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Cloning and Sequencing of a Putative *C. trachomatis* Ribosomal Protein and Surface Labelling of the 18 kDa Chlamydial Binding Protein

By

Gary John Gray

A Thesis

Submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Medical Microbiology and Infectious Disease

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Cloning and Sequencing of a Putative *C. trachomatis* Ribosomal Protein and Surface Labelling of the 18 kDa Chlamydial Binding Protein

submitted by Gary John Gray in partial fulfillment of the requirements for the degree of Master of Science

Dr. W.M. Wenman

Dr. R. Kaul

Dr. J.A. Robertson

Dr. K.L. Roy

Date: April 23rd 1990

Dedication

To my grandfather, for all that he was able to teach and show me,

To my parents and to D.C.H. for their support and understanding,

To C.C.K., for letting me share all of this with her.

Abstract

A *Chlamydia trachomatis* 18 kilodalton (kDa) protein has been observed to bind ^{125}I-labelled HeLa cell membranes and has therefore been postulated to be important in the pathogenesis of this organism. For this eukaryotic cell-binding protein to play a role in attachment and subsequent infection, it must be surface-exposed on the infectious elementary body (EB). This supposition is supported by work showing the ability of EB membranes to be taken up by host cells nearly as efficiently as whole EBs. Surface labelling experiments have failed to demonstrate unequivocally the location of this protein. For this reason, an investigation of the surface localization of the 18 kDa chlamydial binding protein was undertaken.

Monospecific polyclonal antibodies to the 18 kDa protein bound purified whole EBs in dot-blot analysis, indicating that their reacting epitope, the 18 kDa protein, is surface exposed. Immunogold labelling of purified whole EBs yielded the same result, with the antibodies binding on the surface of the EBs. In immunogold labelling of EBs and RBs in ultrathin sections, these antibodies labelled only the periphery of the EBs, and the RBs not at all.

Cloning and expression of a putative 18 kDa binding protein from *C. trachomatis* serovar L_2 which binds ^{125}I-labelled HeLa cell membranes has been reported from this laboratory. Computer comparison has revealed homology of the N-terminus of this protein to ribosomal proteins L6 (*Escherichia coli*) and L10 (*Bacillus stearothermophilus*). The present work was undertaken to

determine the precise role of this putative binding protein and to establish the significance of this protein's apparent close relatedness to ribosomal proteins L6 and L10.

Clones of various sizes from different restriction digests were selected from gene banks of serovars D, J and K genomic DNA using a fragment of the clone encoding the putative binding protein (pCT161/18) as a probe. Restriction mapping and sequence determination of these clones revealed limited homology with the pCT161/18 restriction map and sequence. Southern hybridization of genomic DNA from serovars D, J, K, and L_2 revealed that pCT161/18 is a cloning artifact, resulting from the recombination of at least two unrelated pieces of chlamydial DNA.

The new clones were found, by Southern hybridization, to be representative of the genomic restriction pattern. The largest clone encoded an open reading frame (ORF) which directed the synthesis of a protein of 23 kDa apparent molecular weight. This protein was 66.1% homologous to the L10 protein, and 60.2% homologous to the L6 protein in primary structure. In addition, this protein, designated CL6, cross-reacts immunochemically with a mixture of polyclonal antibodies to ribosomal proteins L3 and L6 of *E. coli*. Further structural and antigenic homologies between these three proteins (as determined by computer analysis), together with the above-mentioned primary structural homology, indicate that CL6 is a ribosomal protein homologous to the ribosomal proteins L6 and L10.

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List of Abbreviations

BHIB	brain-heart infusion broth
Bis	N,N'-methylene-bis-acrylamide
BSA	bovin serum albumin
Ci	Curie
CPM	counts per minute
CsCl	cesium chloride
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EB	elementary body
EDTA	ethylene diamine tetraacetate
EtBr	ethidium bromide
FSB	final sample buffer
GBq	gigaBecquerel
HBSS	Hank's balanced salt solution
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
kV	kilovolt
LGV	lymphogranuloma venereum
MAb	monoclonal antibody
MOMP	major outer membrane protein
NP40	nonidet-P40
OA	ovalbumin
ORF	open reading frame

PAGE ..... polyacrylamide gel electrophoresis

PBS ..... phosphate buffered saline

RB ..... reticulate body

REB ..... tris-acetate

RF ..... replicative form

RNase ..... ribonuclease

SDS ..... sodium dodecyl sulfate

SSC ..... sodium chloride-sodium citrate

TAES ..... N-tris(hydroxy-methyl)methyl-2-aminoethanesulfonic acid

TE ..... tris-EDTA

TEB ..... tris-EDTA-borate

TEMED ..... N,N,N',N'-tetramethylethylene diamine

TES ..... tris-EDTA-sodium chloride

Tris ..... tris(hydroxymethyl)-aminomethane

TSA ..... tris saline

TSN ..... tris saline-NP40

UV ..... ultraviolet

x g ..... times gravitational force

X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

YT ..... yeast-tryptone medium

1. Introduction

1.1 Characteristics and development of Chlamydia species

The order Chlamydiales consists of one family, Chlamydiaceae, comprising one genus, *Chlamydia*, and three species, *Chlamydia trachomatis*, *Chlamydia psittaci* and *Chlamydia pneumoniae* (1,2). As obligate intracellular parasites, the chlamydiae demonstrate fundamental differences from most bacteria. They have a smaller size, a smaller genome, a longer generation time, and they require an exogenous energy supply for growth (3). Long thought to be viruses due to their sometimes intracellular nature, chlamydiae differ from viruses in several important ways. The former contain both DNA and RNA, they divide by binary fission, they contain ribosomes, they have a defined cell envelope similar to that of gram negative bacteria, and they are sensitive to certain antibiotics (3).

Chlamydiae are pathogenic and are implicated in a broad spectrum of disease in animal and avian species (4,5,1). *C. psittaci* is found principally in birds and lower mammals, occasionally causing the respiratory infection psittacosis in humans (5). Various strains are separated into avian and mammalian branches based on the lack of serological cross reaction between the two groups. Further subdivision has not been possible to date due to the extreme heterogeneity of this species (1). *C. pneumoniae* is a new species describing the TWAR (TaiWan Acute Respiratory disease) strains, which have been isolated from cases of acute respiratory infection in humans (2). *C. trachomatis* is the most clinically-relevant species in North America, being the leading cause of sexually transmitted disease (2,5). This species is subdivided into three biovars: mouse,

lymphogranuloma venereum (LGV), and trachoma. This subdivision is based on behavior in natural hosts (host range and preferred site of infection), behavior in laboratory animals (pathogenesis in mice and primates), and behavior in cell culture (1).

The biovars of *C. trachomatis* found in humans are further subdivided into serovars based on serology (6). The LGV biovar is made up of three serovars, L_1, L_2, and L_3. These organisms are responsible for a sexually transmitted invasive infection of the lymph nodes. The trachoma biovar is made up of twelve serovars, A-K. Serovars A, B, Ba, and C are associated with endemic trachoma (conjunctivitis) in certain underdeveloped countries. The remaining eight serovars (D-K) are most frequently isolated as causative agents of genital tract infections in adults and neonatal conjunctivitis (inclusion conjunctivitis) or pneumonitis in babies born to infected mothers (5,7). The trachoma and LGV serovars are further grouped by serological cross-reactivity into complexes B (serovars B, Ba, D, E, F, G, L_1 and L_2) and C (serovars C, J, A, H and I) with two serovars (K and L_3) forming a serological 'bridge' between these complexes.

The *Chlamydia* species may be differentiated by various parameters. *C. trachomatis* strains are sulfonamide-sensitive with glycogen-containing inclusions that stain with iodine, while *C. psittaci* and *C. pneumoniae* are sulfonamide-resistant and their inclusions do not contain glycogen (and hence do not stain with iodine). Further differentiation is accomplished serologically (1).

Relatedness within chlamydial species (as determined by DNA-DNA hybridization) is greater than 94%, except for the mouse biovar, which demonstrates only 30 to 60% homology to the other two *C.*

trachomatis biovars (8,9). In contrast, relatedness between chlamydial species, measured by the same technique, is 10% or less, much less than the 20% expected for members of the same genus (8,10). Nonetheless, these species are clearly related based on common antigens, common developmental cycle and developmental forms, and similar metabolic and biological activities, supporting their placement in a single genus (3,11).

