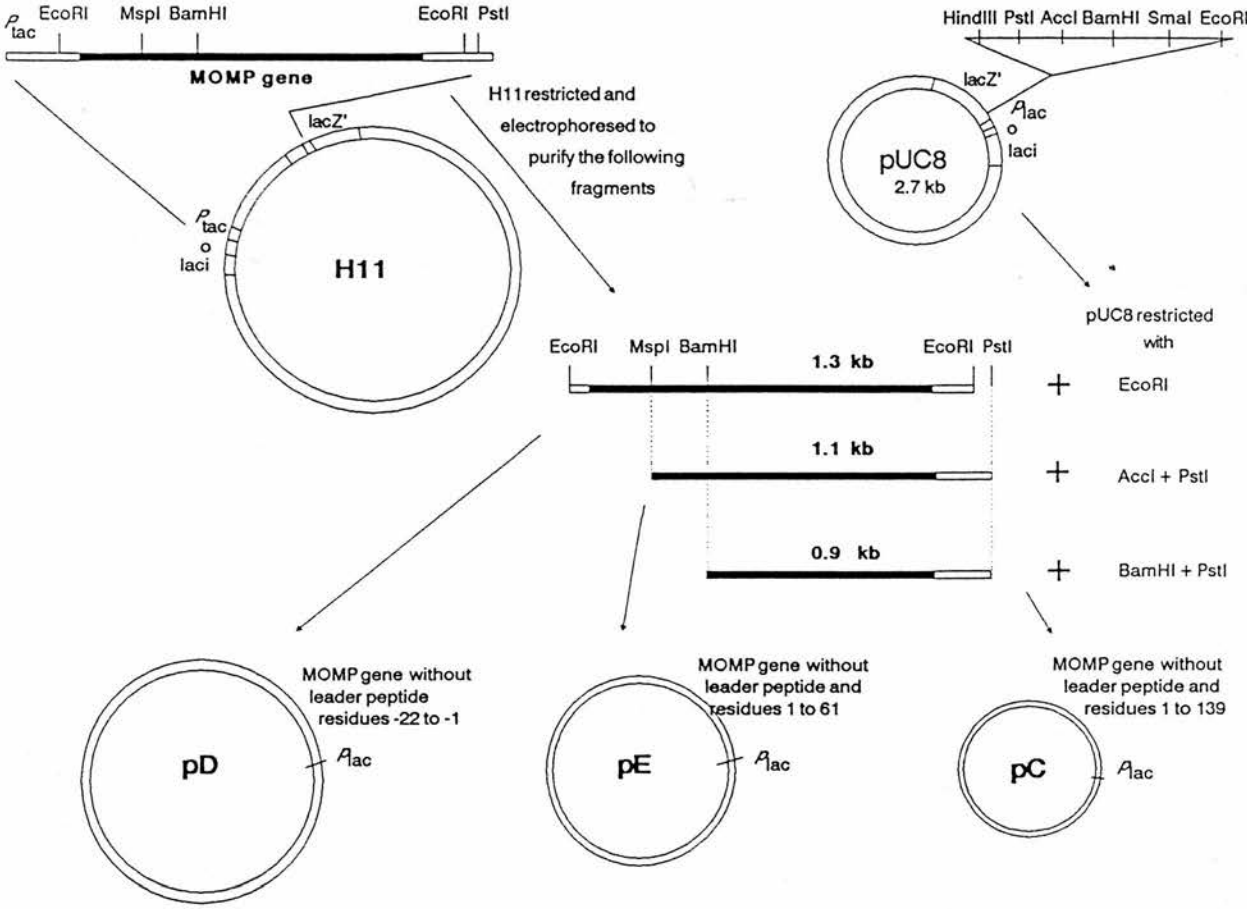
```
                        <----Vector|Insert---->
      EcoRI     SacI        (SmaI)Primer 288C
5'  ------- --------KpnI ---                              3'
... ATG AAT TCG AGC TCG GTA CCC TTG CCT GTA GGG AAC CCA ...
```

Induction of the M13 mptac18 recombinant, H11

Infectious phage H11 at titres of $5x10^{11}$ to $5x10^{12}$ plaque forming units per ml were obtained from culture supernatants. This was used to infect TG-1 cells at a multiplicity of infection of 100:1, 50:1, 10:1 and 1:1. Infected cells were grown to logarithmic phase and then incubated overnight with and without IPTG (final concentration 0.5 mM). Cells were harvested by low speed centrifugation (6500g 5 minutes) and resuspended in sample buffer for SDS-PAGE and immunoblot analysis. Initial results did not show expression of MOMP gene product in detectable quantities (data not shown). Consequently, the MOMP gene was subcloned into plasmid expression vectors.

Cloning in plasmid expression vectors: pUC8, pRIT5 and pRIT2T

Constructs according to the strategy outlined in Figures 7.4 and 7.5 were made. Briefly, pUC8 was linearised with *Eco*RI and treated with calf intestinal phosphatase to inhibit recircularisation. H11 was cut with *Eco*RI and the 1.3kb insert was isolated by preparative agarose gel electrophoresis and purified by the GENECLEAN reagent. Treated vector and insert were mixed in 3:1 molar ratio and ligated to give plasmid pD that should code for the whole mature MOMP attached to several



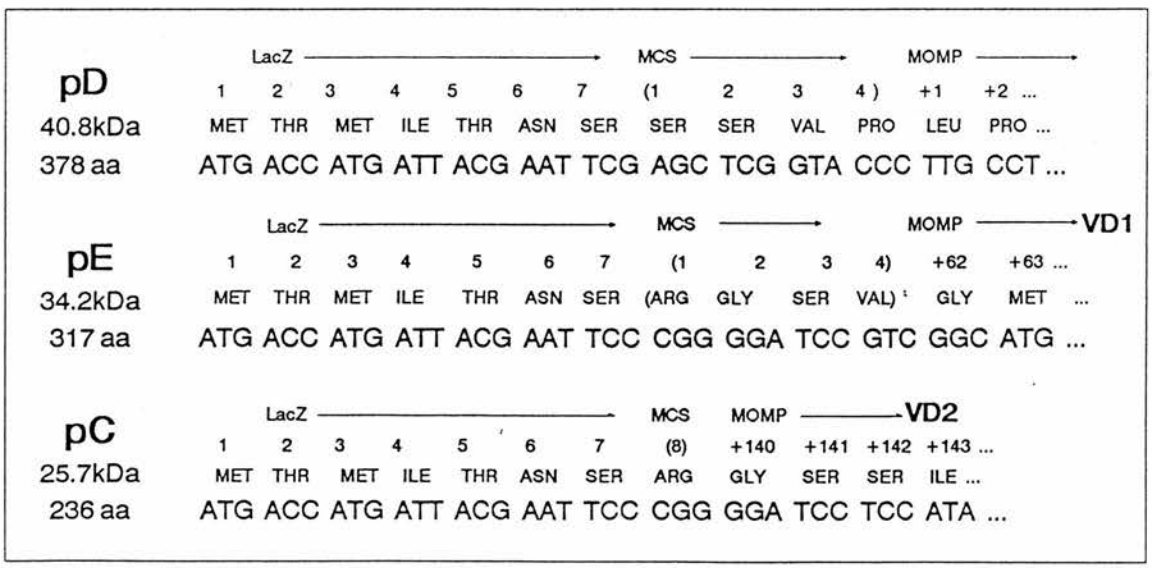


Figure 7.4 Construction of pUC8 recombinants, pD, pE and pC

residues of β-galactosidase and of the MCS (Figure 7.4). Similarly, *Eco*RI-restricted and phosphatased pRIT5 and pRIT2T vectors were ligated with the 1.3kb insert to give plasmids pB and pG respectively (Figure 7.5). Recombinant plasmid pC was produced by cloning the 0.9kb *Bam*HI-*Pst*I fragment from H11 into pUC8 vector cut with *Bam*HI and *Pst*I. This *Bam*HI-*Pst*I fragment was also ligated into the compatible sites of pRIT5 and pRIT2T to produce pA and pF respectively.

The construction of pE involved ligating the *Msp*I-*Pst*I fragment from H11 into pUC8 vector cleaved with *Acc*I and *Pst*I (Figure 7.3). Restriction endonucleases *Acc*I and *Msp*I produce sticky ends which are compatible. pRIT5 and pRIT2T do not possess *Msp*I compatible sites but carry a blunt end *Sma*I restriction site. pE also carries a *Sma*I site a few bases upstream of the *Msp*I/*Acc*I insertion site. Consequently, pE and the pRIT vectors were restricted with *Sma*I and *Pst*I and the relevant fragments isolated by preparative agarose gel electrophoresis and recovered with GENECLEAN. Vector and insert were ligated and produced hybrid sequences of protein A and MOMP genes in the correct reading frame within plasmids pH and pI respectively (Figure 7.4).

The DNA ligations were transformed into competent *E.coli* strains using standard procedures (Chapter 2). Recombinants based on pUC8 were selected by the loss of β-galactosidase activity on IPTG-Xgal agar plates; those based on the pRIT vectors were selected for antibiotic resistance on ampicillin agar plates. The recombinant plasmids were isolated by the modified alkaline-SDS method (Chapter 2) and characterised by restriction endonuclease analyses to determine the presence and orientation of the MOMP inserts. Putative positive clones were further characterised by the detection of rMOMPs using SDS-PAGE and immunoblotting. The results are summarised in Table 7.1 and in the next two sections. Recombinant clones, pD5 and pC2, were found to produce rMOMPs. Further analyses concentrated on these two clones to optimise methods for purification.

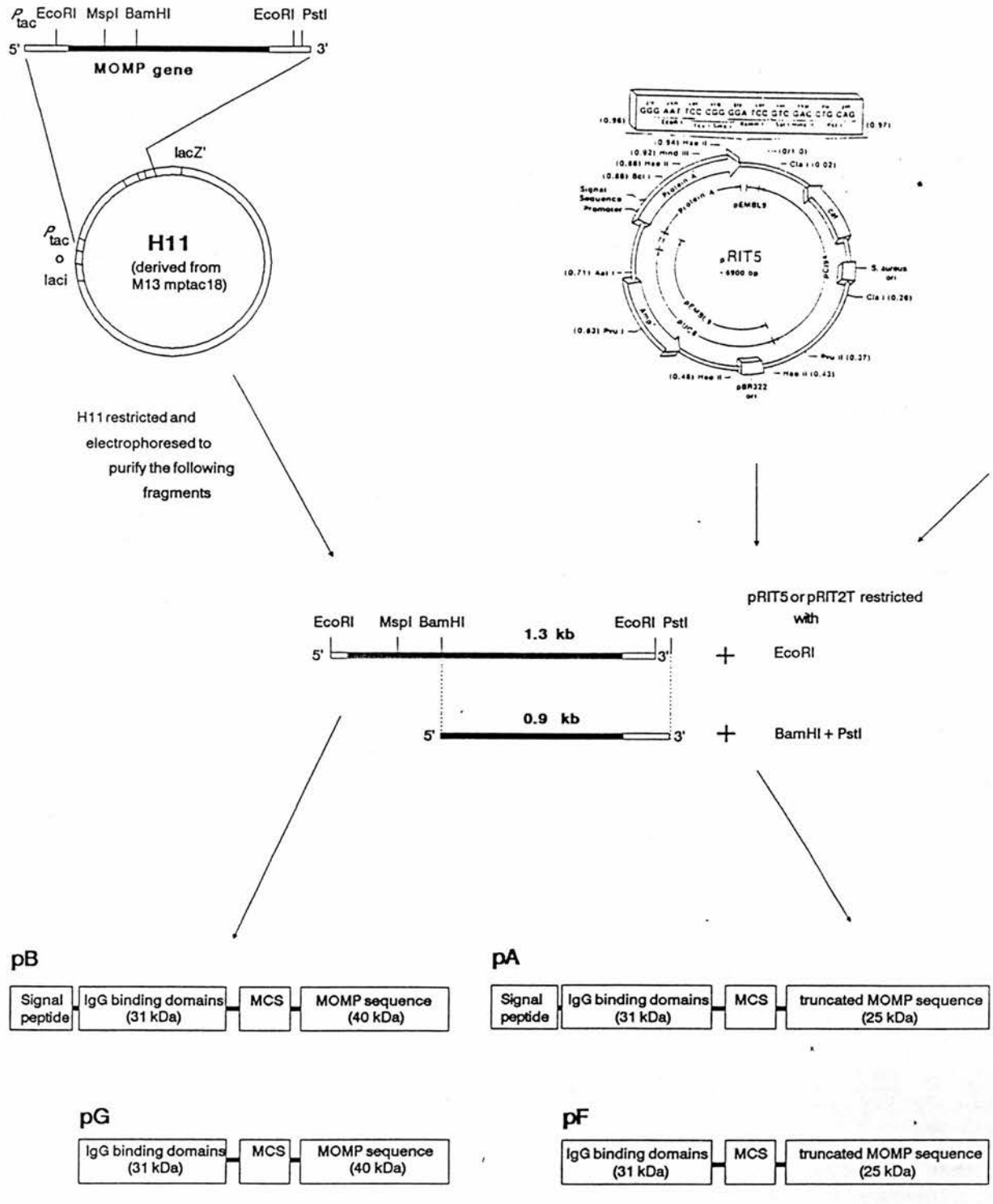
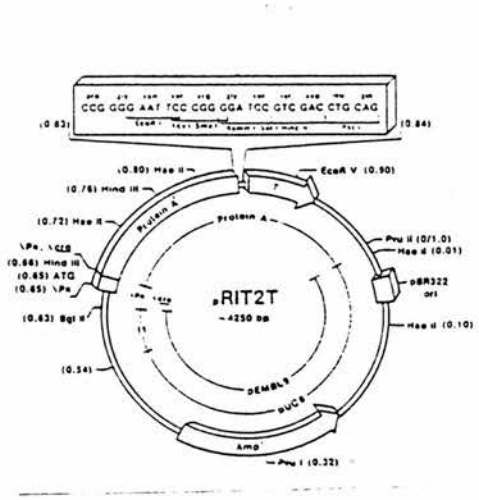
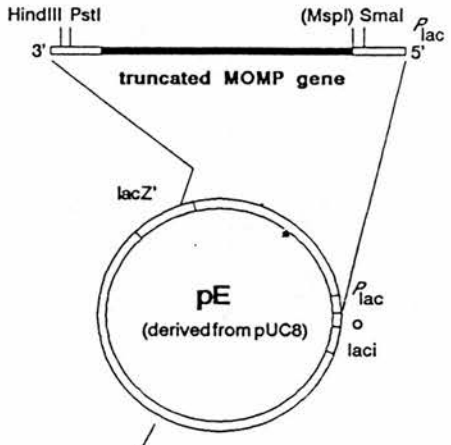


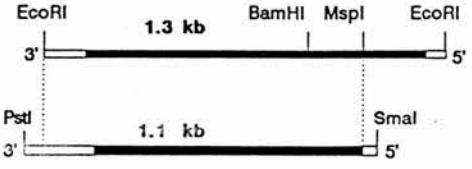
Figure 7.5 Construction of pRIT5 recombinants, pA, pB and pH and pRIT2T recombinants, pF, pG and pI.



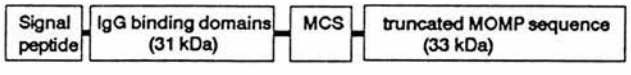


pE restricted and electrophoresed to purify the SmaI-PstI fragment

pRIT5 or pRIT2T restricted with SmaI + PstI



pH



pI

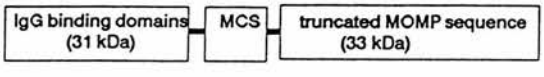


Table 7.1 Summary of recombinant MOMP constructs.

Vector	Recom-binants	Insert fragment	size	origin	Cons-truct	Host cell	Isolation	RE Analysis	SDS-PAGE Analysis~		Cell location	ImmunoBlotting	Purification	Remarks
pUC8	pD	EcoRI-EcoRI	1.3 kb	H11	+	JM101	+	+	40.3 kDa	(40.8)	intracellular	+	70-90%	bipolar inclusion bodies; immunogenicity tests in progress.
pUC8	pE	MspI-PstI	1.1 kb	H11	+	JM101	+	+	31.3 kDa	(34.2)	(intracellular)	no reaction	n.d.	
pUC8	pC	BamHI-PstI	0.9 kb	H11	+	JM101	+	+	26.7 kDa	(25.7)	intracellular	+	70-90%	bipolar inclusion bodies; immunogenicity tests in progress.
pRIT5	pB	EcoRI-EcoRI	1.3 kb	H11	+	HB101	+	+ *	n.v.	(71)	periplasmic	+ ***	n.d.	
pRIT5	pH	SmaI-PstI	1.1 kb	pE	+	n.d.	n.d.	n.d.	n.d.	(64)	(periplasmic)	+	n.d.	
pRIT5	pA	BamHI-PstI	0.9 kb	H11	+	HB101	+	+	n.v.	(56)	periplasmic	+****	n.d.	
pRIT2T	pG	EcoRI-EcoRI	1.3 kb	H11	+	N99cI+	+	+**	n.d.	(71)	(intracellular)	n.d.	n.d.	
pRIT2T	pI	SmaI-PstI	1.1 kb	pE	+	n.d.	n.d	n.d.	n.d.	(64)	(intracellular)	n.d.	n.d.	
pRIT2T	pF	BamHI-PstI	0.9 kb	H11	+	N99cI+	+	+	n.d.	(56)	(intracellular)	n.d.	n.d.	

n.d. = not done
n.v. = not visible
* wrong orientation or triple insert despite screening 60 colonies.
** wrong orientation.