The developmental cycle of these organisms is unique and complex. As intracellular parasites, chlamydiae have evolved morphologically distinct infectious and reproductive forms. The elementary body (EB) is the extracellular infectious form. It is small (0.2-0.3 μm in diameter) and has an electron dense core. The intracellular, non-infectious reticulate body (RB) is larger (approximately 1.0 μm in diameter) and possesses a more evenly dispersed cytoplasm (12-14).

The cell envelopes of both developmental forms consist of an inner cytoplasmic membrane and an outer membrane resembling those of gram-negative bacteria (12). Tamura and Manire (15) have reported morphological and chemical differences between the envelopes of the two forms. In electron micrographs, EB cell envelopes appear as folding, rigid membranes. RB cell envelopes, in contrast, are thin, flattened structures. The protein profiles of the envelopes also differ between forms (12,16). EB outer membranes contain a single predominant protein (about 60% of outer membrane protein mass) of about 42 kilodaltons (kDa) in size (17,16). Proteins of 60 kDa, 74 kDa, 96 kDa, 12 kDa and other minor proteins are also present (16,18). Labelling of these proteins during growth in media

containing ^{35}S-cysteine demonstrates the presence of three "cysteine-rich" proteins (42 kDa, 60 kDa and 12 kDa) in the outer membranes of all *C. psittaci* and *C. trachomatis* strains studied to date (16). Only the 42 kDa cysteine-rich major outer membrane protein (MOMP) is present in RB envelopes, in approximately the same quantity as in EB envelopes (12). Muramic acid has not been detected in the envelopes of either form (17); it is thought that disulfide cross-linking between the cysteine-rich outer membrane proteins lends the EB its rigidity and osmotic resistance. The RB envelope, lacking all but one of the cysteine-rich proteins, possesses less cross-linking and is therefore more pleomorphic and fragile (12,19-21)

As one would expect based on their respective roles in the developmental cycle, these two forms differ functionally. The EB displays cytotoxic effects in cell culture (22) and also demonstrates hemagglutinating ability (23,24) and eukaryotic cell membrane binding ability (25,26) not seen in RBs. The RB demonstrates metabolic functions such as transport (27) and protein synthesis (28) which are not seen in EBs.

The developmental cycle begins with the attachment of the EB to the host cell membrane. In order for the developmental cycle to proceed further, the EB must be taken up by the cell. Large inocula of EBs have been shown in cell culture to be immediately toxic to host cells, but this toxicity is dependent upon uptake (29). The uptake mechanism appears to be parasite-directed, resulting in rates of entry into host cells 10-100 times that of latex particles or non-invasive *Escherichia coli* (30). Wyrick *et al.* (31) have investigated

the route of entry of chlamydial EBs into epithelial cells in vitro by employing polarized cell culture techniques with transformed cell lines (HeLa) and with primary cell culture (human endometrial gland epithelial cells). They hypothesize that this cell culture system more closely approximates the *in vivo* sytem of nutrient transport polarity, hence maintaining these cells under more physiologically-normal conditions. Using this system, they have demonstrated the association of EBs with clathrin coated pits and vesicles and have therefore concluded that EBs enter their host cells by the receptor mediated endocytosis pathway. In order to counter any bias due to the use of artificially high multiplicities of infection (m.o.i. ; Wyrick *et al.*used an m.o.i. of 2000 EBs per host cell in their work), Hodinka and Wyrick (32) performed transmission electron microscopy of serial sections of cells infected with an m.o.i. of 10. Their work also demonstrated the association of EBs with clathrin coated pits and vesicles. This contrasts with earlier work by Ward and Murray (33), the results of which suggested a microfilament- dependent "zipper mechanism" model in which close association of the EB to the host cell surface is important, but the presence of clathrin coated pits is not. This work may have been hampered by the employment of non-polarized cell culture techniques, as it has been shown (31) that confluent HeLa cell monolayers grown on plastic (non-polarized) possess few clathrin coated pits. It seems then, that the primary route of uptake for chlamydiae into host cells is *via* receptor-mediated endocytosis.

Once ingested, the chlamydiae are enclosed in an endosomal vacuole. Like the obligate intracellular parasite *Coxiella burnetii*,

chlamydiae remain inside this vacuole throughout their intracellular life. Unlike *C. burnetii*, which requires phagolysosomal fusion for transport and metabolism, the chlamydiae are able to prevent phagolysosomal fusion (34). Since *C. psittaci* cell envelopes can prevent phagolysosomal fusion, while heat-killed or antibody-coated *C. psittaci* cannot, this ability is believed due to a heat-labile cell surface component of the chlamydial organism (35). It has been suggested that the failure of *C. psittaci* or *C. trachomatis* organisms to grow in certain host cell strains following random attachment may be due to their failure to prevent phagolysosomal fusion (34).

Soon after entering the host cell, the EB begins to differentiate morphologically into the intracellular RB form. The cell wall becomes less rigid, the formerly electron-dense DNA core disperses, the cytoplasm becomes granular due to ribosome production, and the size increases from 0.3 μm to 1.0 μm in diameter. Approximately 9 hours after infection (in the serovar L_2 system), the differentiation of the EB into the much larger RB is complete. The RB will mature within the vacuole and then divide by binary fission (34).

Approximately 20 hours post-infection, RBs begin to reorganize back into infectious EBs. DNA condensation occurs now in the RB, possibly at multiple sites to give rise to more than one EB by cell division. At this point of chlamydial development the endosomal membrane has expanded to form an inclusion occupying over three quarters of the cell volume. This size expansion is unexplained, but may be a result of chlamydial-directed lipid synthesis (34).

Productive chlamydial development inevitably destroys the host cell. Cellular organelles degenerate and necrose, the host

ribosomes and polysomes are lost, the endoplasmic reticulum dilates and vesiculates and the microvilli disappear. EBs may simply be released due to rupture and lysis of endosomal and cytoplasmic membranes, or lysosomal degeneration and release of enzymes may result in autolysis and release of EBs (36). In non-productive chlamydial development, persistent and latent infections arise and are believed transferred between eukaryotic cells at division (37).

1.2 Factors in the adherence of bacteria and viruses

Adherence is an important early event in the pathogenesis of bacterial and viral infections in animals and man. Many bacteria and all viruses possess surface macromolecules which promote adherence to specific receptors present on some animal cells but not others. This selective adherence leads to host and tissue specificities in infections with various pathogens.

The adherent properties of bacteria have long been known. As early as 1908 studies were performed by Guyot on the adhesiveness of bacterial cells for erythrocytes (38). In 1964, Labrec *et al.* (39) were one of the first groups to demonstrate the necessity of adherence for successful pathogenesis in bacterial infections. Using the invasive intestinal pathogen *Shigella flexneri* in guinea pig and rhesus monkey animal models and in HeLa cell culture, they observed a group of mutants which could not stably adhere to epithelial cells *in vitro* and were consequently avirulent.

Since this time, many advances have been made in the study of bacterial adherence, particularly in oral microbiology. The focus on oral microbiology has been due to the accessibility of the surface as

well as the problem of persistent colonization despite the constant flow of sterile saliva (40). Advances in the study of adherance with pathogens specific for other body regions have been less forthcoming, due mainly to the inaccessibility of these regions. With the introduction of widespread use of tissue culture, the study of the adherence of gastro-intestinal and urogenital pathogens has surged.

Using cell culture techniques in conjunction with animal models, the successful pathogenesis of many diseases has been correlated to the production of some surface macromolecule mediating adherence. Investigations into the pathogenesis of *Neisseria gonorrheae* indicate that fimbriated organisms are more infective *in vivo* as well as more adherent *in vitro* (41). Fimbriae are non-flagellar filamentous appendages approximately 4.0-10.0 nm in width and 0.5-4.0 μm long. They are generally evenly dispersed across the bacterial surface, are projected outward from the outer membrane, and, except for certain *Corynebacterium* spp., are restricted to gram-negative bacteria (42).

The importance of fimbriae in the pathogenesis of the enteric bacteria has also been demonstrated. *Escherichia coli* (43,44), *Salmonella* spp. (43) and *Proteus mirabilis* (45-47) all demonstrate a positive correlation between fimbriation and *in vitro* adherence and *in vivo* infectivity. For the gram-positive *Streptococcus* spp. involved in oral pathogenesis, the production of an adherent extracellular polysaccharide matrix (dextran) has been positively correlated with *in vitro* adherence and *in vivo* infectivity (48).

The relationship between expression of these adherence factors and infectivity has been implied by attempts to block the action of

these factors. Several studies (49,50) have demonstrated the ability of homologous antibodies to fimbriae to neutralize the activity of these factors, and these antibodies are protective. As well, these antibodies have been shown to block adherence to host tissues or cells in vitro (51).

The characterization of these adherence factors has largely been accomplished by the study of their interaction with host cell surface macromolecules. In particular, adhesins associated with the fimbriae of bacteria in the family Enterobacteriaceae have been categorized based on their sensitivity to the sugar mannose (or its structural analogues) in blocking adhesion (43). These data suggest that the mannose-sensitive adhesins of the enteric pathogens may act by binding mannose residues on glycosylated host cell surface molecules. Mannose-resistant adhesins also exist which may bind other sugar moities present on the host cell surface (43).

These fimbrial adhesins and the extracellular polysaccharide of the oral streptococci appear to mediate colonization, assisting in the pathogenesis of the organism. Lately, studies of bacterial adherence have focused on those pathogens for whom adherence to and subsequent uptake into the host cell is important. Brunius and Boln (52), in studying the interaction of the invasive pathogen *Yersinia pseudotuberculosis* with the HeLa cell membrane (at 4°C to prevent uptake) noted that the attachment process was protease-sensitive, divalent cation-independent, and involved microvilli. Electron microscopy demonstrated the organisms in intimate association with the glycocalyx of the HeLa cell monolayer.

Further characterization of the adhesin of *Y. pseudotuberculosis* has been accomplished by Isberg *et al.* (53). Using an *in vitro* adherence assay employing HEp-2 cell culture, this group was able to select several recombinant *E. coli* which bound tightly to the monolayer and were subsequently ingested. The recombinant DNA fragment coded for a 103 kDa protein and several incomplete products of smaller size. Since this locus coded for a protein specifying adherence and uptake into host cells, Isberg et al. termed this the invasion, or *inv*, locus. *Y. pseudotuberculosis* organisms with mutations in this locus did not bind host cells.

In contrast to this situation, work by Finlay *et al.* (54,55) with the invasive enteric pathogens *Salmonella choleraesuis* and *S. typhimurium* in polarized cell cultures has demonstrated a temperature-sensitive adherence (no adherence at 4°C) that is correlated with the production of several different proteins. In addition, association with the host cell monolayer was found (by treatment of the bacteria with rifampin and chloramphenicol, respectively) to require RNA and protein synthesis and which is induced by trypsin and neuraminidase sensitive structures present on the epithelial cell surface. Bacteria with mutations in the genes for one or more of the proteins identified did not adhere to or did not invade the polarized monolayer and were avirulent in mice.

A protein adhesin has been identified in *Mycoplasma pneumoniae*, a respiratory tract pathogen, which appears to have a homologue in the related species *M. genitalium*, a genital tract pathogen. The P1 protein of *M. pneumoniae* (170 kDa in size) and a 140 kDa protein of *M. genitalium* cross-react serologically and

possess DNA sequence homology (56). Monoclonal antibodies to P1 have localized this protein to the specialised tip structure of the organism (57), and have inhibited hemadsorption and adherence to hamster tracheal rings (58). Adherence and hemadsorption have been identified as separate properties in *M. pneumoniae* using hemadsorption-negative mutant strains, but these mutants appear unable to initiate any cytopathology (59). Both hemadsorption and adherence of *M. pneumoniae* are sensitive to neuraminidase treatment of the host cell (60,61), suggesting a sialic acid receptor for this pathogen.

As obligate intracellular parasites, both prokaryotes and viruses must replicate within a host cell and therefore must possess some means of attaching themselves to host cell surfaces prior to being ingested (3). Surface proteins involved in adherence to host cells have been identified for most viruses. Virus attachment is mediated by specialised structures referred to as virion attachment proteins (VAPs) (62).

Specificity of virus interactions with host cells has been analyzed in various systems by competitive inhibition with highly purified viral subunits. A good example of this is the adenovirus system in which fiber antigen has been identified by competitive inhibition as the means of attachment to host cells (63).

In both enveloped and non-enveloped viruses, VAPs are present in multiple copies in the virus particle (64). The virion of Semliki Forest virus contains about 240 copies of the glycoprotein VAP (65), there are 12 oligomeric fiber protein VAPs in the virion of adenovirus (64), 300-450 hemagglutinin subunits are present in the

virion of influenza virus (64), and 24 molecules of σ1 protein VAP are found in the virion of reovirus (66).

In the enveloped viruses, protein adhesins are mostly glycoproteins inserted in the virus envelope. These glycoproteins are usually multimeric and are present in the form of spikes 7 to 10 nm in length. When the virions are treated with protease to remove the glycoprotein spikes, infectivity is dramatically reduced. Removal of oligosaccharides from these glycoproteins abolishes virus particle binding to host cells and erythrocytes (64). For some viruses, the local environmental conditions may cause a conformational change in the VAP to permit attachment and entry of virions into host cells (62). For example, the viral hemagglutinins of influenza viruses and orthomyxo- and paramyxoviruses must undergo proteolytic cleavage for viral infectivity (67,68).

The VAPs of the non-enveloped icosahedral mammalian viruses are localized in the outer shell or capsid of the virus (66). The capsid is a geometrically symmetrical structure which is assembled from subunits composed of proteins in a stoichiometrically regular arrangement (3). The proteins making up each subunit are called capsid proteins or outer shell proteins (3). These proteins are not glycosylated but they are responsible, singly or *via* interactions with other capsid proteins, for attachment to host cell surfaces (69). In the reovirus, the minor outer shell protein σ1 has been identified as the VAP (66). In the virus family Picornaviridae, containing rhinovirus, foot and mouth disease virus, coxsackie virus and polio virus among others, attachment functions have been demonstrated to be the domain of the capsid proteins VP1

and VP3 (69). These proteins line the walls of a major "canyon" in the virus capsid. Antiviral compounds have been demonstrated by X-ray crystallography to bind in this region, blocking adsorption of the virus to host cells by altering the conformation of the cleft (70). Most host cell virus receptors identified to date are glycosylated surface proteins (71).

Less information is available regarding the attachment processes involved in the pathogenesis of the obligate intracellular prokaryotes. A putative rickettsial attachment protein 130 kDa in size has been cloned which reacts with neutralizing antibodies and stimulates animals to resist challenge with *Rickettsia rickettsii* (72). The protein is heat-labile, and other workers have shown that antibodies to heat-labile surface proteins of Spotted Fever group Rickettsiae are protective (73). Characterization of specific surface factors involved in the pathogenesis of *Coxiella burnetii* has not been undertaken as yet, but it has been determined that whole cell preparations of the organism and outer membrane subfractions are protective as vaccines (74).

1.3 Chlamydial adherence

The developmental cycle of *Chlamydia* spp., as described above, requires the invasion of and growth within a host cell. The initial EB attachment to the host cell represents a critical point of interaction, and appears necessary for successful invasion (33,34). There are two components to the attachment of any organism or particle to a membrane: non-specific electrostatic forces, and specific interactions with membrane surface receptors (41,34). Isoelectric focusing data

suggest that LGV and trachoma strains possess net negative charges with identical isoelectric points (pI) of 4.64 (75). Host cell membranes also carry a net negative charge at physiological pH, predicting the existence of long range electrostatic repulsive forces between the EB and the host cell membrane (34). This prediction is supported by work showing the enhancement of chlamydial attachment *in vitro* using polycationic compounds such as diethyl aminoethyl (DEAE)-dextran or poly-L-lysine (76-78). Strains of the trachoma biovar show enhanced attachment to HeLa cells by treatment with polycations or by centrifugation onto the monolayer, while LGV strains will efficiently infect cells without these inducements (78,34). Since the net negative charges of these strains are similar, this variation may reflect differences in host cell receptor binding avidity (34).