*** The 33 kDa band was not present in the pRIT5 control.

**** The 53.5, 32.5 and 23 kDa bands not present in pRIT5 control.

~ Note that the calculated Mr for each rMOMP is given within parentheses.

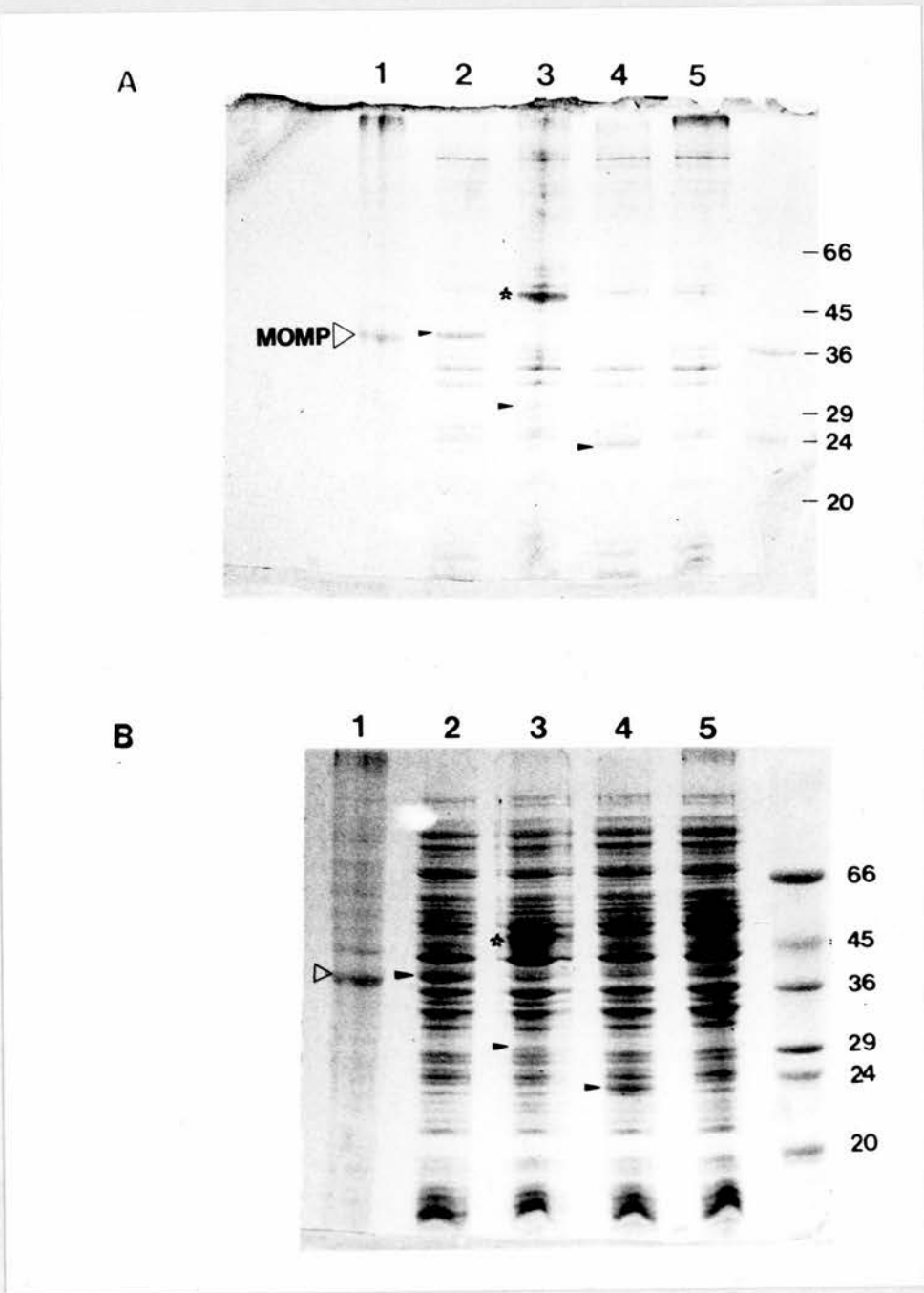
Induction of expression in the pUC8 system

pD, pE, and pC were grown to OD_{600nm} 0.4 in rich medium (L-broth) containing 50 mg/ml ampicillin before induction with 0.1 mM IPTG for 1 hr. Total cell lysates were found to contain extra polypeptide bands at 40kDa, 31kDa and 27kDa respectively (Figure 7.6AB - solid arrowhead). In addition, the profile of pE also possessed a 50kDa band (asterisked). Immunoblotting showed that the 40kDa and 27kDa polypeptides were cross-reactive with anti-MOMP polyclonal antibodies (Figure 7.6C). No clear reaction was observed with the 31kDa or the 50kDa polypeptide.

Recombinant *E.coli* carrying plasmids pD and pC were considered the most promising clones and were selected for further characterisation. Phase contrast light microscopy of both recombinants showed the presence of inclusion bodies as large refractile ovoid aggregates located at the poles of *E.coli* cells. Occasionally, these were found in the central region of the rod, resulting in a bent-shaped rod. In mature cultures, recombinant bacteria were observed to be filamentous and more pleiomorphic, possessing large inclusion bodies at irregular intervals along the length of each filament.

An initial experiment was performed to determine the kinetics of expression. Results showed that rMOMP was expressed in pD without induction with IPTG (1mM in L-broth). Yields were not significantly better with IPTG induction. pC also produced some rMOMP without induction but more was produced on induction for 2h or 16h (overnight). These recombinant bacteria containing rMOMP were subsequently used in the next experiment to determine methods for purifying the rMOMPs (Figure 7.7).

Sonication for 5 minutes in a bath sonicator was sufficient for lysis and release of rMOMP inclusions (Figure 7.7A). However, many *E.coli* components co-sedimented with the rMOMPs at high speed centrifugation (13,000g for 10 minutes). Low speed centrifugation (6500g 3 to 10 minutes) was also able to sediment all rMOMPs from the supernatant containing soluble


A
1
2
3
4
5
MOMP
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45
36
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B
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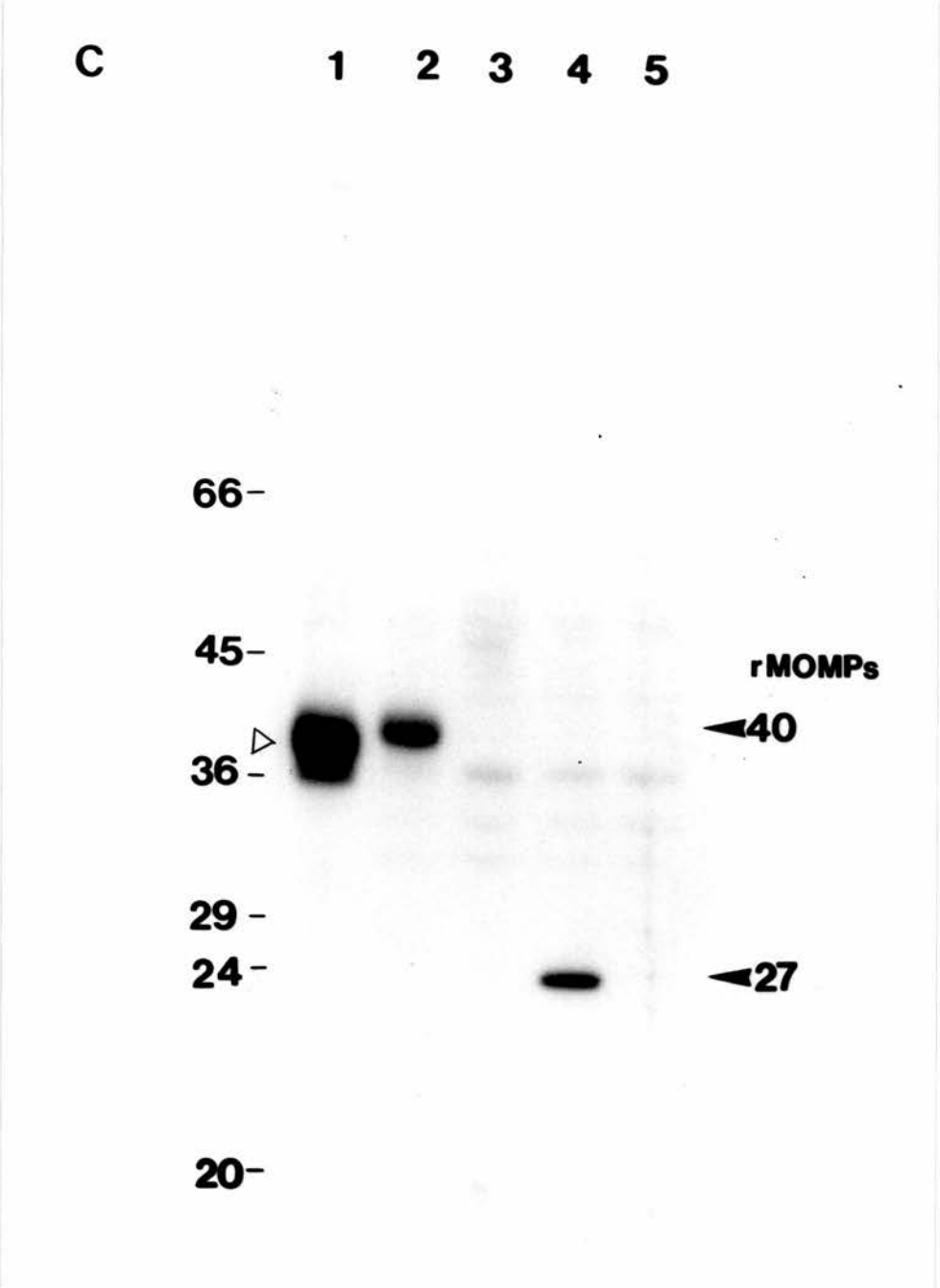


Figure 7.6 SDS-PAGE and immunoblot profiles of pUC8 recombinants. Partially blotted (**Panel A**) and unblotted (**Panel B**) gel stained with Coomassie Blue; autoradiogram of immunoblotted gel replica (Panel C). Total cell lysates of chlamydial EBs (lane 1), and recombinant bacteria carrying plasmids pD (lane 2), pE (lane 3), pC (lane 4) and pUC8 (lane 5) induced with IPTG. The M_r standards are marked in kDa. (Trypsinogen, 24kDa, was not used in the M_r estimations of rMOMPs because it was an outlier in the calibration curve. Subsequent gels using other standards confirmed the M_r of pC rMOMP as 26 to 27kDa.) A polyclonal serum with predominantly anti-MOMP specificity (lane 1) was used for the immunoblot analysis.

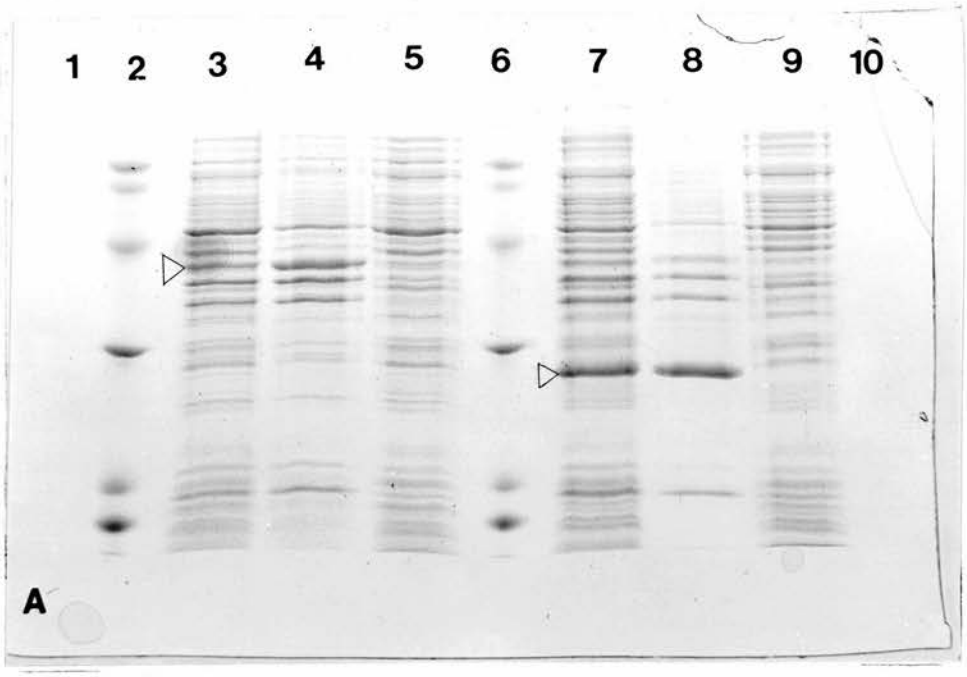
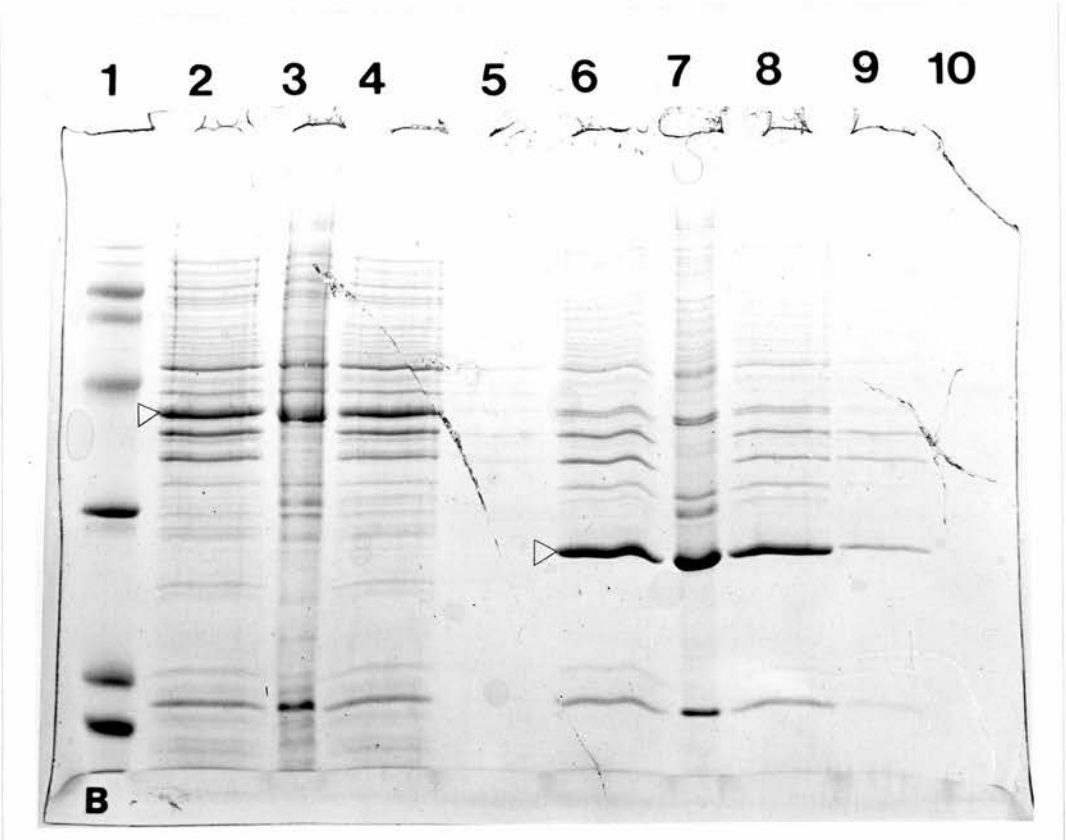
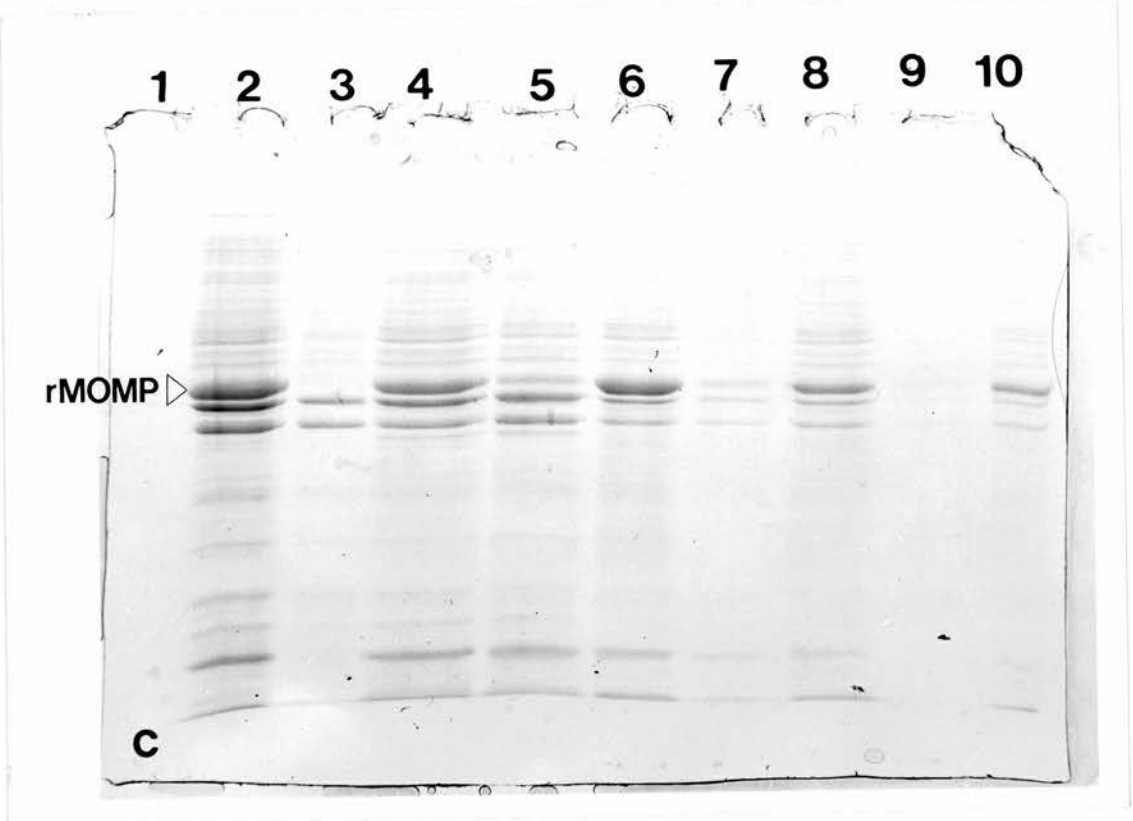