The study of specific interactions of EBs with host cell membrane receptors may be hampered by the use of transformed cell lines such as HeLa (human) and McCoy (mouse) and L (mouse) cell cultures. It has been suggested that, since most clinically important isolates of *C. trachomatis* must be centrifuged onto these monolayers to develop efficient infection, these transformed cells probably lack receptors or other factors used by chlamydiae *in vivo* (34). Indeed, the successful use of both human and mouse transformed cell lines for culture of chlamydiae demonstrates a lack of species specificity shown *in vivo* by the different biovars of *C. trachomatis*. Work done by several groups has demonstrated that *C. trachomatis* will bind to fetal tonsil cells (77), fetal rat astrocytes and neurons (79), human promyelocytes (80), baby hamster kidney cells

and mosquito (*Aedes aegypti*) cells (81). It should be noted, however, that the attachment of EBs to these varied cell cultures did not always lead to uptake and inclusion formation. Of those listed, only fetal rat astrocytes were demonstrated to be competent for inclusion formation (79). Polarized cell culture techniques may show the most promise in future studies of chlamydial adherence to host receptors *in vitro*. Wyrick *et al.* (31) were able to demonstrate association of EBs with clathrin coated pits, indicating binding to specific receptors in these pits, with sub-confluent polarized monolayers of the HeLa transformed cell line.

Several groups have undertaken the study of the kinetics of EB attachment to host cell membranes *in vitro*. Soderlund and Kihlstrom (82) studied adherence of LGV biovar EBs to McCoy cells at 4°C to block uptake. They noted a constant attachment rate over an 8-fold increase in dosage. In contrast to this, Bose and Smith (83) were able to demonstrate positive cooperativity in the attachment of trachoma biovar EBs to HeLa cells. They noted an abrupt increase in adherence rate as the dose was increased. This effect was only seen when freshly isolated EBs were used in the study. In addition, adherence of *E. coli* and *Staphylococcus aureus* was enhanced to HeLa monolayers treated with infectious but not heat-killed LGV biovar EBs, suggesting positive modulation of adherence by an unknown mechanism. Flow cytometric studies by two groups with ^{3}H-labelled LGV biovar EBs in L cell culture have examined the absolute rate of binding (84,85). They found that binding of EBs to host cells was a rapid event, reaching maximum at 60 minutes, whereas ingestion of the EBs is much slower, and is complete by 4 hours. The LGV biovar

EBs were noted to be very infectious, with 90% of attached cells taking up 70% of the EBs bound.

The characterization of the factor or factors responsible for the adherence of EBs to host cells has been carried out in *in vitro* systems by several groups. Essentially, these groups examined the adherence of radio-labelled EBs or EB membranes following their treatment or the treatment of the host cells with blocking factors. Exposure of EBs to heparin (77), heat inactivation (86,87), antiserum (87), chitobiose and chitotriose (82,88) and di- and trisaccharides of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine (88) all inhibited association with host cells. Treatment of EBs with trypsin, chymotrypsin and neuraminidase had no effect on their rate of attachment to host cells (86). These data support the nomination of a heat-labile surface macromolecule containing no sialic acid as the chlamydial adhesin. Lack of interferences by trypsin and chymotrypsin treatment may simply reveal the lack of exposed suitable cleavage sites on these factors, or they are not proteinaceous.

Characterization of the chlamydial receptor on host cells has followed the same path. Treatment of epithelial cell monolayers (mouse and human) with dextran sulfate, trypsin, and wheat germ agglutinin (86,89,87) inhibit EB binding, while treatment with neuraminidase or tunicamycin has no effect (86,89). Contrary to these data, Lee (78) has demonstrated no blockage of EB binding to McCoy cells following trypsin treatment of the host cells, and Kuo *et al.* (76) showed the inhibition of EB attachment to HeLa cells following neuraminidase treatment of the host cells. These

contradictory data may be at least partially explained by variations in methodology: Lee (78) used a lower concentration of trypsin and Kuo *et al.* (76) used a much higher concentration of neuraminidase than other groups have used.

With the introduction of ligand electroblotting techniques, it became possible to identify putative adhesins by their ability to bind ^{125}I-labelled host cell membranes on a nitrocellulose blot of whole EB lysates resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This technique enabled the identification of two membrane binding factors (25,26) which were trypsin sensitive. Their presence only in the EB form of the organism suggested their specific role in infection. These two proteins, 18 kDa and 31 kDa in size, have been identified in all serovars studied to date (90). Antibodies to these proteins have been shown to neutralize infectivity *in vitro* (25). Attempts to localize these proteins at the EB surface by lactoperoxidase-mediated ^{125}I surface labelling have been inconclusive (16,25,26). For instance, weak labelling of an 18 kDa species may be due to a host contaminant associated with purified EB membranes, while the failure of the 31 kDa protein to label may reflect the unavailability of appropriate residues at the surface.

The glycosylation of these specific proteins has not been studied. However, studies of chlamydial protein glycosylation using sensitive staining methods (91) and labelling of EBs with ^{3}H-glucosamine (92) have revealed the potential glycosylation of only the 40 kDa MOMP. These data are indirect and, once again, the problem of host contaminants must be addressed (16).

Further characterization of the 18 kDa putative chlamydial adhesin has been accomplished by Kaul *et al.* (93). This group has cloned and sequenced the gene for this protein from serovar L_2 and has achieved the expression of an 18 kDa clone-directed product which binds ^{125}I-labelled HeLa cell membranes by the ligand-electroblotting technique. Primary sequence comparisons of this protein with the SWISS-PROT protein data bank *via* BIONET (unpublished) have revealed significant homology of the N-terminus of this binding protein with the homologous (94) ribosomal proteins *E. coli* L6 (EL6) and *Bacillus stearothermophilus* L10 (BL10). Work done by Gregory (95) demonstrated that ribosomal proteins of *Streptococcus mutans*,an oral pathogen, are antigenic and that dental caries-free patients exhibit significantly higher levels of naturally-occurring secretory and serum antibodies to preparations of ribosomes of *S. mutans*. This may indicate some cross-reactivity of binding proteins with ribosomal proteins (96). Indeed, ribosomal preparations have shown some efficacy as potential vaccines against *Candida albicans* (97,98).

1.4 Ribosomal proteins

Ribosomes are an integral part of both eukaryotic and prokaryotic cellular machinery. In the prokaryotes, which usually exhibit much higher growth rates than eukaryotes, ribosomes can account for more than 40% of the dried mass of the cell (99). At high growth rates, ribosome production is proportional to the square of the growth rate (100).

The ribosomes of most bacterial species are similar in size and composition (94). The entire ribosome, with a sedimentation coefficient of 70S, can be broken into two smaller subunits of 30S and 50S size (101). Two-thirds of the mass of the 30S subunit is accounted for by a single 16S ribosomal RNA (rRNA) molecule. The remaining structure of the 30S subunit is provided by ribosomal proteins (r-proteins) of which there are approximately 20 (100,101). In the 50S subunit, two rRNA molecules of 23S and 5S size again make up two-thirds of the total mass, with approximately 30 structural r-proteins making up the remainder (100,101).

The structure and function of the *E. coli* ribosome has been the focus of most ribosome research in the past 30 years, with some work also being done with *B. stearothermophilus* (100,102). R-proteins in these bacteria are designated as S (30S subunit, or small) and L (50S subunit, or large) proteins. Immunological evidence has identified a great deal of homology between *B. stearothermophilus* and *E. coli* r-proteins (94). Based on evolutionary considerations and the importance of the ribosome in cell growth it is reasonable to expect that, generally, there will be one-for-one homology among the ribosomal proteins of eubacteria (as among the rRNAs), with each of the proteins from one species being represented by a structural and functional homologue in any other species (94). This expectation of homology has been exploited in the development of a prokaryotic phylogenetic tree based on relatedness of ribosomal RNA (rRNA) sequences between species (103).