Figure 7.7. Purification of rMOMP inclusion bodies from recombinant *E.coli* carrying plasmids pD and pC: SDS-PAGE analyses of various treatments. **Panel A.** Total cell lysates from clones pD and pC (lanes 3 and 7 respectively). These recombinant bacteria were sonicated (5 minutes in bath sonicator) and centrifuged (13,000g at 20°C for 10 minutes) to produce a pellet (lanes 4 and 8 respectively) and a supernatant (lanes 5 and 9 respectively). M_r standards (lanes 2 and 6) are 116, 93, 77, 67, 45, 30, 17 and 12kDa from the top.



Panel B. The respective post-sonication pellets (Panel A lanes 4 and 8) were resuspended and sampled for SDS-PAGE (lanes 2 and 6). In lanes 3 and 7, corresponding samples were not treated with 2-ME or boiled. The resuspendates were centrifuged (6,500g at 20°C for 8 minutes) to yield pellets (lanes 4 and 8) and supernatants (lanes 5 and 9). M_r standards in lane 1 are the same as that in Panel A.



Panel C. The post-sonication pellet (lane 2) as described for Panels
and B was resuspended in PBE and centrifuged (6,500g for 3 minutes)
produce PBE I supernatant (lane 3) and pellet (lane 4). This wash
pellet was resuspended in PBE-1% Sarkosyl (PBES) and re-centrifuged
yield the PBES supernatant (lane 5) and pellet (lane 6). This pell
was then resuspended in PBE-1M urea (PBEU) and similarly centrifuged
give the PBEU supernatant (lane 7) and pellet (lane 8). Finally t
PBEU pellet was resuspended in PBE and re-centrifuged to give the
II supernatant (lane 9) and pellet (lane 10).

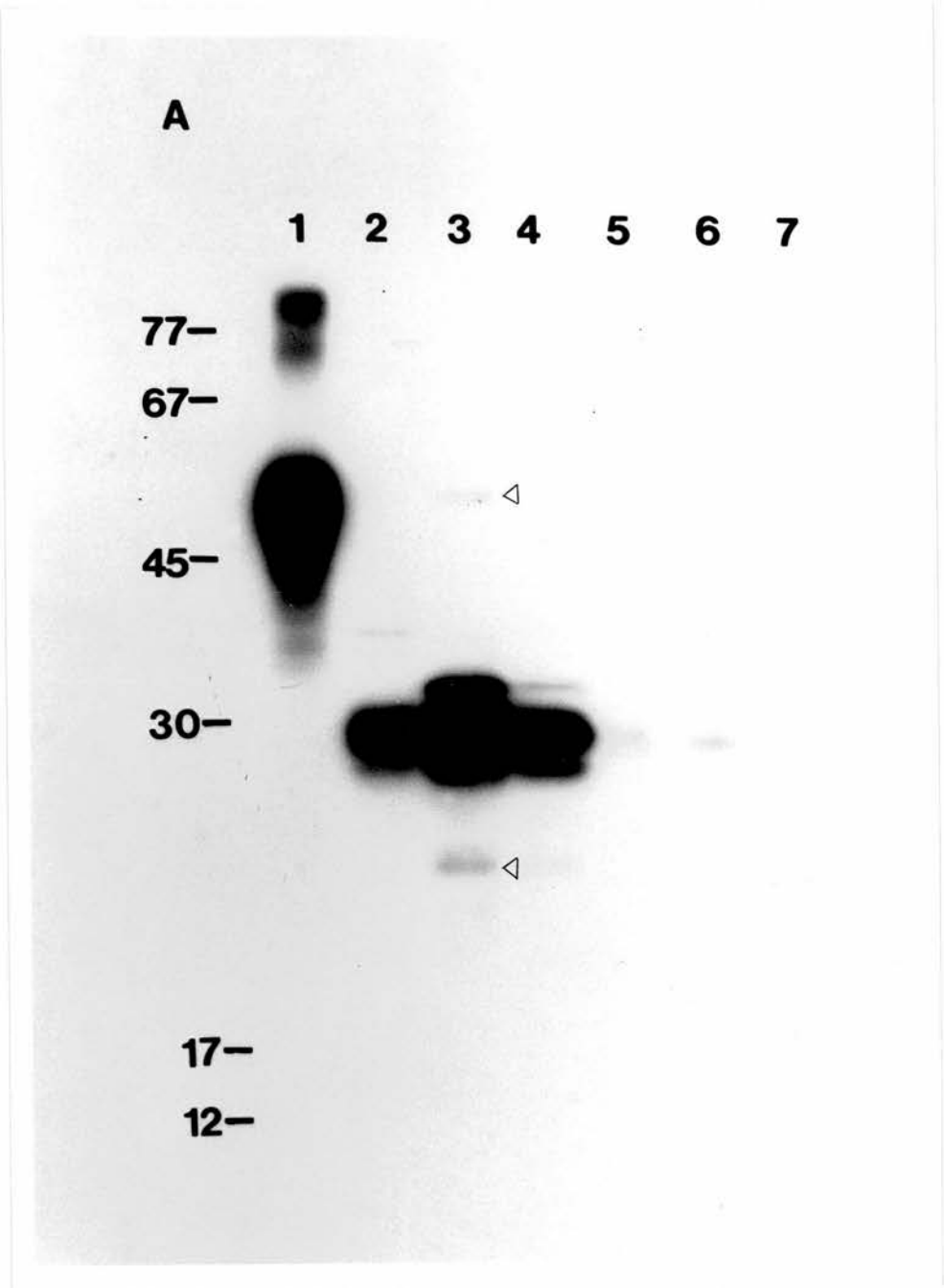
intracellular proteins (Figure 7.7B). Other contaminants, presumably outer membrane components of *E.coli* and other cell debris, which co-sedimented with the rMOMP inclusion bodies could be washed off by resuspension in 10mM sodium phosphate buffer pH7.4 containing 1 mM EDTA (PBE) followed by re-centrifugation. Sequential washes with PBE, with PBE containing 1% Sarkosyl and with PBE containing 1M urea were able to reduce the level of background contamination (Figure 7.7C).

Alternatively, recombinant bacteria were treated with lysozyme (1 mg/ml) in a Tris buffer (50mM pH 8.0) containing 100mM NaCl and 1mM EDTA for 20 minutes at room temperature. Spheroplasts thus formed were collected by low speed centrifugation. Brief sonication (3x30seconds) of lysozyme-treated bacteria was sufficient to release inclusion bodies but this did not improve the quality of crude rMOMP precipitate and introduced lysozyme as an additional protein contaminant (data not shown).

It was also noted that the SDS-PAGE profiles of crude rMOMP inclusion bodies resuspended in sample buffer with the omission of reducing agent, 2-mercaptoethanol, and the boiling step (Figure 7.7B lanes 3 and 7), were slightly different from that of reduced and boiled material (Figure 7.7B lanes 2 and 6). Several bands present in the profile of the reduced and boiled sample were absent in that of unreduced and unboiled sample. Instead, bands of significantly lower mobility appeared in the latter. Unreduced and unboiled rMOMP, however, only migrated slightly ahead of the reduced and boiled rMOMP.

Induction of expression in the pRIT5 system

Selected clones carrying the desired recombinant plasmid grew to early stationary phase (OD_{600nm} about 1) in rich medium (L-broth) containing 50μg/ml ampicillin in 4-5 h. Cells were harvested and periplasmic proteins obtained by osmotic shock treatment (see Chapter 2; manufacturer's instructions). The periplasmic lysate was analysed by SDS-PAGE and immunoblotting


A
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77–
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45–
30–
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12–

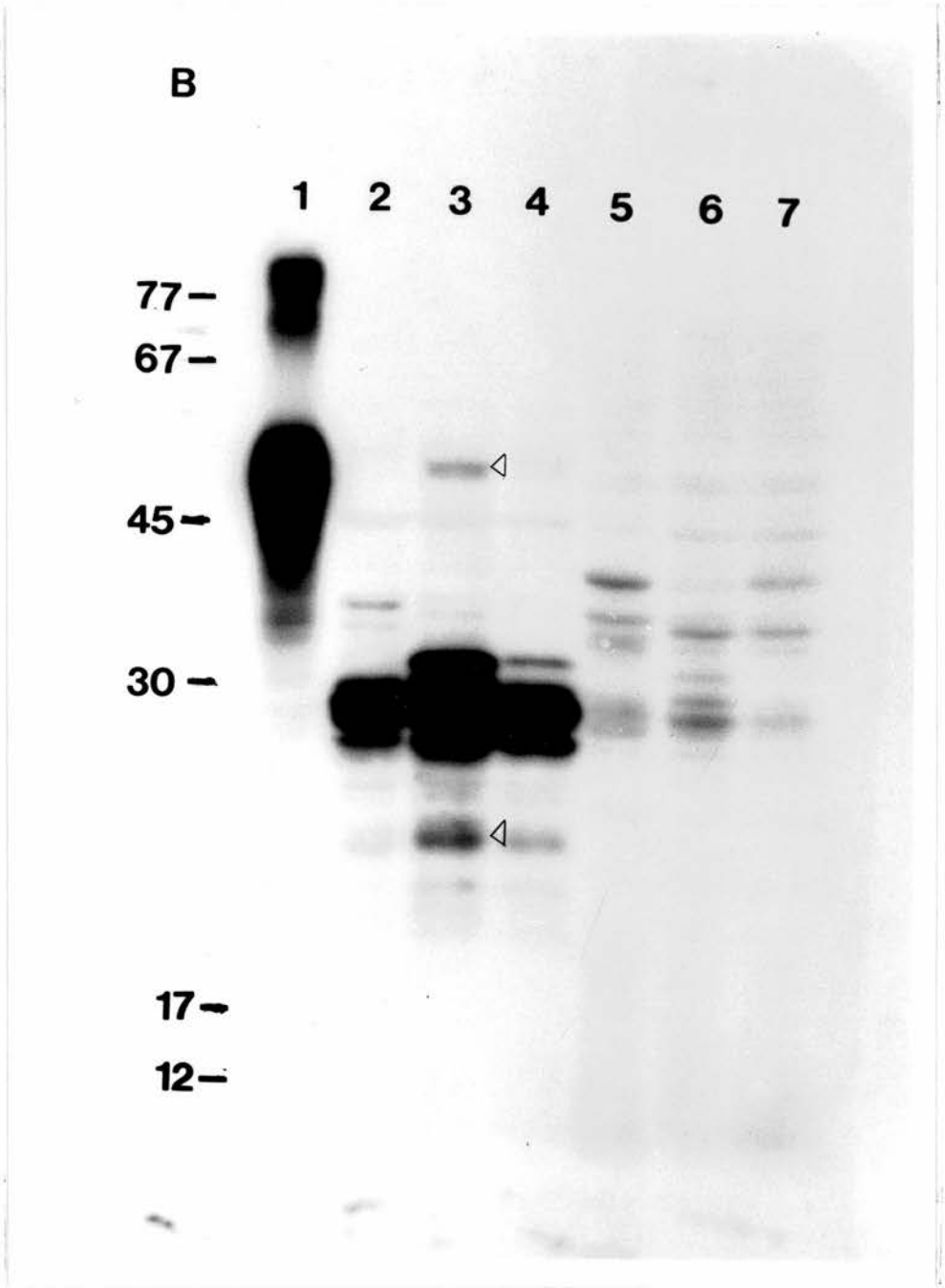

Figure 7.8. Immunoblot analyses of Protein A fusion products synthesised by recombinant clones pA and pB. **Panel A** is an autoradiogram of a blot probed only with radio-iodinated rabbit anti-sheep $F(ab)_2$ antibodies. **Panel B** is an autoradiogram of the same blot probed with polyclonal serum with strong anti-MOMP specificity followed by radio-iodinated anti-sheep $F(ab)_2$ antibodies. Key: Native Protein A (lane 1), periplasmic fractions of pRIT5 (lane 2), pA4 (lane 3) and pB15 (lane 4), remaining cell pellet of the corresponding clones (lanes 5 to 7 respectively). The M_r standards are marked in kDa.

(Figure 7.8). The SDS-PAGE profiles were very complex and recombinant polypeptides could not be distinguished from host cell components when compared with the control cells carrying the parent plasmid, pRIT5 (data not shown). The recombinant polypeptides were identified by immunoblotting using radio-iodinated rabbit immunoglobulins.

Native Protein A (Sigma) from *S.aureus* strain Cowan I migrated as several bands with the predominant band having an apparent M_r of about 52 kDa (Figure 7.8 lanes 1). Truncated Protein A expressed by the parent plasmid pRIT5 was associated with the periplasmic fraction and produced a dominant IgG-reactive band at approximately 29 kDa flanked by two minor bands at 30 kDa and 27.5 kDa. Bands similar in mobility to the triplet were also found in the periplasmic profiles of both pA and pB. In addition, the profile of pA included a 32kDa band and two other fainter bands at 53.5 kDa and 23 kDa (Figure 7.8 triangles). The two faint bands became slightly darker when the blots were re-probed with anti-MOMP antibodies (Figure 7.8B - triangles). Additional bands in the profile of pB, however, were all weak and some also became darker upon re-probing with anti-MOMP antibodies (33kDa and 23kDa) . Faint reactive bands, particularly those in the cell pellets, may represent non-specific reactions as a result of prolonged exposure to sheep anti-MOMP antibodies (lanes 5-7).

DISCUSSION

This chapter describes several approaches used to express the MOMP gene from *C.psittaci* OEA strain S26/3. One viral expression vector, M13 mptac18, and three different plasmid vectors, pUC8, pRIT5 and pRIT2T, were used. Of the ten different expression constructs made, several which expressed rMOMPs were chosen for further characterisation. One construct (plasmid pD) codes for the complete mature MOMP fused at the N-terminus to a short sequence (11 amino acids) of β-galactosidase and the multiple cloning site. It is the second report, after Allan *et al* (1984), of the expression of a complete mature chlamydial MOMP, and the first for *C.psittaci*.

A truncated form of MOMP from plasmid pC was also successfully produced. Amino acid residues 1 to 139 containing CD1, VD1, CD2 and a few residues of VD2 were deleted from this recombinant polypeptide. These rMOMPs should be useful for future investigations into the immunoprotective role of MOMP in ovine enzootic abortion.

Cloning in expression vector M13 mptac18

The first attempt at expression in *E.coli* using the M13 mptac18 construct, H11, was not successful. Despite attempts to vary several parameters including the multiplicity of infection, the duration of induction and the concentration of the inducer, no expression was detected either by SDS-PAGE or immunoblotting (data not shown). Nevertheless this attempt resulted in the cloning of the MOMP gene insert into a multiple cloning site with useful restriction sites flanking the desired sequence. This sequence was thus easily manipulated into the three plasmid expression vectors.

Expression of rMOMP using the pUC8 system

The characteristics of rMOMPs derived from the pUC8 system for expressing "fusion" recombinants with a short segment of β-galactosidase sequence are given in Figure 7.4 and Table 7.1. Both pD and pC expressed rMOMPs with M_r estimated in SDS-PAGE gels, 40.3 and 26.7 kDa, which agreed very well with the predicted values, 40.8 and 25.7 kDa, respectively. Both rMOMPs cross-reacted in the immunoblot test with anti-MOMP polyclonal antibodies showing that at least some antigenic determinants have been reproduced. These determinants detected by immunoblotting are probably linear, denaturation-resistant and/or "renaturable" epitopes.

Owing to the lack of anti-MOMP monoclonal antibodies that are directed to conformational epitopes, it was not possible to determine if the rMOMPs coded for by pD and pC had refolded into similar tertiary structures as the native MOMP. Initial characterisation of the rMOMPs showed that in an unreduced

state, the rMOMPs migrated in SDS-PAGE gels in a monomeric form. This indicated that most recombinant polypeptides were not cross-linked with each other, unlike MOMPs in the outer membrane of EBs (Bavoil *et al*, 1984; Hatch *et al*, 1984). The slightly higher mobility of unreduced rMOMPs compared to reduced rMOMPs is indicative of a tighter conformation, suggesting the possibility that intra-chain disulphide bonds may have formed despite the lack of inter-chain bonding. Whether these disulphide linkages are randomly paired or similar to that in the native MOMP is not known. It may be possible, without elucidating the disulphide linkage pattern of either the native or recombinant MOMP, to obtain an idea of how similar these linkages are by performing comparative limited proteolysis on the unreduced purified polypeptides, and by analysing the fragments under non-reducing and reducing conditions with SDS-PAGE or liquid chromatography.

Bavoil *et al* (1984) have shown that MOMP from *C.trachomatis* serotype L2 can be purified in a form which allows its putative porin-like activity to be demonstrated *in vitro*. If the proteolytic patterns of rMOMP and native MOMP are similar, it may be possible to reconstitute rMOMPs into artificial membranes, perhaps with the inclusion of chlamydial LPS to mimic the native situation as far as possible. Such a model will be useful for studying the relationship of structural domains of MOMP with functions such as permeability and structural rigidity, the interaction of MOMP with other outer membrane components, as well as its folding pattern in the membrane and possibly its high resolution structure.

With regard to the ultimate objective of determining whether MOMP is an immunoprotective antigen in OEA, the initial results showed that rMOMP polypeptides can be produced for vaccine testing in far larger quantities and with considerable ease compared to isolating MOMP from cell-cultured chlamydiae. For example, typical yields of chlamydial organisms from six 25 ml Falcon flask monolayers range from 0.5 to 2 mg protein. Since each EB contains an estimated 10-20% MOMP by mass, about 200 μg

to 400 μg of MOMP can be purified from six monolayers at maximum efficiency. The procedure typically requires 1 day for seeding the inoculum, 3 days for IDU treatment, 4-7 days for the chlamydial culture to mature, 1 day for harvesting and purification, 1 day for detergent extraction, a total of 10 to 13 days. In contrast, an OD_{600nm} 0.4 culture of recombinant *E.coli* yields up to 4000 μg per 150 ml of rMOMP without optimisation. It takes 1 day for the overnight culture and 1 day for the induction and the isolation of rMOMP inclusion bodies using half the number of centrifugation steps and having no requirement for ultracentrifuges.

Presently, rMOMPs from plasmid pD and pC are being produced in milligram quantities for immunogenicity tests. If antibodies that cross-react with native MOMP can be elicited, these rMOMPs will be tested as potential recombinant subunit vaccines in sheep. Both the complete rMOMP and the truncated rMOMP when tested will also permit an assessment of the relative importance of the N-terminus and C-terminus domains of MOMP in immunoprotection.

It is not understood why the rMOMP of pE failed to react with anti-MOMP antibodies. It is unlikely that an out-of-frame ligation resulting from an illegitimate annealing of the AccI and MspI sticky ends could have produced the observed 31 kDa rMOMP, a size close to the predicted 34 kDa, because stop codons were present near the ligation site in the other two reading frames. The origin of the additional band around 50kDa is not known. Until DNA sequencing of the joint using primer 844C is carried out, it would be premature to speculate about the explanation for these observations. It should be noted that pUC8 carries the β-lactamase gene which codes for pre-β-lactamase of approximately 30 kDa in SDS-PAGE gels.

Expression of rMOMP using the pRIT system

The size of native Protein A has been estimated to be 42 kDa by gel filtration and 56 kDa by disc electrophoresis in polyacrylamide gels containing 0.1% SDS (Bjork *et al*, 1972;

Movitz *et al*, 1979). From the sequence data of Protein A (Uhlen *et al*, 1984b and erratum), the mature protein contained 488 amino acids (and 36 amino acids as the signal sequence) giving a calculated M_r of 53,697 Da. SDS-PAGE estimates by Uhlen *et al* (1984a) were in the range of 52 to 53 kDa. The result of 52 kDa in this study agreed well with the electrophoretic results. It is not known what the higher M_r IgG-reactive bands in the Protein A track in Figure 7.8 might be. Heterogeneity in Protein A has been observed before (Uhlen *et al*, 1984a) but the size variation is less than 10 kDa.

The recombinant *E.coli* HB101 cells carrying pRIT5, pA and pB produced IgG-reactive bands in the periplasmic fraction and not in the spheroplasts showing that the polypeptide chains of the C-terminus truncated Protein A (tProtein A) and its fusion products have been recognised as substrates for translocation by the *E.coli* secretory apparatus. The signal sequence of 36 amino acids (4 kDa), or similar size fragments, are probably cleaved off in the process because the sizes of the tProtein A bands produced by pRIT5 corresponded to the expected M_r range (Table 7.1 and 7.2). Although alternative cleavage sites of the signal sequence may have produced the triplet of tProtein A, Uhlen *et al* (1984a) have suggested proteolytic degradation as a likely explanation. While homogeneous species of full length tProtein A are secreted directly into the medium by Gram-positive staphylococcal strains, sequestration of such polypeptides in the periplasmic space of *E.coli* may have resulted in exposure to the action of periplasmic or membrane-bound proteases. Signal peptides released into the periplasmic space by signal peptidase in the process of translocation are known to be rapidly degraded presumably by enzymes such as Protease IV, a membrane-bound signal peptide peptidase (Novak and Dev, 1988 and references therein). Recombinant polypeptides may be substrates for such proteases.

The expected size of the fusion product from construct pA was calculated to be 55.9 kDa (Table 7.2) which may be represented by a weak band at 53.5 kDa on the blot profile.

Table 7.2 Characteristics of Protein A, truncated recombinant Protein A and its fusion products.

Protein	Origin	Observed Mr (kDa)	Calculated Mr (kDa)	Remarks
Native Protein A	Staphylococcus aureus (Cowan I strain)	42 (gel filtration)* 56 (disc electrophoresis)* 52-54 (SDS-PAGE)*	53.697	36 aa (signal sequence) + 488 aa (mature protein)
truncated Protein A (tProtein A)	pSPA16/ HB101 (Uhlen et al, 1984b)	30-31	30.963	+1 ... +268 +274 ... lys asp asp pro gly asn ser STOP ... ... AAA GAC GAT CCG GGG AAT TCT TGA ... Protein A gene + pUR222 MCS + pBR322
truncated Protein A (tProtein A)	pRIT5/ HB101	27-30	31.921	+1 ... +271 +283 ... pro gly asn ser arg gly ser val asp leu gln pro ser STOP ... ... CCG GGG AAT TCC CGG GGA TCC GTC GAC CTG CAG CCA AGC TAG CTT ... Protein A gene + pUR222 MCS + pEMBL9 MCS
Fusion polypeptide from pB EcoRI-EcoRI insert in pRIT5	pB/ HB101	n.o.	71.0	+1 ... +271 +1 MOMP ... +367 ... pro gly asn ser ser ser val pro leu pro ... ... CCG GGG AAT TCG AGC TCG GTA CCC TTG CCT ... Protein A gene + pUR222 MCS + M13 mptac 18 MCS + MOMP sequence
Fusion polypeptide from pB with insert in wrong orientation	pB15/ HB101	32.5	31.5	+1 ... +271 +279 ... pro gly asn ser tyr ala asn gly phe STOP ... ... CCG GGG AAT TCT TAT GCG AAT GGA TTT TAG AAA ... Protein A gene + pUR222 MCS + 3' end of the MOMP gene
Fusion polypeptide from pA BamHI-PstI insert in pRIT5	pA/HB101	57.5 (32.7+23.4)	55.9	+1 ... +271 +277 +140 MOMP ... pro gly asn ser arg gly ser ser ile ... ... CCG GGG AAT TCC CGG GGA TCC TCC ATA ... Protein A gene + pUR222 MCS + pEMBL9 MCS + truncated MOMP gene

* see text for references.
n.o. not obtained.

other bands at 32.5 and 23 kDa could be degradation products of this putative fusion protein. However, the amount of fusion or degradation products detected by SDS-PAGE or blotting was very low compared to the bands at 30, 29 and 27.5 kDa. These appeared to co-migrate with the tProtein A bands produced by the parent plasmid pRIT5. The possibility that this pA *E.coli* preparation may have been contaminated by clones bearing the parent plasmid was thought to be unlikely because the original clone had been re-streaked several times to ensure that a pure clone was obtained. Further work is necessary to characterise these bands which were also present in the pB recombinant.

It was not known why recombinants isolated from the pB constructs were found to carry only inserts in the wrong orientation when characterised by REA. Several had triple inserts but none had the insert in the right orientation despite screening nearly 60 colonies. These triple inserts were arranged in the following orientation, wrong-right-wrong, and resulted in a stop codon in the tProtein A reading frame as shown in Table 7.2. The expected size of this recombinant polypeptide is 31.5 kDa and may be represented on the blot by the 33 kDa band not present in the pRIT5 lane (Figure 7.8).

The difficulty in characterising these recombinant products was due to the lack of suitable probes which can distinguish the presence of MOMP epitopes on putative fusion or degradation products from the IgG-binding domains of Protein A. Consequently, the construct pF, although successfully isolated, was not re-transformed into *E.coli* N4830-1 cells for the production of fusion polypeptides. Isolation of construct pG was not attempted again after the initial failure to obtain insert in the correct orientation (Table 7.1). Ligated plasmids pH and pI were also not transformed into *E.coli* HB101 and N99cI^{+} cells respectively for the same reason.

Errors in the polymerase chain reaction

The polymerase chain reaction uses the *Taq* polymerase which, unlike *E.coli* DNA polymerase I, does not possess a

"proof-reading" 3'->5' exonuclease activity. Consequently, the fidelity of replication is lower. Typical estimates of the error rate range from 1 nucleotide per 800 bases (Dunning *et al*, 1988) to 1 per 2500 (Larrick *et al*, 1989). It is therefore essential to sequence the coding region of the rMOMP plasmid constructs to determine if the MOMP sequences have been accurately replicated. In addition, the limited proteolytic peptide profiles of the rMOMPs in comparison with that of the native MOMP should provide some indication as to the degree these rMOMPs have been properly transcribed and translated. (As this thesis was being prepared, chymotryptic profiles of pD and pC rMOMPs were obtained, showing an identical set or a subset of bands to that of MOMP respectively.)

Potential application of the strategy to express other MOMPs

The experimental strategy, as exemplified by the construction of H11, demonstrated that MOMP sequence can be amplified very conveniently and very quickly in quantity by PCR for insertion into expression vectors. In that process, the appropriate restriction sites (in the case of H11, the *Pst*I site) can be engineered into the insert to facilitate subsequent manipulation. Useful cloning sites can be tagged on by cloning into the appropriate MCS of the plasmid vector (in the case of pH and pI, relying on the MCS restriction site *Sma*I of pE).

The success of the PCR in amplifying various MOMP sequences for DNA sequencing (as discussed in Chapter 6) and for expression (as described in this chapter) suggests that rMOMP constructs derived from other strains of *C.psittaci* and *C.trachomatis* can be readily made by a similar procedure. The MOMP primers, 288C and 289C, have been used for the amplification of MOMP sequences from nearly all *C.psittaci* strains available in the institute (S.Baxter, personal communication). Primer 612C was also successfully used for *C.psittaci* strains, S26/3, A22 and Cal10.

From the comparison of various MOMP nucleotide sequences displayed in Figure 6.5, it is likely that universal chlamydial MOMP primers can be designed for PCR amplification of the MOMP coding sequence of *C.psittaci* and *C.trachomatis* strains (see Figure 8.1; Dutilh *et al*, 1989; Griffais and Thibon *et al*, 1989). Amplified sequences will contain the variable regions characteristic of each strain which may be differentiated by REA.

Signal sequence and recombinant MOMPs

Measures were taken as part of the overall strategy to avoid the expression of the signal peptide so that the recombinant MOMP product will not be a substrate for the secretory apparatus of *E.coli*. It was thought that this may circumvent any toxic effect related to the possible insertion of rMOMPs into the outer membrane.

For secreted polypeptides in general, the signal sequence binds the signal recognition protein (SRP) resulting in a translational block at the ribosome until the complex binds the 'docking protein' which in turn catalyses the release of the SRP-induced block (Walter *et al*, 1981). Removal of the signal sequence may thus preclude any negative feedback mechanisms on expression caused by saturation of the secretory system of host *E.coli*. It may also result in the accumulation of recombinant polypeptides within cytoplasmic inclusion bodies which may facilitate subsequent purification.

Although the rMOMP constructs of the pUC8-based system do not possess *E.coli* signal sequences, the additional N-terminus residues partially originated from the N-terminus of β-galactosidase and partially corresponded to the MCS (Figure 7.4). These residues, MTMITNSSSVP, MTMITNSRGSV and MTMITNSR (of pD, pE and pC respectively), however, do not possess even the minimal structures for translocation across membranes (Perlman and Halvorson, 1983; von Heijne, 1985) and, therefore, were not expected to bind SRP. Indeed the data for pD and pC confirmed that they were not exported.

Purification of rMOMP inclusion bodies

It is generally thought that insoluble recombinant proteins accumulate in *E.coli* in the discrete form of inclusion bodies (Marston, 1987). Since MOMP occurs in the outer membrane as a structural protein, its solubility was expected to be low. The highly refractile inclusion bodies observed in recombinant bacteria bearing pD or pC, but not in bacteria bearing the parent plasmid, are very similar to those in the phase contrast photomicrograph of Marston (1987). It is likely that these inclusion bodies contained aggregates of rMOMP. The presence of these inclusion bodies appeared to distort the normal rod-shaped morphology of some bacteria. It is possible that the large size of these bodies may also have interfered with the normal processes of cell division, causing the formation of undivided rods with a filamentous appearance.

Preliminary experiments in exploring useful methods of purifying these aggregates indicated that sonication with or without lysozyme pretreatment can release them for sedimentation by low speed centrifugation. Washes in buffer can selectively remove contaminating host material to yield sufficiently pure rMOMP for immunogenicity tests. Further experiments will be required to determine the optimum conditions and procedures for a greater degree of purification. Since both recombinant polypeptide chains appeared not to be covalently cross-linked to each other by disulphide bridges, it may be possible to solubilise these rMOMPs for further purification by liquid chromatography. Purified products may be renatured in the attempt to regain the native configuration of MOMP.

The ease with which large quantities of rMOMPs can be produced suggests that the necessary amounts in a sufficiently purified state can be achieved. This will enable further experiments to determine the role and efficacy of MOMP in protection against ovine enzootic abortion, which is the main objective of this cloning exercise.

CHAPTER 8
GENERAL DISCUSSION

A summary of the findings of this project and the reasoning behind the experiments that were carried out are presented in this chapter. The significance of these findings is also discussed from a broader perspective, in terms of mechanisms of chlamydial pathogenesis, abortion and immunity, of diagnosis and detection and of the molecular aspects of MOMP. Ways of advancing research in the field of ovine enzootic abortion are also suggested where appropriate.

SUMMARY

Identification of chlamydial antigens

With respect to the aim of identifying the antigens of OEA isolates of *C.psittaci* as recognised by the ovine immune system, an extensive list of nearly two dozen antigens out of more than 80 separate bands in SDS-PAGE has been compiled from immunoblotting analyses of sera from sheep of different immune status (Table 3.1). The more commonly visible bands included high M_r antigens from 85kDa and above, a 60kDa antigen (possibly the 60kDa cysteine-rich protein), a 50kDa antigen (detected in OEA strains only), an immunodominant 39-40kDa antigen corresponding to the MOMP in SDS-PAGE gels, and several bands at 30kDa and 18kDa (possibly the adhesins).