Investigations into ribosome structure and function in *Chlamydia* spp. have dealt only with cloning and characterization of

rRNA genes (104) and operons (105). Based on rRNA sequence homologies, the chlamydiae have been identified as eubacterial in origin (106). To date, no work has dealt with the ribosomal proteins of the chlamydiae.

1.5 The scope of this project

This thesis will examine the surface exposure of the 18 kDa adhesin in *C. trachomatis*. I will attempt to localize the 18 kDa binding protein by dot-blot analysis and by immunogold-labelling techniques.

The remainder of the work presented here will deal with the elucidation of the relationship of the cloned 18 kDa binding protein to ribosomal proteins EL6 and BL10. I report here the cloning, sequencing and partial characterization of a putative ribosomal protein from *C. trachomatis* serovar J which possesses complete homology with the amino-terminal region of the 18 kDa protein cloned by Kaul *et al.* (93).

2. Materials and Methods

2.1 Bacterial strains and media

C. trachomatis serovars L_2 (L_2/434/Bu), J (J/UW-36), D (D/UW-3), and K (K/UW-31) were grown in HeLa 229 culture as described by Kuo *et al.* (105). Briefly, infectious EBs were incubated with HeLa monolayers for 2 h at room temperature, the medium was discarded and replaced with fresh culture media containing 10% fetal calf serum. Flasks were kept in an incubator at 37°C and an atmosphere of 5% CO_2. Cultures were harvested at 40 h for EBs, using sterile glass beads to disassociate the adherent eukaryotic cells. This cell suspension was sonicated and submitted sequentially to differential centrifugation (500 x g for 15 min then 43,000 x g for 30 min through 35% Renografin [E.R. Squibb & Sons]), discontinuous Renografin gradient centrifugation (40 to 52% Renografin at 43,000 x g for 1 h), and centrifugation through 10mM N-tris(hydroxy-methyl)methyl-2-aminoethanesulfonic acid (TAES;Sigma Chemical Co.) to remove the Renografin (31,000 x g for 30 min). The EB pellet was resuspended in TAES to a final protein concentration of 5 mg/mL. Cultures were harvested in a similar fashion at 18 h for RBs.

E. coli strains JM83 and DH5αF' were used for propagation of plasmids and M13 bacteriophage, respectively. JM83 was maintained and grown on yeast tryptone (YT) medium and agar. Selective pressure for bacteria carrying plasmids was maintained by the addition of ampicillin (to 100 μg/mL). DH5αF' was maintained and grown on minimal medium (+ 20% glucose). Transfected cells were grown on YT plates without selective pressure. DH5αF' cells

were used for M13 plating because they possess a stable F' episome, necessary for infection.

E. coli strain p678-54 was maintained and grown on YT medium and agar and was used for minicell studies of expression. This mutant strain exhibits a defect in cell division, producing tiny cells containing ribosomes, enzymes, and copies of multicopy plasmids (if transformed) but no chromosomal DNA (108). Brain-heart infusion broth is used to chase the radiolabel in these experiments.

These cells were made competent for transformation or transfection essentially according to the method of Hanahan (109). An overnight culture (5 mL) of the appropriate strain (JM83 and p678-54 in YT; DH5αF' in minimal medium) was inoculated into 500 mL of YT and shaken for 1.5-2 h at 37°C ($O.D._{660}$= 0.3-0.4). The culture was then stored at -20°C for 15 min and pelleted by centrifugation at 1300 x g for 5 min. The pellet was resuspended in 100 mL of 50mM $CaCl_2$, 10 mM Tris-HCl (pH 8.0), stored at -20°C for 15 min and pelleted again at 1300 x g for 5 min. This pellet was resuspended in 20 mL of 50 mM $CaCl_2$, 10mM Tris-HCl, 10% glycerol (pH 8.0), frozen in a dry ice/isopropyl alcohol bath, and stored at -76°C. For use, these cells were thawed on ice.

2.2 Plasmid and bacteriophage strains

All cloning and sequencing work was accomplished using the plasmids pUC 18 and pUC 19 and the bacteriophages M13mp18 and M13mp19 (110). Plasmids were maintained in *E. coli* JM83, while bacteriophage were maintained in DH5αF' (section 2.2).

The recombinant clone described by Kaul *et al.* (93), designated pCT161/18, was also maintained in JM83 as described above.

2.3 Enzymes and reagents

Restriction enzymes were supplied by Boehringer Mannheim, Bethesda Research Laboratory, and New England Biolabs. All restriction endonuclease digestions were carried out under the conditions prescribed by the manufacturers.

All radioisotopically labelled compounds were obtained from Amersham Inc. These include [α^{32}P]dATP (~3000 Ci/mmol), ^{125}I (4.0 GBq/mL), and [^{35}S]methionine (>800 Ci/mmol). Other enzymes for molecular biology (T4 DNA ligase, Klenow fragment, calf alkaline phosphatase, etc.) were obtained from Boehringer Mannheim. The Sequenase reagents and enzyme for dideoxy sequencing were purchased from United States Biochemical. Primers for sequencing were supplied by Boehringer Mannheim and the Regional DNA Synthesis Facility (University of Calgary). The specific primer GGR#1 was a gift from Dr. K.L. Roy. Oligonucleotides for random primer labelling of DNA were also supplied by the Regional DNA Synthesis Facility (University of Calgary). *In vitro* transcription-translation kits were purchased from Amersham Inc. X-omat AR X-ray film was obtained from Eastman Kodak Co.

Nitrocellulose for electrophoretic transfer of DNA and proteins was purchased from Schleicher and Schuell, Inc. Filters for colony hybridization and plaque lifts were supplied by Millipore. Acrylamide, N,N'-methylene-bis-acrylamide (bis), ammonium persulfate, N,N,N',N'-tetramethylethylenediamine (TEMED), sodium

dodecyl sulfate (SDS) and electrophoresis purity grade agarose for gel electrophoresis were all obtained from Bio-Rad. Protein molecular weight markers were purchased from Pharmacia and contained: phosphorylase B (94 kDa), bovine serum albumin (BSA; 67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa). Lambda *Hin*dIII DNA size markers were supplied by Boehringer Mannheim. All other reagents and chemicals were obtained from Sigma Chemical Co. or Fisher Scientific Ltd., or where otherwise noted in the methods.

2.4 Preparation of Chlamydial antisera

Hyperimmune rabbit antisera to purified *C. trachomatis* L_2 EB were prepared as described previously (111). The EBs (1-2 mg of protein) were mixed with an equal volume of complete Freund's adjuvant (Difco) and injected intradermally into 4 to 6 week old New Zealand white rabbits. Subsequent intradermal injections were on weeks 2, 3 and 5 with incomplete adjuvant. Rabbits were bled at weeks 4 and 6. The rabbit blood was centrifuged (500 x g for 10 min) and the serum was removed and stored at -70 °C.

Monospecific polyclonal antibodies were prepared as described previously (112). Briefly, whole serovar L_2 EB lysates were prepared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to nitrocellulose. Proteins were visualized by amido-black staining, and strips containing proteins of interest were excised. The excised strips were incubated with polyclonal rabbit anti-EB sera for 2 h, washed thrice with 50 mL buffer containing 50 mM Tris, pH 7.5, 5 mM EDTA,

150 mM NaCl and 0.1% NP40, and then eluted with 0.2 M glycine-HCl, pH2.8. This eluant was immediately neutralized with 1 M Tris pH 8.8. 1% BSA in PBS was added as a stabilizer to eluted antibodies. These strips were reused four times for antibody and elution. Preparation and characterization of the monoclonal antibodies (MAb) to the species-specific domain of the 40 kDa MOMP has been described previously (113).

A mixture of polyclonal antibodies to *E. coli* ribosomal proteins L3 and L6 was provided to us as a gift by M. Nomura (University of California, Irvine).