A degree of variability in the specificity of the antibody response among sheep of similar immune status was observed, similar to the findings of McClenaghan *et al* (1986). A possible explanation might be the previous exposure of such sheep to other strains of *C.psittaci* such as intestinal strains which do possess cross-reactive epitopes (Figure 4.2B). However, in ewes that had been vaccinated with purified EBs and completely protected against experimentally induced OEA, a strikingly consistent and vigorous response to the putative MOMP was detected. MOMP was thus implicated as an immunoprotective antigen and a candidate for vaccine efficacy tests (Chapter 3).

Biochemical characterisation of MOMP

Although the original aim was to characterise various antigens of *C.psittaci*, subsequent work was thus focussed on MOMP. At the close of this project, data had accumulated which confirmed the identity of this putative immunoprotective antigen as MOMP, such as its predominance in the outer membrane, its susceptibility to proteolytic digestion, its surface exposure, its ability to form disulphide-linked oligomers *in vitro* and finally, its N-terminal amino acid sequence (Chapter 4). These properties were consistent with the MOMP of all known chlamydial strains. In particular, the N-terminus sequence was identical to that of MOMP from *C.trachomatis* serovar L2.

Isolation of MOMP and assessment of vaccine efficacy

Some of these characterisation experiments showed that MOMP could be highly enriched by a two-step detergent extraction procedure modified after Caldwell *et al* (1981), Caldwell and Schachter (1982) and Bavoil *et al* (1984) using anionic detergent, Sarkosyl and reducing agent, DTT. Immunoblotting with convalescent sheep serum showed that this subcellular preparation contained predominantly MOMP as the antigenic molecule although LPS was also present. Electron microscopy revealed that the preparation consisted mainly of membrane fragments of fine knob-like particles arranged in an array which appeared hexagonal at some locations. Such an appearance was also detected on the OM of intact EBs. Other minor but conspicuous structures were rosettes, some of which were attached to membrane fragments (Chapter 4).

Since further attempts to solubilise this subcellular preparation of MOMP with octylglucoside and to purify MOMP using column chromatography resulted in very poor yields, the two-step procedure was adopted for preparing MOMP for a vaccination-challenge experiment in pregnant ewes. A further advantage of this preparation was the ultrastructural evidence that it contained MOMP in a native state. This feature was thought vital at that stage of research because nothing was

known about the nature of the immunoprotective epitopes in OEA. This subcellular vaccine was tested with a purified EB preparation identical to that which had initially led to the identification of MOMP as the potentially immunoprotective antigen. Results showed that both vaccines protected ewes from infection and abortion in a single dose. Vaccinated ewes produced a statistically significant improvement in the lamb survival ratio compared with the control animals which were sham-inoculated with a placebo vaccine containing uninfected culture cells (Chapter 5).

Having demonstrated that the protective components of all previous OEA vaccines resided on the EB outer membrane, it was reasoned that MOMP, being the major component of the preparation, is likely to carry immunoprotective epitopes. Much evidence in the literature, already discussed in the discussion sections of the appropriate chapters, supported this as a reasonable hypothesis worthy of testing. One constraint was the limited quantities of MOMP that could be obtained. A recombinant DNA approach to express MOMP in *E.coli* was adopted because it could facilitate the following. Firstly, if MOMP was indeed immunoprotective, then it would be needed in large quantities for probing the immune mechanisms operating in chlamydial infections. Secondly, further manipulation of the MOMP molecule to isolate and characterise the regions responsible for eliciting such immune mechanisms may be required. Thirdly, studies on the genetic regulation of such a highly synthesised and developmentally critical protein as MOMP will lead to a better understanding of the biology of chlamydiae.

Sequencing and expression of the MOMP gene

A final and practical reason for adopting the recombinant DNA approach was because the MOMP gene had been isolated and cloned by then (Herring et al, 1989). The efforts of this author were hence channelled towards the restriction mapping and DNA sequencing of this gene for the purpose of devising ways of expressing this gene in *E.coli* (Chapter 6). Partly as a result

of the extensive analysis of the sequence in comparison with other known chlamydial MOMPs, the procedures and strategies for expressing recombinant MOMP were successfully carried out, as detailed in Chapter 7. The sequence analyses showed that even if MOMP were not immunoprotective, it is still of value as an immunological probe because it contains variable domains analogous to other chlamydial MOMPs which have been shown to be antigenically important sites. Computer predictions also showed the presence of potentially amphipathic α-helical regions in MOMP, thought to be ideal candidates for T-cell epitopes.

Initial characterisation and purification studies showed that rMOMP is produced in vast quantities compared to native MOMP from chlamydial EB cultures. This would provide the quantities of sufficiently pure MOMP needed for immunological analyses such as enzyme-linked antibody assays and T-cell proliferation assays, for isolating specific T-cell clones from MOMP- or EB-vaccinated ewes, and for generating monospecific anti-MOMP polyclonal or monoclonal antibodies.

Experiments need to be carried out to identify possible ways of renaturing rMOMP. In this respect, efforts to characterise the putative hierarchy of disulphide-linkages in native MOMP are essential in the absence of mAbs recognising conformational epitopes on native MOMP. A knowledge of this pattern, which has so far not been assigned for any chlamydial MOMP, will provide an assay criterion for the degree of similarity of rMOMP to native MOMP. At the same time, such an approach should also assist the elucidation of the three-dimensional structure of MOMP in the native state.

The EM study in this thesis has correlated ultrastructure with biochemical content and immunological characteristics. Further work on optical diffraction and image enhancement of the regular arrays of fine particles on the OM that was observed may facilitate the determination of the folding pattern of MOMP in the intact OM. This may provide vital clues as to how the

various MOMP domains are involved in structural and permeability functions that are critical in the developmental cycle of chlamydiae.

This project has laid the foundation and provided the tools for the probing the immune mechanisms operating in the immunity of sheep to OEA strains of *C.psittaci*. It has also provided the material for testing the hypothesis that MOMP is necessary and sufficient for eliciting immunity in ewes. Moreover, these possibilities also have important repercussions for the wider field of *C.trachomatis* and *Chlamydia* TWAR (*C.pneumoniae*) infections by providing in the sheep a model system to study systemic, conjunctival, intestinal, synovial and placental infection, "latency" or "persistence", lymph node responses and lymphocyte traffic, humoral and cell mediated mechanisms of immunity. Some aspects of the findings briefly or not mentioned above are discussed in conjunction with broader issues in the sections that follow.

THE DISEASE

Infectious cycle and characteristics of OEA

Buxton *et al* (in press) have recently suggested a probable sequence of events during the infectious cycle of OEA strains of *C.psittaci*. Susceptible ewes become initially infected by ingesting and/or inhaling *C.psittaci* from a contaminated environment during the previous or current lambing season (Wilsmore *et al*, 1984b; Blewett *et al*, 1982). Infection is established first in the tonsils (Jones and Anderson, 1988) and may subsequently migrate via the lymph or blood to an unknown site where it can exist in a "latent" or "persistent" state (McEwen *et al*, 1951a; Munro and Hunter, 1981; Wilsmore *et al*, 1984b). Blood-borne chlamydiae may then gain access to the placentome via the localised haemorrhages (haematomas - Figure 1.1) that occur naturally in the hilar zone of the placentome from the 60th day of gestation. Around the 90th day of gestation, the infection establishes itself at the placental trophoblastic epithelia lining these haemotomas (Studdert, 1968) and gradually spreads to contiguous epithelia. Maternal

and fetal immune systems respond to the chronic nature of the infection. Eventually, the overwhelming infection results in insufficiency of maternal-fetal transfer, fetal death, abortion or premature birth of non-viable lambs. Ewes once aborted, are immune to the disease in subsequent pregnancies although they may continue to shed organisms (Stamp *et al*, 1950; Aitken, 1983). However, it is not known if the shed organisms are of the same strain as the causative organism.

Thus, ovine enzootic abortion differs in several aspects from other well-studied human chlamydial infections such as trachoma, genital disease and their respective laboratory animal models in that infection only affects the pregnant ewe at a specific stage of pregnancy, involves localised pathology that results in abortion and provides lasting immunity against reinfection in subsequent pregnancies. Infected pregnant ewes are also relatively symptomless and do not appear to suffer adverse effects before or after the abortion.

Mechanism of pathogenesis

With respect to the intriguing timing and localisation of chlamydial pathology at the placenta, it seems plausible to speculate that some host cell receptor whose expression is coupled to the regulation of pregnancy may explain tropism and the temporal behaviour of the infection. The initial identification of chlamydial adhesins described in Chapter 4 may provide some basis for further research on the identification of host cell receptors that may be involved in mediating tropism. Alternatively, it is speculated that the tissue tropism of the OEA EBs may involve some metabolic aspect of trophoblastic cells that favour chlamydial growth and replication.

Another possible explanation for the timing of placental colonisation is that the complex balance between the apparently quiescent organisms at some unknown location with the host immune system is altered by the immunosuppression that is known to occur during pregnancy. This may trigger the recrudescence of infectious organisms which migrate to the placenta. The latter

concept is attractive because it is already known that gamma-interferon has bacteriostatic activity on the replication of *C.psittaci* in macrophages (Byrne and Faubion, 1982; Byrne and Krueger, 1983; Rothermel *et al*, 1983). As explained in Chapter 1, bacteriostatic and bactericidal antibiotics, although of therapeutic value in other chlamydial infections, are not considered a viable approach against OEA. Hence, no attempt will be made to discuss this topic except to pose intriguing questions such as the following. If chlamydial organisms do not possess peptidoglycan, where is the alternative site of inhibition by penicillin? Are penicillin-binding proteins involved and what are their functions?

Mechanism of abortion

The mechanism involved in the induction of abortion in ewes by OEA strains of *C.psittaci* is not clearly understood. Since *C.psittaci* is a Gram-negative bacterium, the LPS that is present in its outer membrane may be involved. Both smooth and rough LPS have been shown to induce abortion in pregnant laboratory animals (Zahl and Bjerknes, 1943; Rieder and Thomas, 1960 and references therein). This endotoxin-induced abortion can be prevented by antisera directed against LPS or the Lipid A moiety of LPS but not by the polysaccharide moiety (Rioux-Darrieulat *et al*, 1978). Moreover, antagonists of serotonin such as chlorpromazine or methysergide can partially prevent abortion suggesting that LPS may interrupt pregnancy by inducing the release of serotonin or some related substance (Parant and Chedid, 1964). This protection by serotonin antagonists can be potentiated with anti-LPS antiserum (Rioux-Darrieulat *et al*, 1978). Cortisone, however, did not prevent endotoxic abortion in mice despite being highly effective against endotoxic shock (Rieder and Thomas, 1960). These data indicate that the toxic and abortive effects of LPS may be mediated by different biological pathways.

Ewes infected by OEA strains of *C.psittaci* do not exhibit the classic symptoms of endotoxic shock. It is speculated that the syndesmochorial placentation in sheep may limit the amount of chlamydial LPS released into the circulation, thereby reducing endotoxic reactions such as disseminated intravascular coagulation (DIC), renal cortical necrosis and vasomotor shock. Secondly, the infection appears to be chronic and does not resemble the rapid onset of abortion brought about by endotoxin in mice and rabbits (Zahl and Bjerknes, 1943; Rieder and Thomas, 1960). Whether chlamydial LPS directly causes ovine abortion awaits further investigations. It is possible, however, that it plays only a minor role (if any) in a complex chain of events leading to abortion and that the primary lesion is caused by the colonisation and multiplication of infectious EBs at the placenta.

A recent study of infected sheep during late gestation has demonstrated that such lesions may cause changes in the pattern of placental hormone secretion (Leaver *et al*, 1989). In infected ewes compared to uninfected control ewes, progesterone, which maintains pregnancy, declined prematurely while oestradiol 17β and prostaglandin E2, which stimulate uterine responses at parturition, appeared much earlier. Interestingly, oestradiol 17β and prostaglandin E2 have been demonstrated to enhance the growth of *C.trachomatis* in cell culture (Bose and Goswami, 1986; Sugarman and Epps, 1982; Ward and Salari, 1980). If this hormonal aspect of OEA proves important, then any prophylactic agent should be targetted at a stage before colonisation of the placenta to prevent abnormal changes in the hormonal balance that favour chlamydial replication.

In contrast to OEA, the zoonotic infection of pregnant women by ovine abortion strains of *C.psittaci* is acute, and results in abortion and post-partal symptoms of DIC and renal involvement (Chapter 1). It is reminiscent of the dual but separate effects of endotoxin on pregnant laboratory animals mentioned above. While it is not known whether chlamydial LPS is

involved in abortion, LPS is likely to play a role in causing DIC, although thromoboplastic products of degenerating tissues at the site of infection may be a contributory factor (reviewed by Lester and Roth, 1977; Wong *et al*, 1985; Johnson *et al*, 1985). The spread of LPS endotoxin and thromboplastic material is probably facilitated by the haemochorial placentation in pregnant women. In this respect, the mAbs isolated with anti-LPS activity (Chapter 4) may be further characterised to compare or contrast their efficacies in preventing LPS-induced or *C.psittaci*-induced abortion in a mouse model or in sheep.

IMMUNITY

Immune mechanisms

Little is known about the immune mechanisms operating in post-abortion ewes that provide long-term protection against subsequent abortion. Despite this, vaccines have been developed and successfully used in the control of OEA (McEwen *et al*, 1951b and 1955; McEwen, 1954; McEwen and Foggie, 1954 and 1956; Foggie, 1973). The immunity to OEA afforded by vaccination is thought to last at least three years (McEwen and Foggie, 1956) but annual vaccination is recommended for current vaccines. Unfortunately, even less is known about how the vaccine works.

Both humoral and cellular responses are known to occur during infection (Huang *et al*, in press; Buxton *et al*, in press; Wilsmore *et al*, 1984b; Dawson *et al*, 1986a and 1986b). CF antibodies, however, are not thought to be involved in immunity (McEwen and Foggie, 1954; Dawson *et al*, 1986b; Buzoni-Gatel *et al*, 1987). Antibody specificities as determined by immunoblotting do not provide evidence as to the involvement of antibodies in protection but showed that generally, post-abortion ewes carried a wider range of antibody specificities compared to vaccinated ewes (Chapter 3). Although serum neutralisation of OEA *C.psittaci* infectivity has been demonstrated *in vitro* (I.E.Anderson, unpublished data), recent data do not show any evidence that ammonium sulphate-

precipitated antibodies from post-abortion ewes when passively transferred to pregnant ewes can protect against challenge with live organisms (G.E.Jones, personal communication).

With the availability of essentially unlimited amounts of rMOMPs and the possibility of a high degree of purification, it may be possible to overcome the problems of cross-reactivity and effects associated with using LPS-containing whole organism preparations in the DTH skin tests and lymphocyte transformation (or proliferation) assays of Wilsmore *et al*, (1984) and Dawson *et al* (1986a; 1986b). Such tests of the involvement of cell-mediated immunity should form an adjunct to future vaccination-challenge experiments involving rMOMPs or other formulations.

Current experiments to assess the induction of lymphocyte proliferation *in vitro* by fractionated chlamydial antigens should shed light on the relative role of each antigen (C.M.McCafferty and D.M.Haig, personal communication) but may not indicate the importance of the cellular response *in vivo*. Nonetheless, the role of each antigen may be correlated with the immunoblotting data of this thesis, and potentially immunoprotective antigens in addition to MOMP may be identified. However, progress is hampered by the lack of sufficient antigenic mass essential for such proliferation assays.

Computer algorithms predict that the MOMP sequence contains T-cell epitopes (Chapter 6). Since T-cell responses do not usually distinguish between native and denatured forms of a protein (Gell and Benacerraf, 1959; Mills, 1986), the non-native but biochemically defined rMOMP will be useful for priming sheep lymphocytes and for selecting specific T-cell lines. These cell lines can be characterised for function and phenotype, and correlated with their protective efficacy in transfer experiments described below. They can be further characterised to identify the specific T-cell epitopes on the

MOMP sequence by testing synthetic peptides of the predicted sequences (Chapter 6) or a panel of truncated rMOMPs (such as those described in Chapter 7) in *in vitro* proliferation assays.

Experimental transfer of cellular immunity of the type performed by Buzoni-Gatel *et al* (1987) on mice will be difficult but not impossible to perform on sheep although inbred sheep are not available. Experimental sheep can be matched by haplotyping or alternatively, ewes can be induced to produce identical twins, triplet, quadruplet or quintuplet lambs for such transfer of immunity (Smith *et al*, 1986). A sibling vaccinated with purified EBs or subcellular MOMP preparations may be used as a source of primed T-cells for transfer to the unimmunised syngeneic sibling which can be mated and challenged with live chlamydiae. Similarly, T-cells from post-abortion siblings may be used. Phenotypic markers for ovine helper and cytotoxic/suppressor T-cells are available and it is possible to separate them by fluorescence-activated cell sorting (FACS) or in larger numbers, by panning. Thus the role of each T-cell sub-population may be analysed in sheep in a manner analogous to the experiments of transferring cell-mediated immunity in mice (reviewed by Williams, 1988 and Rank, 1988).