2.5 Dot-blot analysis

Dot-blot analysis was performed essentially as described previously (114). Briefly, whole *C. trachomatis* EBs from B-complex (L_2 and D), C-complex (J) and bridge (K) serovars were dot-blotted on nitrocellulose (pre-wet with PBS) in volumes of 50 µL (10µg/mL) with or without prior incubation (5 min at 37°C) with 2% SDS in PBS. These were filtered for 20 min under gravity, then vacuum was applied. The wells were blocked by gravity filtration with 2% OA in PBS(containing 0.05% Tween 20) and washed once with 200 µL of PBS + 0.05% Tween 20 under vacuum. Monospecific antibodies were then added (100µL in blocking solution) and filtered by gravity for 40 min before vacuum. Wells were then washed 3 x with PBS + 0.05% Tween 20 as above. ^{125}I-Protein A (5 x10^5 CPM in blocking solution) was added to each well and filtered by gravity for 20 min before applying vacuum. The wells were then washed 3 times as

above using PBS + 0.05% Tween 20. The blot was rinsed with PBS containing 0.1% NP40 and autoradiographed for 8 hours at -70°C.

2.6 Immunogold labelling

The immunogold labelling technique has been previously described (115). Carbon coated copper grids were floated on drops of serovar L_2 EB suspension (5 μg protein/mL) for 30 min to allow absorption of the EB to the grids. The grids were then washed thrice for 5 min each with 1% BSA in PBS, and floated on the appropriate antibody (diluted with 0.5% BSA in PBS) for 30 min. Washing was then repeated followed by incubation with 10 nm colloidal gold/protein A suspension (E.Y. Labs, Inc., San Mateo, CA.). The grids were washed with 1% BSA in PBS thrice, once with distilled water and viewed by transmission electron microscope (Phillips EM300) at 80 kV. In a parallel set of experiments, 18 h (RB) and 48 h (EB) cultures of L_2-infected HeLa cells were first fixed with 1.5% glutaraldehyde and then scraped from the flasks, dehydrated, and embedded in Lowicryl K4M (JBS Supplies, Pointe Claire, P.Q.). Ultrathin (~60 nm) sections of L_2-infected HeLa cells were picked up on uncoated nickel grids, processed and viewed exactly as described above. Normal mouse serum was substituted for antibody as a negative control in each experiment.

2.7 Gel electrophoresis of DNA and proteins

DNA restriction fragments were resolved on 6% polyacrylamide gels (for fragments less than 2.0 kb in length) or on 0.75% agarose gels (for fragments greater than 2.0 kb in length; 116) in REB (tris-

acetate) buffer. REB-PAGE (REB-polyacrylamide gel electrophoresis) was performed at 400V per gel. Agarose gel electrophoresis was performed at 40V. DNA bands were visualized by staining with ethidium bromide and viewing with ultraviolet light (300nm).

Resolution of proteins was accomplished by SDS-PAGE (117). The stock concentration of acrylamide: bisacrylamide was 29.2%: 0.8% and was used for both REB-PAGE (DNA resolution, see above) and SDS-PAGE. Polyacrylamide concentration of the stacking gel was 4.5% and the separating gel was 12.5%. Proteins in each sample were denatured in 20 μL final sample buffer. Samples were boiled for 5 min prior to loading and approximately 25-30 μg of protein sample or molecular weight standards were loaded onto each lane. The samples were electrophoresed at 40 mA/gel and the gels were stained with Coomassie Brilliant Blue R-250 stain. Gels were destained in 35% methanol, 10% acetic acid.

All polyacrylamide gels were run with 0.75 mm spacers in a Bio-Rad Protean II gel apparatus. Agarose gels were of the horizontal submerged type.

DNA sequencing polyacrylamide gels of 4% and 6% were poured from stock acrylamide with an acrylamide: bisacrylamide concentration of 38%: 2% (118). Running buffer for these gels was 60 mM TEB. Long gels (19 cm wide x 85 cm tall, 0.25 mm thick) were pre-run at 75 Watts for 1 h prior to loading, then run at 75 Watts. Short gels (34 cm wide x 40 cm tall, 0.25 mm thick) were pre-run at 60 Watts for 1 h prior to loading, then run at 50 Watts. A 6 mm aluminum plate was placed over the front glass plate to distribute the heat evenly during the run.

2.8 Immobilization of DNA and proteins

After resolution by gel electrophoresis, both DNA and proteins were immobilized on nitrocellulose.

The transfer of DNA to nitrocellulose has been described by Southern (119). After electrophoresis and visualization of the DNA, the agarose gel was washed 2 x 15 min in 0.25 M HCl. The DNA was then denatured in 0.5 M NaOH, 1.5 M NaCl 2 x 30 min and neutralized in 0.5 M Tris (pH 7.5), 3.0 M NaCl 2 x 30 min. The gel was placed on Whatman 3MM paper which drew 10 x SSC from a reservoir, and a nitrocellulose sheet (pre-soaked in 2 x SSC) is placed on top of the gel. Two more pieces of Whatman paper, cut to size and also pre-soaked, were placed on top of this. A stack of paper towels is added on top of the nitrocellulose and Whatman paper to draw the 10 x SSC upward overnight. As the buffer flows upward, the DNA is transferred to the nitrocellulose. Subsequent baking of the nitrocellulose in a vacuum oven at 80°C for 2 h will ensure that the DNA is firmly bound to the nitrocellulose.

The electrophoretic transfer of proteins to nitrocellulose has been described by Towbin *et al.* (120). After electrophoresis, a piece of nitrocellulose, pre-cut to size and pre-soaked in transfer buffer, is placed on the gel. The gel and nitrocellulose are sandwiched between two scotch-brite pads by two plastic grids held together with rubber bands. The entire apparatus is placed in a transfer tank vertically so that field lines are perpendicular to the plane of the gel and nitrocellulose. The transfer is run for 30 min at 400 mA, and the efficiency of the transfer can be determined, markers positioned and

proteins fixed to the nitrocellulose by staining the nitrocellulose blot with amido black stain. For ligand electroblotting experiments in which ^{125}I-HeLa cell membranes were used to probe the transferred proteins (25), a 14-16 h transfer to nitrocellulose done exactly as above but with 50 mM phosphate buffer (pH 7.0) at 195 mA gave best results.

Immobilization of DNA on nitrocellulose filters after colony or plaque lifting in preparation for subsequent hybridization was as follows (121). Filters were laid on plates containing colonies or plaques, and the filter position marked with pin pricks. Plaque-bearing plates were refrigerated for 5 min to maintain the integrity of the soft agar layer while lifting. Filters were carefully lifted off the plates and inverted for 10 min on Whatman 3MM sheets soaked with 0.5 M NaOH, avoiding entrapment of air bubbles. The filters were then transferred to Whatman sheets soaked with 1 M Tris-HCl pH 7.5, 1.5 M NaCl (to neutralize the blots) for 10 min. The last treatment was 30 min on Whatman sheets soaked with a 0.4 mg/mL protease (from *Streptomyces griseus*) solution and 2 min in chloroform. The filters were then baked in a vacuum oven at 80°C for 2 h to ensure bonding of the DNA to the nitrocellulose.

2.9 Iodination of protein A and HeLa cell membranes

Protein A and HeLa cell membranes were labelled with ^{125}I by the lactoperoxidase method (122,25,26).

Protein A (100 μg) was labelled in a reaction mixture containing 1 mCi of ^{125}I, 20 μL of a 0.1 mM KI solution, 1 unit of lactoperoxidase in phosphate buffer (0.2 M, pH 7.0), 20 μL 40% H_2O_2,

in a preincubation total volume of 135 μL. The reaction was incubated at room temperature for 7 min, and more lactoperoxidase (1 unit) and H_2O_2 (20 μL of 40%) were added. The incubation was continued for an additional 10 min and then NaN_3 (20 μL of a 25% solution), KI (25 μL of a 0.1 mg/mL solution) and BSA (25 μL of a 1% solution in phosphate buffer) were added. This whole reaction mixture was applied to a column of Sephadex G25 over an ion exchange resin (AG-1X8 acetate form) and eluted with 1% BSA. Fractions of 1 mL were collected and the reaction efficiency determined by precipitation with trichloroacetic acid (TCA) and monitoring in a LKB RackGamma II gamma counter.