Potential sites of immunity

Vaccination or immunity acquired from a previous abortion may inhibit chlamydial infection at the following stages: induction of mucosal immunity to prevent the initial infection at the tonsil or other sites; prevention of migration to unknown site(s) of putative "latency"; suppression of recrudescence from these sites; prevention of haematogenous migration to the placenta; or bolstering of the humoral and cellular response at the placenta to limit the multiplication and spread of infectious EBs and to lessen the effects of pathology (Figure 1.2). It is presently premature to speculate about the relative importance of each potential locus of action. Nevertheless, the advent of highly sensitive techniques such as PCR (discussed below) that can potentially detect single genomes, suggests the possibility that some of these potential sites of action may be

tested. The progress or lack of progress of chlamydial infection as monitored by PCR detection may be compared in susceptible, vaccinated and convalescent ewes.

Limitations of current vaccine

The minimum criterion for a satisfactory vaccine is the ability to limit the extent of the pathology at the placenta or to delay it sufficiently so that the ewe can give birth to viable lambs. All early vaccines improved the lamb survival ratio by reducing the number of abortions due to chlamydial infection to levels which were acceptable to farmers. For example, an early study involving 110 flocks showed the reduction in incidence from 5 - 6% in unvaccinated flocks to 1 - 2% in vaccinated flocks (Littlejohn *et al*, 1952). Despite extensive use, the vaccine does not eradicate the disease within a flock (Aitken *et al*, 1986). It is possible that current vaccines do not protect against infection and operate close to the minimum criterion such that any reduction in vaccine efficacy due to insufficient antigenic matter, change in animal husbandry and stress, or slight antigenic variation of the pathogen in the field, could easily lead to vaccine breakdown. A potential alternative to the inactivated vaccine is the temperature-sensitive live vaccine developed by Rodolakis (Rodolakis, 1983; Rodalakis and Bernard, 1984) but its usefulness or stability in the field has yet to be demonstrated.

In recent vaccination experiments (Anderson *et al*, submitted; this thesis), various criteria of vaccine efficacy have been used including placental infection, abortion rate and lamb survival ratio. It should be noted that no adequate criterion of infection can be obtained without a clearer understanding of the stages of chlamydial infection in ewes. Similarly, no criterion for quality assessment in commercial vaccines is adequate so long as the critical antigens that provide immunoprotection are not known.

Current methods of titrating infectious EBs for vaccine production suffer a drawback in that OEA *C.psittaci* EBs have a strong tendency to aggregate. Assays using polyclonal antibodies to whole organisms or the monoclonal antibody to LPS (Chapter 4) are not useful because of the LPS activity. Unlike LPS which can be over-produced and found on the membranes of the infected cell, the MOMP content per organism is likely to be fairly constant (Richmond and Stirling, 1981; Karimi *et al*, 1989). Whether MOMP is the immunoprotective antigen or not, polyclonal antibodies raised against the rMOMP may be used as assay for antigenic mass in the vaccine production of current whole organism vaccines. Alternatively, the PCR technique can be adapted in a quantitative assay to "count" genomes. Other complications to the question of vaccine efficacy is the problem of antigenic variation in the field which is discussed next.

DIAGNOSIS, DETECTION AND TAXONOMY

Serological tests for diagnosis

Currently, there is no simple and reliable serological test which can identify infections caused by ovine abortion *C.psittaci* as opposed to infections caused by *C.trachomatis*, *Chlamydia* TWAR (*C.pneumoniae*) or avian *C.psittaci*. The limitations of a recent epidemiological survey on the extent of zoonotic infection by OEA *C.psittaci* illustrates the problem (Hobson and Morgan-Capner, 1988). The traditionally used complement fixation (CF) test, which relies on chlamydia-specific antibodies as serological markers of infection, lacks sensitivity and specificity for OEA *C.psittaci*. Presently, 40% of all sheep fetopathies are undiagnosed. Whether these are caused by OEA *C.psittaci* is not known. Another complication is that several serologically cross-reactive but biologically different types of *C.psittaci* can infect sheep. One aspect in the control of OEA thus appears to be the development of better tests for distinguishing OEA *C.psittaci* from other disease types as well as for identifying susceptible or infected animals.

5' end of MOMP ORF

S26/3	ATG AAA AAA CTC TTG AAA TCG GcA
A22/M	ATG AAA AAA CTC TTG AAA TCG GcA
CAL10	ATG AAA AAA CTC TTG AAA TCG GcA
GPIC	ATG AAA AAA CTC TTG AAA TCG GcA
L2	ATG AAA AAA CTC TTG AAA TCG GtA
A	ATG AAA AAA CTC TTG AAA TCG GtA
B	ATG AAA AAA CTC TTG AAA TCG GtA
C	ATG AAA AAA CTC TTG AAA TCG GtA
UnivPri	**5'-ATG AAA AAA CTC TTG AAA TCG G-3'**

5' end of processed MOMP

S26/3	tTG CCT GTa GGG AAc CCa GCT GAA CCA
A22/M	tTG CCT GTa GGG AAc CCa GCT GAA CCA
CAL10	tTG CCT GTa GGG AAc CCa GCT GAA CCA
GPIC	tTG CCT GTa GGG AAt CCa GCT GAA CCA
L2	cTG CCT GTg GGG AAt CCt GCT GAA CCA
A	cTG CCT GTg GGG AAt CCt GCT GAA CCA
B	cTG CCT GTg GGG AAt CCt GCT GAA CCA
C	cTG CCT GTg GGG AAt CCt GCT GAA CCA
UnivPri	**5'-YTG CCT GTR GGG AAY CCW GCT GAA CC-3'**

3' end of MOMP ORF

S26/3	GCT CAC aTg AAt GCt CAA TTC aGa TTC TAA
A22/M	GCT CAC aTg AAt GCt CAA TTC aGa TTC TAA
CAL10	GCT CAC aTg AAt GCt CAA TTC aGa TTC TAA
GPIC	GCT CAC gTa AAc GCt CAA TTC aGa TTC TAA
L2	GCT CAC gTa AAt GCa CAA TTC cGc TTC TAA
A	GCT CAC gTa AAt GCa CAA TTC cGc TTC TAA
B	GCT CAC gTa AAt GCa CAA TTC cGc TTC TAA
C	GCT CAC gTa AAt GCa CAA TTC cGg TTC TAA
Consen	**5'-GCT CAC RTR AAY GCW CAA TTC MGV TTC TAA-3'**
UnivPri	**3'-CGA GTG YAY TTR CGW GTT AAG KCB AAG ATT-5'**

Figure 8.1. Comparison of selected nucleotide sequences of primers based on these sequences may be reasonably expected to amplify any chlamydial MOMP. The ambiguity code used is based on the IUB recommendations (Nomenclature Committee, 1985, European Journal of Biochemistry, **150**, 1-5). Consen = consensus. UnivPri= universal primer. *C.psittaci* strains are S26/3 (OEA), A22/M (Pickett *et al*, 1988a), Cal10 meningopneumonitis strain and GPIC guinea pig inclusion conjunctivitis strain (Zhang *et al*, 1989a). *C.trachomatis* strains are serotypes L2 (lymphogranuloma venereum strain), A, B and C (Stephens *et al*, 1986; 1988b).

Antigens useful for serological tests should not contain genus- or species-specific epitopes, thus ruling out LPS and MOMP. The 50kDa antigen of OEA *C.psittaci* appears to be specific to OEA isolates (Figure 4.2B) and should be further investigated for its potential as a serological marker. However, such potentially type-specific antigens may not elicit a detectable antibody response at the time of serum sampling. Nevertheless, work is in progress to develop a competitive ELISA using type-specific monoclonal antibodies directed against OEA MOMP (J.Dooley, personal communication). Recombinant MOMPs specifically designed to contain desired epitopes should provide useful antigenic material for future development of such tests.

MOMP and MOMP gene as a locus for taxonomy and detection

MOMP is a useful locus for taxonomic classification of *Chlamydia* spp. It is present in all types of chlamydiae. Each MOMP bears distinctive epitopes at various taxonomic levels which have their basis in the pattern of variation and conservation within the amino acid sequence of the protein and hence, in the nucleotide sequence of the gene (Chapter 6 and references therein). Fortuitously, the conserved sequences flank the variable sequences thereby allowing the possibility of PCR amplification of the variable gene sequences using a limited set of oligonucleotide primers. Recently, Dutilh *et al* (1989) have amplified short segments of conserved sequences of *C.trachomatis* MOMP genes. Figure 8.1 shows a set of 'universal' primers that may be used for amplifying any MOMP gene.

More recently, S.Baxter (PhD thesis in preparation) has shown that amplified MOMP gene sequences can be analysed by restriction endonuclease profiling, by Southern blot hybridisation using specific probes, or by DNA sequencing. A classification table of such nucleotide sequence data for each chlamydial strain may be quickly built up. Furthermore, the PCR technique is extremely sensitive, often reaching the ultimate in detection by amplifying single molecules of DNA. Tests may

be performed on material obtained from swabs, mouth rinses, bodily secretions and excretions without having to culture the organism (reviews by Bell, 1989; White *et al*, 1989).

The expression work described in this thesis illustrates the possibility of cloning other PCR-amplified MOMP sequences into expression vectors to produce polypeptides. By designing the appropriate primers tailed with restriction sites, the VSs of new strains can be cloned into expression vector systems such as the pRIT Protein A fusion system. Recombinant polypeptide bearing specific epitopes of the corresponding VD attached to Protein A can be conveniently purified and chemically or enzymatically cleaved to produce MOMP peptide epitopes for antibody detection in an ELISA system that can also type the chlamydial infection.

Such is the versatility and power of the PCR method that if a standard protocol can be agreed upon and adopted by various workers in this field, an additional basis of chlamydial systematics providing a comprehensive classification of diverse *C.psittaci* isolates can reasonably be expected in the near future. The functional and immunological significance of MOMP suggest that a classification based on the MOMP gene may provide a useful indicator of host specificity, the type of disease caused or the degree of cross-protection of vaccines.

Variation in OEA *C.psittaci*

Despite indications of limited cross-protection in sheep (Aitken *et al*, 1981) and in mouse models (Russo et al, 1979; Johnson and Clarkson, 1986), OEA isolates of *C.psittaci* do not appear to be distinguishable by the following criteria: virulence and cultural characteristics (Anderson, 1986a; 1986b); SDS-PAGE, immunoblot and restriction endonuclease profiles (McClenaghan and Herring, in preparation; Chapters 3 and 4; McClenaghan *et al*, 1984); MOMP gene sequencing, Southern hybridisation and hybrid duplex RNA analysis (S.Baxter, PhD thesis in preparation). Thus the basis of variation, if any,

	OmpA	MOMP	BRHO	PhoE	OmpF	OmpC	NGOmpI
N=	346	389	262	351	362	368	326
Ala	37	54	30	31	34	29	33
Cys	2	7	0	0	0	0	0
Asp	22	21	10	31	27	32	17
Glu	13	11	11	14	14	11	13
Phe	9	18	13	21	19	19	15
Gly	38	30	26	37	49	48	37
His	5	4	0	1	1	1	12
Ile	16	22	15	14	14	10	7
Lys	19	20	7	25	19	17	22
Leu	23	35	39	23	23	27	20
Met	6	7	10	9	5	4	2
Asn	19	19	3	27	32	32	16
Pro	19	17	12	3	5	4	6
Gln	17	13	4	17	13	21	15
Arg	13	13	7	12	12	13	16
Ser	16	25	14	21	16	17	24
Thr	23	30	19	24	22	24	19
Val	27	21	23	16	26	25	29
Trp	5	10	8	3	2	4	5
Tyr	17	12	11	22	29	29	18

Table 8.1. Composition of prokaryotic outer membrane proteins (precursor proteins). OmpA, PhoE, OmpF and OmpC are *E.coli* outer membrane proteins. The composition of bacteriorhodopsin (BRHO) of *Halobacterium halobium* and OmpI of *Neisseria gonorrhoeae* (NGOmpI) are also displayed with MOMP of *C.psittaci* S26/3.

remains to be determined in order to facilitate epidemiological studies into the incidence of OEA and to determine the valency and composition of improved vaccines.

MAJOR OUTER MEMBRANE PROTEIN

Structure and Function of MOMP

Transmembrane proteins may be categorised by the nature of the membrane-spanning segments in relation to other protein domains (Paul and Rosenbusch, 1985 and references therein). For example, cytochrome b5 has soluble domains anchored to the membrane by a short peptide while others such as glycophorin possess two soluble domains separated by a single

membrane-spanning peptide. Yet others like the reaction centre of photosynthetic bacteria possess more bulky membrane-spanning domains. Outer membrane proteins in bacteria such as bacteriorhodopsin and enterobacterial porins OmpF, OmpC and PhoE are subclasses of compact transmembrane proteins where the polypeptide chain is thought to traverse the membrane several times, exposing only small peptide segments to the surface. The porins are very hydrophilic and possess a high degree of β-sheet structure. The polypeptide chain of PhoE, for example, is thought to traverse the OM in an anti-parallel β-sheet pattern exposing eight segments to the cell surface, each segment about 40-45 residues long (van der Ley *et al*, 1987a; 1987b and references therein). By contrast, bacteriorhodopsin is highly hydrophobic, possesses a completely α-helical structure with seven hydrophobic helices forming the transmembrane segments (Engelman *et al*, 1980).

MOMP, itself possessing porin-like function, appears to resemble these porins of *Enterobacteriaceae* in amino acid composition (Table 8.1). These porins have M_r in the 36 to 45kDa range (Hall and Silhavy, 1981 - review). Their signal peptides are of similar length, 20-21 residues with 2 to 3 Lys residues at the N-terminus. Enterobacterial porins, for example, exist as trimers; different porins can also form hetero-trimers (Gehring and Nikaido, 1989). OmpF forms a regular two-dimensional array on the surface of the OM, reminiscent of the ultrastructure of the MOMP-enriched OM preparations (Chapter 4). Comparison of PhoE of different enterobacterial species showed that they possessed 4 variable domains but much smaller in length than and located differently from the MOMP VDs (van der Ley *et al*, 1987a).

While MOMP may possess many features of porins, it also performs a vital structural role in the chlamydial OM, analogous to the peptidoglycan of other Gram-negative bacteria. *E.coli* porins, OmpF, OmpC and PhoE, and *Neisseria gonorrhoeae* protein I (major outer membrane protein/porin) (EMBL databank) do not possess cysteine residues whereas *E.coli* OmpA, a

structural OM protein possesses two (Morona *et al*, 1984). These porins are passive diffusion channels whereas the permeability function of MOMP appears to be regulated by redox potential.

Although up to about five major outer membrane proteins are present in the OM of *E.coli* (Hall & Silhavy, 1981), MOMP is pre-dominant in the OM of *C.psittaci* and is thought to be involved in a supramacromolecular network of disulphide linkages with other chlamydial cysteine-rich proteins (CRPs). It seems reasonable to suggest that MOMP is a fairly unique OM protein that combines structural and permeability functions. It is likely that MOMP is a porin that has been adapted to perform a structural role. Evidently, the structure-function relationship of this intriguing protein deserves a closer examination. In this respect, the availability of the MOMP gene cloned into an *E.coli* plasmid vector facilitates the construction of porin-MOMP hybrids which may reveal the functional aspects of the MOMP sequence (see van der Ley *et al*, 1987b). Comparative renaturation and reconstitution of MOMP, rMOMP and its truncated versions as porins in artificial vesicles may possibly be used to identify the channel forming sequences in the polypeptide chain. Detergent extraction, analysis of the solubilised components and cross-linking experiments of the type reported by Birkelund *et al* (1988) may reveal the molecular composition and architecture of the OM.

Immunogenicity of MOMP and vaccine development

While it is clear that MOMP possesses many B-cell epitopes of different specificities, there is no experimental evidence for the presence of T-cell epitopes on MOMP. The demonstration of T-cell epitopes on the MOMP sequence is important because these epitopes may stimulate helper T-cells *in vivo* (Chapter 6 - discussion). Helper T-cell immunity is necessary not only for the initial antibody response but is also essential for an anamnestic (memory) response to the relevant B-cell epitopes. Additionally, the specific helper T-cell may possess cytolytic activity on antigen-bearing cells expressing class II major histocompatibility complex (MHC) molecules (Berzofsky *et al*,

1987). Identification of such epitopes in MOMP or other antigens by the methods described earlier in this chapter may result in the inclusion of such epitopes in improved vaccines. Moreover, some cytotoxic T-cell epitopes of influenza virus nucleoprotein have been predicted using the "Rothbardian" motifs described in Chapter 6 (Bastin *et al*, 1987), suggesting that some of the predicted epitopes on MOMP may also stimulate MHC class-I-restricted cytotoxic T-cell activity. An efficient cytotoxic T-cell response is generally thought to be required for the clearance of persistently infected cells in viral infections and may be important in chlamydial infections.

The general trend in the quest for a vaccine against *C.trachomatis* infection has been to locate specific MOMP sequences recognised by protective mAbs and to synthesise them either chemically (Conlan *et al*, 1988 and 1989) or by recombinant techniques (Baehr *et al*, 1988; Stephens *et al*, 1988b; Pickett *et al*, 1988b) as target antigens for synthetic peptide or recombinant subunit vaccines. The motivation for this selectiveness originates from the findings of early vaccine trials in man and primate models. These indicate that immunity is serovar-specific but short-lived while immunopathological reactions upon reinfection are genus-specific (Wang *et al*, 1967; Mordhurst, 1967; Grayston *et al*, 1985 - review). However, recent data show that purified MOMP does not induce deleterious ocular DTH responses in experimental monkeys (Taylor *et al*, 1988) and that a 57 kDa genus-specific antigen is likely to be responsible (Morrison *et al*, 1989).

The approach in the case of OEA has been different. It is possible to trace a logical progression of vaccine development beginning with crude whole organisms from infected tissues, egg culture and cell monolayer culture to the purified EB and the subcellular OM fraction of this thesis. The protective components of the EB has thus been narrowed down from more than a dozen antigens to a few including MOMP located in the OM. The next step would be to test individual antigens, particularly

MOMP, as subunit vaccines. The cloning and expression of the OEA MOMP gene is expected to facilitate the testing of the hypothesis that MOMP bears immunoprotective B- or T-cell epitope(s). If MOMP is indeed immunoprotective, modification of the MOMP gene constructs to express selected domains of MOMP can be conveniently performed. This would allow the critical epitopes to be identified, if necessary, for the development of peptide vaccines. At the same time, novel and improved methods of presenting immunogens to the ovine immune system form an important part of vaccine design. In this respect, the recombinant plasmids, pD and pC, have recently been transformed into *Salmonella typhimurium aroA* mutants which are known to be useful carriers of foreign antigens that can elicit humoral and cellular immune responses (Brown *et al*, 1987). Initial data showed good expression of rMOMP (J.J.Oliver, A.J.Herring and S.Dunbar, personal communication).

It is envisaged that testing such vaccines in the well established situation of OEA will not only result in improved OEA vaccines but will also converge with and contribute to the research in tackling the serious and relatively complicated disease situations of blinding trachoma or sexually transmissible diseases caused by *C.trachomatis*.

CONCLUDING REMARKS

Although it has been nearly 40 years since the causative agent of OEA has been identified as *C.psittaci*, much still remains to be known about the pathogenesis of the disease. The immune mechanisms responsible for protection in post-abortion or vaccinated ewes are presently not understood although both antibody and cell-mediated responses are thought to be involved. The reasons for the re-emergence of the disease in the past two decades are not clear. The characterisation of the antigenic structure of OEA *C.psittaci* has been initiated only in recent years. The work reported in this thesis has extensively catalogued the antigens of the organism and provided the basis

for further characterisation of individual antigens to identify their function and their possible involvement in immunity. In particular, this project has carried the work of characterising MOMP, a dominant antigen strongly implicated as having immunoprotective properties, to the point where it can be tested for vaccine efficacy in the form of a recombinant subunit vaccine.

From the sequence analyses and other data, it was inferred that MOMP is a mosaic of B-cell and T-cell epitopes, and rMOMPs would provide the important tools for probing the immune mechanisms that are responsible for protection. Moreover, the introduction of the PCR technique into the field of chlamydiology has opened up exciting possibilities in the taxonomy and typing of *C.psittaci* isolates, in the detection of chlamydial organisms and the definition of the pathogenetic events of OEA, and in the manipulation of the MOMP gene and the manufacture of specific polypeptide segments.

Finally, it is hoped that research will continue in the characterisation of MOMP and other chlamydial antigens identified in this project and to relate the fascinating structures observed in the outer membrane preparations to their function in the biology of chlamydiae. An increasing understanding of chlamydiae and the diseases they cause will no doubt establish the basis for rational design of chlamydial vaccines in the future. With regard to OEA, better vaccines will not only increase sheep production but will also lead to the improved health and well-being of the sheep and the sheep farming community.

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APPENDIX 1: NUCLEOTIDE AND AMINO ACID SEQUENCE SYMBOLS

The letter codes for amino acids and nucleotide ambiguity proposed by the International Union of Biochemistry (IUB) (Nomenclature Committee, 1985, Eur. J. Biochem., 150: 1-5) were used in this thesis. Occasionally, three letter codes for amino acids were used.

Nucleotides

A	Adenosine		
C	Cytosine		
G	Guanine		
T/U	Thymine/Uracil		
M	A or C	aMino group	
R	A or G	puRine	
W	A or T	Weak hydrogen bonding	
S	C or G	Strong hydrogen bonding	
Y	C or T	pYrimidine	
K	G or T	Keto group	
V	A or C or G	not T	V follows T and U
H	A or C or T	not G	H follows G
D	A or G or T	not C	D follows C
B	C or G or T	not A	B follows A
N	G or A or T or C	aNy	

Amino acids				Back translation
A	Ala	Alanine	GCT,GCC,GCA,GCG	GCX
B	Asp,Asn		GAT,GAC,AAT,AAC	RAY
C	Cys	Cysteine	TGT,TGC	TGY
D	Asp	aspart(D)ic acid	GAT,GAC	GAY
E	Glu	Glutamic acid	GAA,GAG	GAR
F	Phe	ph(F)enylalanine	TTT,TTC	TTY
G	Gly	Glycine	GGT,GGC,GGA,GGG	GGX
H	His	Histidine	CAT,CAC	CAY
I	Ile	Isoleucine	ATT,ATC,ATA	ATH
K	Lys	lysine (K before L)	AAA,AAG	AAR
L	Leu	Leucine	TTG,TTA,CTT,CTC,CTA,CTG	TTR,CTX,YTR,YTX
M	Met	Methionine	ATG	ATG
N	Asn	asparagiNe	AAT,AAC	AAY
P	Pro	Proline	CCT,CCC,CCA,CCG	CCX
Q	Gln	g(Q)lutamine	CAA,CAG	CAR
R	Arg	aRginine	CGT,CGC,CGA,CGG,AGA,AGG	CGX,AGR,MGR,MGX
S	Ser	Serine	TCT,TCC,TCA,TCG,AGT,AGC	TCX,AGY,WSX
T	Thr	Threonine	ACT,ACC,ACA,ACG	ACX
V	Val	Valine	GTT,GTC,GTA,GTG	GTX
W	Trp	tr(W)ptophan	TGG	TGG
X	Xxx	unknown		XXX
Y	Tyr	tYrosine	TAT,TAC	TAY
Z	Glu,Gln		GAA,GAG,CAA,CAG	SAR
*	End	terminator	TAA,TAG,TGA	TAR,TRA,TRR

APPENDIX 2

PUBLICATIONS AND ABSTRACTS IN CONFERENCES AND SYMPOSIA

A2.1 Sequence analysis of the major outer membrane protein gene of an ovine abortion isolate of Chlamydia psittaci. A.J.Herring, T.W.Tan, S.Baxter, N.F.Inglis and S.Dunbar. FEMS Microbiol. Lett. 65, 153-158.

A2.2 Antibody response of the ovine lymph node to experimental infection with an ovine abortion isolate of Chlamydia psittaci. H-S.Huang, T.W.Tan, D.Buxton, I.E.Anderson and A.J. Herring. Vet. Microbiol. (in press)

A2.3 Efficacy against ovine abortion of an experimental vaccine containing purified elementary bodies of Chlamydia psittaci. I.E.Anderson, T.W.Tan, G.E.Jones and A.J.Herring. Vet. Microbiol. (submitted).

A2.4 Protection of sheep against Chlamydia psittaci abortion with an experimental subcellular vaccine. T.W.Tan, A.J.Herring, I.E.Anderson and G.E.Jones. Infection and Immunity (in preparation).

A2.5 What is the role of Tween 20 in immunoblot assays? T.W.Tan and A.Taylor. Abstract in the annual conference of the Association of Veterinary Teachers and Research Workers (AVTRW), March 1988, Scarborough.

A2.6 Specific antibody responses in sheep vaccinated with an inactivated preparation of an ovine abortion isolate of Chlamydia psittaci. T.W.Tan, I.E.Anderson, G.E.Jones and A.J.Herring. Abstract in the annual conference of the AVTRW, March 1988, Scarborough.

A2.7 Immunological responses of the ovine lymph node to Chlamydia psittaci. H-S. Huang, T.W.Tan, I.E.Anderson and D.Buxton. Abstract in the annual conference of the AVTRW, March 1988, Scarborough.

A2.8 Immunoblotting analysis of the humoral immune response in sheep infected with the ovine abortion strain of Chlamydia psittaci. T.W.Tan, A.J.Herring, M.McClenaghan, H-S.Huang, I.E.Anderson, N.F.Inglis, G.E.Jones and D.Buxton. In Proceedings of the European Society for Chlamydia Research, Societa Editrice Esculapio, Bologna, Italy. (1988) Volume 1, p136. (Abstract).

A2.9 Zoonosis by ovine abortion strains of Chlamydia psittaci: comparative studies and experimental vaccination in the natural host. T.W.Tan, A.J.Herring, G.E.Jones, I.E.Anderson, I.W.Smith and D.Buxton. Abstract in the proceedings of the First Asia-Pacific Congress of Medical Virology, November 1988, Singapore.

A2.10 Immunization of sheep against chlamydial abortion with an experimental subcellular vaccine. T.W.Tan, I.E.Anderson, G.E.Jones and A.J.Herring. Abstract in the annual conference of the AVTRW. March, 1989. Scarborough.

A2.11 Development of improved vaccines against ovine abortion caused by Chlamydia psittaci. T.W.Tan, A.J.Herring et al. Abstract submitted to the Herpes Vaccine Research Trust (UK). Awarded jointly the Annual Prize of the Trust on 7th January 1989.

'EMS Microbiology Letters 65 (1989) 153–158
'ublished by Elsevier

'EMSLE 03747

Sequence analysis of the major outer membrane protein gene of an ovine abortion strain of *Chlamydia psittaci*

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Received 16 June 1989
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Key words: *Chlamydia psittaci*; Outer membrane protein; Cloning; DNA sequencing

. SUMMARY

The major outer membrane protein (MOMP) ene from an ovine abortion strain of *Chlamydia sittaci* (S26/3) has been cloned and sequenced. 'he gene shows the features of other chlamydial MOMPs but comparison with the previously re-orted sequence for the ovine abortion isolate .22/M has revealed substantial sequence diver-ence which is clustered into the same four in-amolecular regions as the sequence variation ound between *C. trachomatis* serovars. Subse-uent restriction enzyme analysis of A22/M DNA as shown that it has an avian-type genomic pro-le and thus the comparison is between types ıther than between strains.

. INTRODUCTION

Enzootic abortion of ewes (EAE) is the most revalent infection causing lamb loss in the UK ıd is due to infection with *Chlamydia psittaci* [1]. he disease was controlled for many years by an inactivated vaccine but recently has re-emerged as a problem thereby focusing attention on the immune mechanisms and possible strain variants involved in the infection [2,3]. It is also now established that the EAE strain can cause abortion in women [4] which lends further importance to the study of this pathogen.

The species *C. psittaci* is a diverse grouping within which, until recently, types have been defined only by their origin of isolation and biological properties. A recent DNA/DNA reassociation study has shown substantial differences within the group suggesting that separate species status for some types may be applicable [5]. Restriction endonuclease (RE) analysis [6,7], micro-immunofluorescence (MIF) [8], and monoclonal antibody studies [9,10] have also been used to demonstrate variation.

Studies on the human pathogen *C. trachomatis* have shown that variable epitopes which determine the serovars defined by the MIF test reside in the major outer membrane protein (MOMP) [11] and that antibodies to this protein can neutralise chlamydial infectivitiy both 'in vitro' and 'in vivo' [12,13]. This dual involvement of MOMP in both immunity and strain variation has prompted us to clone and sequence the MOMP gene from an EAE strain.

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3. MATERIALS AND METHODS

3.1. Sources of chlamydial DNA

Several of the strains used in this work, including *C. psittaci* S26/3 used as the source of DNA for cloning, have been described previously as has the method for DNA extraction [6]. S57/3 is a more recent abortion isolate from the same farm as S26/3. A22/M DNA was the kind gift of Ian Clarke, Southampton University.

Two overlapping plasmid clones (pUC-18) which separately contain the 5′ and 3′ ends of the MOMP gene from *C. trachomatis* serovar L2, together with flanking sequences covering a total of 2.35 kb, were the kind gift of Richard Stephens, University of California, San Francisco.

3.2. Nucleic acid manipulations and cloning

Restriction endonucleases, T4 DNA ligase and calf intestinal phosphatase (CAP) were obtained from Boehringer Corporation (London) Ltd. or Gibco BRL Ltd. (Paisley, Scotland) and used as recommended by the manufacturer. DNA fragments from agarose gels were recovered using 'Geneclean' (Bio101, Calfornia) and labelled with ^{32}P by the random priming procedure [14]. 'Southern' transfers and hybridisations were carried out as described by Meinkoth and Wahl [15] using 'Hybond N' membranes (Amersham Int., U.K.).

Genomic library construction in the bacteriophage λ vector EMBL-3 was carried out by the methods described by Kaiser and Murray [16]. A partial *Mbo-1* digest of S26/3 DNA was treated with CAP and ligated into *Bam*HI-digested vector. After packaging and initial growth in *E. coli* strain C600, recombinants were selected by growth on *E. coli* strain Q359 r_m^- m_k^+ su_{11}^+ 80^RP2, a P2 lsyogen. The library was then screened with the L2 MOMP DNA probes using standard methods [17].

Sub-cloning into bacteriophage M13 vectors mp18 and mp19 using *E. coli* strain JM101 as a host was performed as described by Messing [18] using DNA fragments recovered from agarose gels. Subsequent sequencing was performed using the dideoxynucleotide chain termination method [19] using [^{35}S]dATP (Amersham Int. Ltd., U.K.) as a label.

4. RESULTS AND DISCUSSION

The *C. psittaci* MOMP gene was identified b first constructing a genomic library with S26/ DNA in EMBL-3 and screening with two DN probes spanning the whole *C. trachomatis* H MOMP gene. It had been previously demo strated that, while there is less than 10% relate ness between the two chlamydial species, there a conserved regions in the MOMP gene [20]. single clone was identified which reacted wi both probes. Subsequent blotting studies show that this hybridisation could be localised to a 2 kb *Sst-1* fragment. This fragment was then su cloned into M13 mp19 to avoid problems plasmid instability encountered previously wi this gene [21]. The resulting clone was mapp using several restriction enzymes compatible wi the polylinker cloning sites and further sub-clon into M13, mp18 and mp19 for sequencing.

The deduced nucleotide sequence for stra S26/3 and its translation are shown in Fig. 1. Tl sequence shows the features previously demo strated for the MOMP gene [22]. These inclu typical promoter elements (although with a long spacing between the cognate elements than found in *E. coli*), an open reading frame of tl expected length, containing 7 cysteine residue and an inverted repeated with a structure simil to that of typical rho-independent transcriptic terminators (inverted repeat followed by 8 T r sidues) [23]. Provisional analysis upstream of tl first promoter region has confirmed the presen of a double promoter as reported for *C. trachom tis* [24] (data not shown).