HeLa cell membranes were first isolated by removing the cells from the flask with glass beads in 10 mL of Hank's balanced salt solution (HBSS) and pelleting them at 350 x g for 5 min. The pellet was resuspended in 5 mL swelling buffer (10 mM Tris pH 8.0, 2 mM $MgCl_2$) and incubated on ice for 5 min, sonicated at low intensity for 25-30 sec, and the debris pelleted at 700 x g for 10-15 min at 4°C. The supernatant was spun at 93,000 x g for 60 min at 4°C, and the pelleted membranes were resuspended in 100 μL phosphate buffered saline (PBS) with sonication.

The 100 μL HeLa cell membrane preparation was ^{125}I-labelled as above, in a pre-incubation total volume of 160 μL with 2-3 μL of ^{125}I (4.0 GBq/mL). The final labelled membranes were sedimented in a microfuge for 10 min, washed in 2% BSA, sedimented again and resuspended in 200 μL 200 mM phosphate buffer pH 7.0.

2.10 Labelling of DNA fragments

Specific DNA fragments used as probes in clone selection were labelled with [α^{32}P]ATP in one of three ways.

a) Nick translation (123)

The DNA (~1 μg) was dissolved in 50 μL of a reaction mixture containing nick translation buffer (5 μL) and free deoxynucleoside triphosphates (dNTPs;1 μL each of a 1 mM solution), one of which is [α^{32}P]dATP. The DNA was nicked randomly by incubation with DNaseI (1mg/mL) for 2 min, providing 3'-OH ends. *E. coli* DNA polymerase I was used to initiate DNA synthesis from these ends (at 16°C for 90 min) resulting in incorporation of [α^{32}P]dATP into the newly synthesized strand. The reaction was stopped by the addition of 2 μL of 0.5 M EDTA and incubation at 65°C for 10 min.

b) Fill in labelling (124)

DNA fragments with 3'-recessed ends were generated by digestion with certain enzymes (such as EcoRI and HindIII). These fragment ends (0.5-2.0 μg) in reaction buffer were filled in by the action of T4 polymerase with free nucleotides (2 μL of each in 1 mM solution). The reaction was incubated for 5 min at 37°C, a cold chase nucleotide was then added corresponding to the radiolabelled nucleotide used, and the incubation was continued at 37°C for an additional 10 min. The reaction was stopped by incubating at 70°C for 5 min.

c) Random primer labelling (125)

DNA fragments were denatured at 95-100°C and annealed with random hexanucleotide primers. In reaction buffer with free nucleosides present (1 uL each of 3 cold 1 mM solutions and 20 μCi

of a radiolabelled nucleoside) these oligonucleotides primed the synthesis of complementary strands. The reaction required 2 units of Klenow fragment and incubation at room temperature for at least 5 h for maximal incorporation of radiolabel into the newly synthesized strand.

For all of these methods, separation of labelled DNA from free nucleotides was required. This was accomplished using a small (~ 8.0 mL) Sephadex G75 column (Pharmacia).

2.11 DNA hybridization and protein blotting

DNA was immobilized on nitrocellulose, by Southern transfer or colony and plaque lifts, for subsequent analysis by hybridization to a radiolabelled DNA probe. The procedure for hybridization analysis of immobilized DNA has been described by Southern (119) and was identical for Southern transfers and colony and plaque lifts. Baked filters were pre-wet in 6 x SSC before prehybridization. Prehybridization fluid was made up of deionized formamide (5.0 mL), 20 x SSC (2.5 mL), 1 M Tris-HCl pH 7.5 (0.5 mL), 50 x Denhardt's (1.0 mL; 1% Ficoll, 1% polyvinylpyrrolidone, 1%BSA), and 0.4 mL of 2.5 mg/mL salmon sperm DNA (boiled for 5 min and placed on ice immediately) for blocking, in a total volume of 10.0 mL. Formamide was deionized in a mixed-bed ion exchange resin (1:1 of AG50W-X8: AG1-X8) by stirring for 30 min at room temperature. The filters (with immobilized DNA) prehybridized in this solution for 2 h at 37°C with agitation. The hybridization fluid was identical to the pre-hybridization fluid, with a volume of probe (boiled for 5 min and placed on ice immediately) representing 1 x 10^6 counts per minute

(c.p.m.) displacing the same volume of water in the final 10.0 mL volume. The filters hybridized for 16-17 h at 37°C with agitation and were then washed 2 x 20 min with 2 x SSC, 0.2% SDS at 65°C, and 2 x 20 min with 0.2 x SSC at 65°C. The filters were then air dried, Saran wrapped while still damp, and autoradiographed.

Proteins were immobilized on nitrocellulose electrophoretically for subsequent reaction with antibodies (immunoblotting). The amido black stained nitrocellulose blot was first blocked with 2% BSA in TSA for 1 h at room temperature with agitation, then reacted with the appropriate antibody (diluted 1:100 in 1% BSA) overnight. The blot was washed 6 x 15 min with TSA, then reacted with 1 x 10^6 c.p.m. of ^{125}I-protein A in 50 mL 2% ovalbumin (OA) for 2 h at room temperature. The blot was then washed 6 x 15 min with TSN, dried, and autoradiographed.

Ligand electroblotting technique was carried out as described by Wenman and Meuser (25). Whole EB lysates were resolved on SDS-PAGE and transferred electrophoretically to nitrocellulose overnight in phosphate buffer. The amido black stained nitrocellulose blot was blocked with 4% BSA in TSA for 1 h, placed in 2% BSA in TSA, and reacted to 200 µL of ^{125}I-labelled HeLa cell membranes for 2 h at room temperature with agitation. The blot was then washed 10 x 15 min with TSN at 37°C, dried, and autoradiographed.

2.12 DNA isolation

Chromosomal DNA was isolated from purified EBs as described previously (126). EBs were pelleted at 17,000 rpm for 30 min and

the pellet resuspended for lysis in 50 mM Tris-Cl pH 8.0, 30 mM EDTA, 25% sucrose. To this suspension, proteinase K and Sarkosyl were added to final concentrations of 200 μg/mL and 0.8%, respectively. The lysis was aided by a 15 min incubation at 55°C followed by 45 min at 37°C. The lysate was phenol/chloroform extracted and centrifuged at 5,000 x g for 10 min. The DNA in the aqueous layer was further purified by CsCl density gradient centrifugation (η= 1.3860, density= 1.55 g CsCl/mL) at 310,000 x g for 17 h. The viscous DNA was drawn off the gradient and dialyzed in 3 changes of DNA buffer at 4°C to remove CsCl.

Both plasmid and bacteriophage replicative form (RF) DNA were isolated by the alkaline lysis method of Birnboim and Doly (127). For large-scale preparations, a 500 mL overnight culture in YT medium of the *E. coli* strain harboring the recombinant plasmid (100 μg/mL ampicillin for plasmid selection) was pelleted at 1300 x g for 5 min. The pellet was resuspended in 10 mL of "Solution 1" and incubated at room temperature for 5 min. Lysis was completed by the addition of 20 mL of "Solution 2" and cooling on ice for 10 min. The cell DNA and bacterial debris were precipitated by the addition of 15 mL of 5 M potassium acetate (pH 4.8) on ice for 10 min and the lysate cleared by centrifugation at 32,000 x g for 20 min at 4°C. The DNA was precipitated from the supernatant by adding 0.6 volumes of isopropyl alcohol, mixing and incubating at room temperature for 15 min. After centrifuging for 30 min at 7,800 x g at room temperature, the pellet was washed with 70 % ethanol, dried and resuspended in 8 mL TE. Covalently closed circular DNA (plasmid and bacteriophage RF) was further purified by cesium chloride (CsCl) ethidium bromide

(EtBr) density gradient centrifugation (1 g CsCl/mL DNA, 0.8 mg EtBr/mL CsCl solution, 310,000 x g for 17 h). The plasmid or RF band was removed from the gradient, the EtBr extracted with isopropyl alcohol, and CsCl dialysed out in 3 changes of DNA buffer at 4°C.

For small-scale, rapid isolation of plasmid DNA, 1.5 mL of an overnight culture was pelleted by centrifugation in an Eppendorf tube in a microfuge for 1 min at 4°C. The pellet was treated in a scaled-down version of the above procedure and the DNA precipitated with ethanol after phenol/chloroform extraction to remove contaminating proteins. The precipitated DNA was resuspended in 50 µL of TE (pH 8.0) containing 20 µg/mL DNase-free pancreatic RNase.