The first *C. psittaci* MOMP sequence was r ported by Pickett et al. for strain A22/M, al designated as an ovine abortion isolate [25]. Th the S26/3 sequence is shown in Fig. 1 align with that of A22/M. The variation between S26/ and A22/M at the amino acid level is shown Fig. 2 which also includes data for the L2 *trachomatis* MOMP [22]. There is substantial co servation evident in the three sequences especial at the amino and carboxyl termini of the *C. ps taci* genes. However, in the central part of tl molecule there are 4 regions in which S26/3 a A22/M display considerable variation. The i

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                        -35 REGION                          -10 REGION                              SD
S26/3    -85  ACTTGGACGTG GTGCCG CCAGAAGAGCAAATTAGAATAGCGAG CACAAA AAGAAAAGATACTAAGCATAATCTTTA GAGG TGAGT
A22/M

         -22  Met Lys Lys Leu Leu Lys Ser Ala Leu Leu Phe Ala Ala Thr Gly Ser Ala Leu Ser Leu Gln Ala Leu Pro
S26/3      1  ATG AAA AAA CTC TTG AAA TCG GCA TTA TTG TTT GCC GCT ACG GGT TCC GCT CTC TCC TTA CAA GCC TTG CCT
A22/M      1

           3  Val Gly Asn Pro Ala Glu Pro Ser Leu Leu Ile Asp Gly Thr Met Trp Glu Gly Ala Ser Gly Asp Pro Cys
S26/3     73  GTA GGG AAC CCA GCT GAA CCA AGT TTA TTA ATC GAT GGC ACT ATG TGG GAA GGT GCT TCA GGT GAT CCT TGC
A22/M     73                                                                                    A

          27  Asp Pro Cys Ser Thr Trp Cys Asp Ala Ile Ser Ile Arg Ala Gly Tyr Tyr Gly Asp Tyr Val Phe Asp Arg
S26/3    145  GAT CCT TGC TCT ACT TGG TGT GAT GCT ATC AGC ATC CGC GCA GGA TAC TAC GGA GAT TAT GTT TTC GAT CGT
A22/M    145            G                C  C  T

          51  Val Leu Lys Val Asp Val Asn Lys Thr Ile Thr Gly Met Gly Ala Val Pro ... ... ... Thr Gly Thr Ala
S26/3    217  GTA TTA AAA GTT GAT GTG AAT AAA ACT ATC ACC GGC ATG GGT GCA GTT CCT ... ... ... ACA GGA ACC GCA
A22/M    217                                 T T  G          C     AC      ACG CAG GCT       T  A

          72  Ala ... Ala Asn Tyr Lys Thr Pro Thr Asp Arg Pro Asn Ile Ala Tyr Gly Lys His Leu Gln Asp Ala Glu
S26/3    280  GCA ... GCT AAT TAC AAA ACT CCT ACG GAT AGA CCC AAC ATC GCT TAC GGC AAA CAC TTA CAA GAC GCC GAA
A22/M    289  AGT AAT A       C G CC  GAA G A  AT  GC       G                  A  GG   T A G       T   A   G

          95  Trp Phe Thr Asn Ala Ala Phe Leu Ala Leu Asn Ile Trp Asp Arg Phe Asp Ile Phe Cys Thr Leu Gly Ala
S26/3    349  TGG TTC ACC AAT GCA GCT TTC CTC GCA TTG AAT ATC TGG GAT CGC TTT GAT ATT TTC TGC ACA TTA GGC GCT
A22/M    361       T T A          C      A   C   A   C   T              C  C              C       G   A

         119  Ser Asn Gly Tyr Phe Lys Ala Ser Ser Ala Ala Phe Asn Leu Val Gly Leu Ile Gly Val Lys Gly ... Ser
S26/3    421  TCT AAT GGG TAC TTC AAA GCT AGT TCT GCG GCA TTC AAC CTC GTT GGT TTG ATT GGT GTT AAA GGA ... TCC
A22/M    433   C       A             T A       G   T            T G          A   A   G T   TC   CT ACC AG

         142  Ser Ile Ala Ala ... ... ... Asp Gln Leu Pro Asn Val Gly Ile Thr Gln Gly Ile Val Glu Phe Tyr Thr
S26/3    490  TCC ATA GCA GCT ... ... ... GAT CAG CTT CCC AAT GTA GGC ATC ACT CAA GGA ATC GTT GAA TTT TAT ACA
A22/M    505   A  CC T T A C GAG CTT CCA ATG   A       T   C          T   C       T G T   G

         163  Asp Thr Thr Phe Ser Trp Ser Val Gly Ala Arg Gly Ala Leu Trp Glu Cys Gly Cys Ala Thr Leu Gly Ala
S26/3    553  GAT ACA ACA TTC TCT TGG AGT GTA GGT GCA CGC GGA GCT TTA TGG GAG TGT GGT TGT GCG ACT TTA GGA GCA
A22/M    577   C    T    T          C              T                  A                A                T

         187  Glu Phe Gln Tyr Ala Gln Ser Asn Pro Lys Ile Glu Met Leu Asn Val Val Ser Ser Pro Ala Gln Phe Val
S26/3    625  GAG TTC CAA TAC GCT CAG TCT AAT CCT AAA ATT GAA ATG TTG AAT GTA GTC TCC AGC CCA GCA CAA TTT GTG
A22/M    649                          A              G         G  C C   C   C ACT   A

         211  Val His Lys Pro Arg Gly Tyr Lys Gly ... ... Thr Ala Phe Pro Leu Pro Leu Thr Ala Gly Thr Asp Gln
S26/3    697  GTT CAC AAG CCT AGA GGA TAC AAG GGA ... ... ACA GCA TTT CCT TTA CCT CTA ACA GCT GGT ACT GAT CAG
A22/M    721  A        A  A       C  T   A     GCT AGC T G AAT               A    G       A  A ACA G A

         233  Ala Thr Asp Thr Lys Ser Ala Thr Ile Lys Tyr His Glu Trp Gln Val Gly Leu Ala Leu Ser Tyr Arg Leu
S26/3    763  GCA ACT GAC ACT AAG TCG GCT ACA ATT AAA TAC CAC GAA TGG CAA GTT GGT TTA GCG CTC TCT TAT CGA TTG
A22/M    793   T  A       C  A  A                        T              A  C C C  C  G       C A

         257  Asn Met Leu Val Pro Tyr Ile Ser Val Asn Trp Ser Arg Ala Thr Phe Asp Ala Asp Ala Ile Arg Ile Ala
S26/3    835  AAC ATG CTT GTT CCT TAC ATT AGC GTA AAC TGG TCA AGA GCA ACT TTT GAT GCT GAC GCT ATC CGC ATC GCT
A22/M    865   T              A  T   G                                          T A             T

         281  Gln Pro Lys Leu Ala Ala Ala Val Leu Asn Leu Thr Thr Trp Asn Pro Thr Leu Leu Gly Glu Ala Thr Ala
S26/3    907  CAA CCT AAA TTA GCT GCT GCT GTG TTA AAC TTG ACC ACA TGG AAC CCA ACC CTT TTA GGA GAA GCT ACA GCT
A22/M    937                AAA T G  AG A T C T    A T  T                 G     C       TC  A C   T A

         305  Leu ... ... ... Asp Thr Ser Asn Lys Phe Ala Asp Phe Leu Gln Ile Ala Ser Ile Gln Ile Asn Lys Met
S26/3    979  TTA ... ... ... GAT ACT AGC AAC AAA TTC GCT GAC TTC TTG CAA ATT GCT TCG ATT CAG ATC AAC AAA ATG
A22/M   1009   G CCC AAT AAT  G  GG   AG G T GTT C A T    T G

         326  Lys Ser Arg Lys Ala Cys Gly Val Ala Val Gly Ala Thr Leu Ile Asp Ala Asp Lys Trp Ser Ile Thr Gly
S26/3   1042  AAG TCT AGA AAA GCT TGT GGT GTA GCT GTT GGT GCA ACG TTA ATC GAC GCT GAC AAA TGG TCA ATC ACT GGT
A22/M   1081

         350  Glu Ala Arg Leu Ile Asn Glu Arg Ala Ala His Met Asn Ala Gln Phe Arg Phe End
S26/3   1114  GAA GCA CGC TTA ATC AAT GAA AGA GCC GCT CAC ATG AAT GCT CAA TTC AGA TTC TAA
A22/M   1153                                  T
                                                          -----> - ->      <- - <-----
S26/3   1171  GGA TTT AGT TTA TAC TAT CCT AAC TTT TTG TCC CGC TA TCAGAACCTAGGAATCTCTGGCTTCTGA TTTTTTTT GCTCTCAT
A22/M   1210                                                        G  G   C  G                    AC  C
                                                          -------> ->      <- <-------
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ig. 1. Nucleotide sequence and translation of the major outer membrane protein from *C. psittaci* S26/3 ovine abortion strain ɔmpared to that of the A22/M strain [22]. Only differences are shown. Dots in the sequences indicate gaps inserted for optimal ignment. Numbering of the nucleotide sequence begins at the start codon while that of the deduced amino acid sequence begins at the putative N-terminal of the processed MOMP.

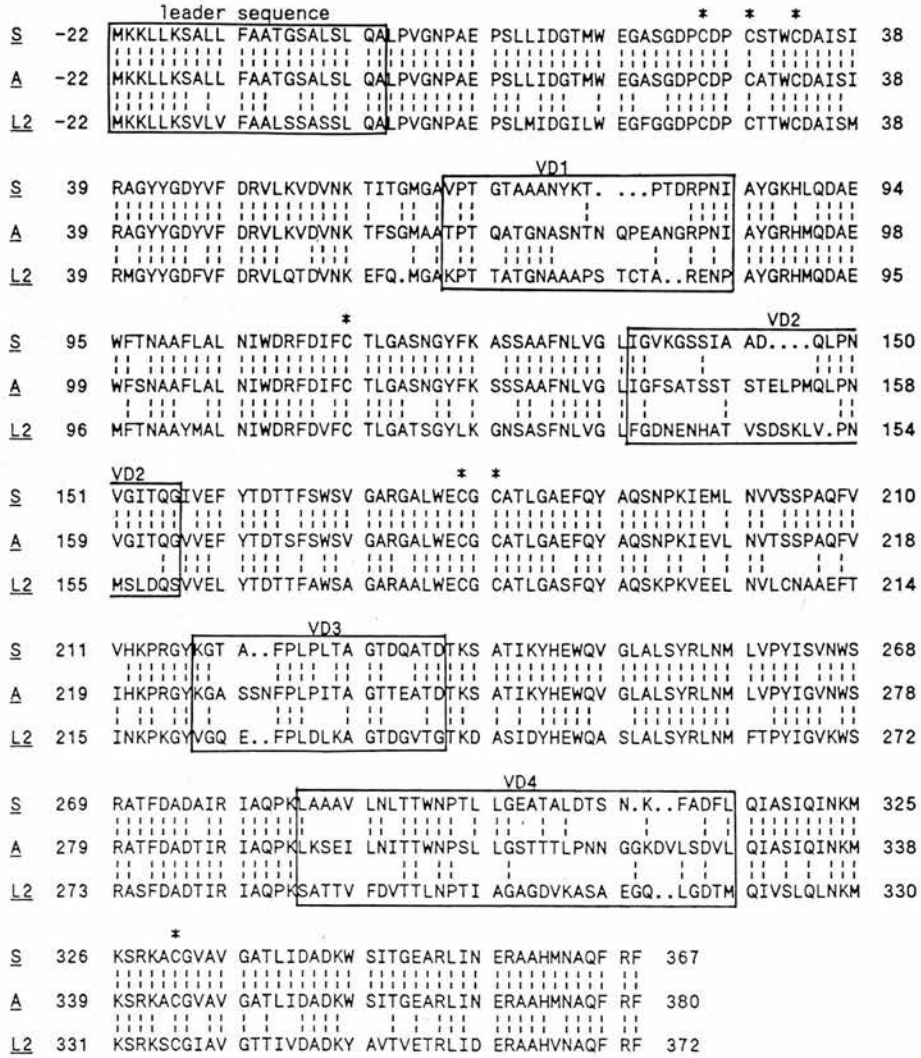


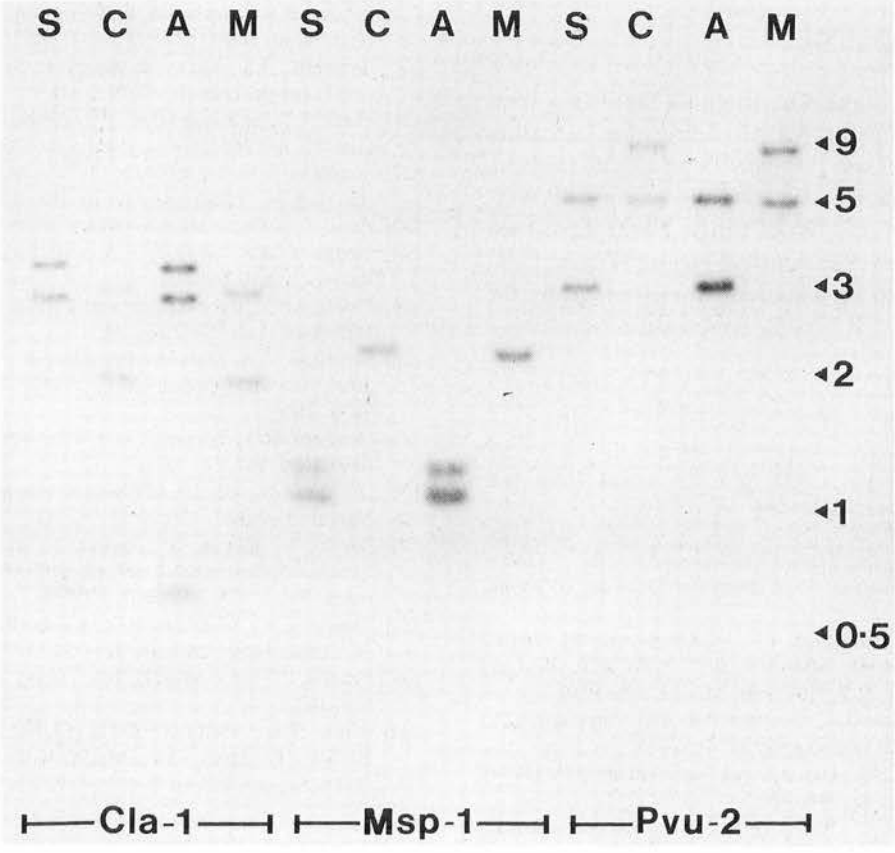
Fig. 2. Comparison of the deduced amino acid sequence of the major outer membrane proteins from *C. psittaci* strains S26/3 (S) an A22/M (A) [22] and *C. trachomatis* serovar L2 [25]. Dots in the sequence indicate the gaps introduced for alignment. The conserve cysteine residues are marked with asterisks and the four variable domains [26] are shown.

tramolecular location of this variation is very similar to that which has already been described by Stephens et al. [26] who determined the sequence differences between the serovars of *C. trachomatis* and described 4 variable domains (VD1-4). Epitope mapping using recombinant proteins and synthetic peptides has shown that important serovar specific and subgroup specific epitopes lie within VD1, VD2 and VD4 [27–29]. This comparison of *C. psittaci* MOMP sequences shows that sequence variation is largely confined to thes same VDs. A similar finding has been reporte recently by Zhang et al. [30] who compared th MOMP sequence of the guinea pig inclusion cor junctivitis strain with that of the avian isolate C 10.

The degree of variation between *C. psitta* S26/3 and A22/M was unexpected. Compariso of a number of isolates from cases of ovine abo tion using RE profiling of total genomic DNA ha

evealed only very minor differences [4,6]. Moreover, no interstrain differences were revealed when ne DNAs from 12 independent abortion strains ere digested with REs (which cut within the MOMP coding sequence) and analysed by Southrn blotting using the Sst-1 MOMP fragment as robe. However, when A22/M was compared with wo abortion strains, including the original A22 olate, in a similar Southern blotting experiment, lear differences were seen, as shown in Fig. 3. he hybridising bands seen in the A22/M strain ere similar to those of the Cal 10 strain. Comarison of the A22/M strain with abortion strains, al 10 and an avian isolate by RE profiling of otal genomic DNA [6] confirmed that A22/M as very similar, but not identical, to Cal 10 (data ot shown). Comparison of the A22/M sequence with that published by Zhang et al. for Cal 10 shows only a 3% difference at the amino acid level.

Since the Cal 10 strain is avian in type as judged by its origin from a case of presumptive ornithosis [31], RE profile [6] and DNA reassociation properties [5], the A22/M strain appears to be avian also. The comparison of MOMP sequences in Fig. 1 above is thus an inter-type rather than an inter-strain comparison. A full history of the A22/M strain is not available but we have established that it should have been identical to A22 which is the original type strain isolated at the Moredun Institute [1]. The isolate has passed through several laboratories and has been grown for many passes in embryonated hen's eggs. It is thus quite possible that it has become con-



g. 3. Southern blot analysis of genomic DNA from strains S57/3 (S) Cal 10 (C) A22 (A) and A22/M (M) digested with Cla-1, sp-1 and *Pvu-11* as indicated and hybridised with a 2.6 kb *Sst-1* fragment containing the complete S26/3 MOMP gene. The position of molecular weight markers is indicated (kilobase pairs).

taminated with an avian strain which has subsequently overgrown.

The above findings only serve to emphasise the urgent requirement for a simple and widely accessible method to classify *C. psittaci* strains. The sequence conservation at the termini of the MOMP gene clearly make it an ideal target for amplification by the polymerase chain reaction (PCR) [32]. Preliminary results using PCR on purified DNA samples have shown that it is possible to amplify the MOMP sequences from a wide range of *C. psittaci* types. Thus our present efforts are directed towards the development of a generally applicable PCR based test for use with clinical samples which can both detect and type *C. psittaci* infections. We are also attempting to express the MOMP gene in *E. coli*.

ACKNOWLEDGEMENTS

We wish to thank Dr. Richard Stephens, University of California, for providing the L2 clones and Dr. Ian Clark, University of Southampton, for A22/M DNA and useful discussions. T.W.T. is in receipt of the Wooldridge Farm Livestock Fellowship from the Animal Health Trust, U.K. S.B. is supported by a studentship provided by the Animal Diseases Research Association.

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