Isolation of single stranded bacteriophage DNA for sequencing experiments was accomplished by precipitation of virus particles with 20% polyethylene glycol (PEG) in 2.5 M NaCl at 4°C from supernatants of overnight 4 mL cultures in 2xYT medium. The precipitated phage were harvested by centrifugation in a microfuge for 15 min. The pellet was resuspended in 300 uL TES, extracted thrice with phenol/chloroform and chloroform alone, and precipitated with ethanol.

Isolation of restriction fragments of plasmid and RF DNA from 0.75% Tris-acetate agarose and 6% REB polyacrylamide gels was done by first visualizing the bands with EtBr under UV light and cutting out the band of interest. The gel piece was crushed and the pieces soaked in DNA elution buffer overnight at 37°C. The DNA was then precipitated from the elution buffer with 2 volumes of ethanol.

2.13 Cloning of restriction fragments

Genomic DNA from EBs of serovars D, J, and K was cut with selected restriction endonucleases with recognition sequences 6 nucleotides in length. These digests were phenol/chloroform extracted and the digested DNA precipitated by addition of two volumes of ethanol at a final salt concentration of 0.3 M sodium acetate. The plasmid vector pUC18 was digested with the corresponding enzyme and treated with calf intestine alkaline phosphatase to minimize recircularization.

Shotgun ligations of digested genomic DNA (passenger) to the linearized pUC18 (vector) were carried out at 16°C overnight or for 4 h at room temperature. The reaction volume was 20 μL with an approximate molar ratio of passenger to vector of 3:1. These ligation mixtures were used to transform competent *E. coli* JM83. Transformation was performed by incubating 100 μL of competent cells with 2 μL of ligation mixture (diluted to 20 μL in TE) on ice for 45 min. This suspension was then heat-shocked at 42°C for 1.5 min and returned to ice for 2.5 min. Ligation of forcign DNA into the polylinker region of the pUC vectors interrupts the *lacZ'* gene but leaves the beta-lactamase gene intact. The transformed cells were incubated for 1 h at 37°C (in an orbital shaking incubator at 220 rpm) to activate the beta-lactamase gene prior to plating on ampicillin-containing YT agar. Cells bearing plasmid were selected for on the ampillin medium, and those bearing recombinant plasmids (carrying passenger DNA) further selected by coating the plate with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and adding IPTG (isopropyl-β-D-thiogalactopyranoside) to the cells prior

to plating. Recombinant plasmids have an interrupted *lacZ'* gene and therefore cells bearing these plasmids cannot transform the X-gal on the plate and these colonies remain white. Cells carrying non-recombinant plasmids can convert the X-gal on the plate and therefore these colonies turn blue in color. Further selection of positive clones from thousands of recombinants was accomplished by colony lifts and hybridization with a radiolabelled probe. The probe used for shotgun cloning from the genomic DNA was constructed by ^{32}P-labelling the insert from the plasmid pCT161/18 (93).

Five positive clones were identified. Using the restriction endonuclease *Pst*I, clones from all three serovars were isolated and named pCTDP1, pCTJP1 and pCTKP1 (for serovars D, J, and K respectively). Using the restriction endonuclease *Eco*RI, a single clone from serovar J was isolated and named pCTJE1. Using the restriction endonuclease *Sac*I, again a single clone from serovar J was isolated and this was named pCTJS1. Regions of interest in the inserts of these clones were subcloned into pUC and M13 vectors for DNA sequencing analysis as described by Messing (128).

2.14 Restriction mapping

Cloned DNA fragments were restriction mapped by single and double digests using various restriction endonucleases. Fragments were separated by agarose or polyacrylamide gel electrophoresis and sized with DNA size standards. Restriction sites were located with respect to the ends of the fragment by radio-labelling one end of the fragment, digesting it, resolving the products by electrophoresis and autoradiographing the gel. Only those fragments extending from the

labelled end were then visualized, assisting the placement of restriction sites relative to each other. All digests were repeated at least once to confirm results.

2.15 DNA sequencing

All DNA sequencing was performed using the dideoxy chain termination method of Sanger et al. (129) with the Sequenase sequencing kit. In this reaction, chain extension is obtained from a DNA primer oligonucleotide annealed upstream of the sequence to be determined. Chain extension is terminated randomly by the incorporation of dideoxy nucleotides in four independent base-specific reactions. The randomly terminated chains, with incorporated [α^{32}P]ATP, are then size fractionated on polyacrylamide gel, the gel is Saran wrapped and autoradiographed.

Sequencing of inserts in single stranded M13 vectors and double stranded pUC vectors has been done (129,130). For both, once the primer had been annealed to the DNA the chain extension reaction was carried out followed by the termination reaction according to the conditions recommended by the manufacturer of the Sequenase sequencing kit. Prior to primer annealing, double stranded DNA (in the case of directly sequencing the double stranded pUC vector) was first denatured in 0.25 M NaOH (130). Following denaturation and precipitation in 0.2 M ammonium acetate and 2 volumes of ethanol, annealing of the primer was carried out at 37°C for 20 min. For most sequencing, the 17 nucleotide universal primer (5'-GTAAAACGACGGCCAGT-3') was used. For elucidation of insert sites close to the 3' end of this primer, a 15 nucleotide primer (5'-

TCCCAGTCACGACGT-3') hybridizing 18 bases further upstream on the vector was used. For sequence extension in large clones, a specific synthetic oligonucleotide primer, GGR#1, was obtained as a gift from Dr. K.L. Roy.

2.16 Sequence analysis

Sequence compilations, open reading frame (ORF) identification and translation and restriction map construction were all performed with DNA Strider software for the Macintosh computer(C. Marck, Service de Biochimie, Centres d'Etudes Nucleaires de Saclay, France). Peptide alignments were performed with Microgenie (Beckman) on the IBM. Codon usage and pI determinations were performed using IBI Pustell DNA Sequence Analysis software for the Macintosh computer.

2.17 Plasmid-directed synthesis of proteins

Recombinant expression products were analyzed by *in vitro* transcription-translation and minicell methods, essentially as described previously (131,108). *In vitro* transcription-translation experiments were carried out according to the protocol supplied by the manufacturer of the kit used. Labelled and unlabelled experiments were performed for recombinant and non-recombinant plasmids. TCA-precipitated protein products were dissolved in 30 µL FSB prior to resolution on SDS-PAGE.

E. coli strain p678-54 was used for mini-cell expression experiments. The recombinant plasmid and pUC18 (non-recombinant control) were transformed into competent p678-54, and

grown in 200 mL (each) 2xYT overnight. Large cells were pelleted at 1300 x g for 5 min, and minicells were pelleted from this supernatant at 7,800 x g for 10 min. The minicells were resuspended in 1 mL of BSG and further purified from large cells on sucrose gradients. Gradients were prepared by freezing a sucrose solution (12.5% sucrose in BSG) in 25 mL (30 mL Corex tubes) and 12.5 mL (15 mL Corex tubes) volumes for 8 h at -76°C, then thawing at 4°C overnight. Minicells were spun once on the 25 mL gradient (2,000 x g for 20 min) and twice on the 12.5 mL gradient (2,000 x g for 20 min). Bands of minicells were drawn off with a Pasteur pipet, pelleted at 7,800 x g for 10 min, and resuspended in 1 mL BSG prior to loading on the next gradient. The final minicell band was pelleted as before and resuspended in 1 mL of labelling medium. The optical density (O.D.) of this suspension was determined at 660 nm, and 0.2 O.D. units of recombinant and non-recombinant minicells were diluted, in duplicate, to 1 mL in Eppendorf tubes with labelling medium. These were pre-warmed for 15 min at 37°C, 50 μCi of [^{35}S]methionine was added to one set of cells (recombinant and non-recombinant). To the other set of cells was added 500 μL brain-heart infusion broth (BHIB). The cells were incubated at 37°C for an additional 45 min, 500 μL BHIB was added to the radiolabelled cells, and all cells were cooled on ice for 3 min. The cells were pelleted in a microfuge for 3 min then resuspended in 40 μL FSB before resolution by SDS-PAGE.

In vitro transcription-translation samples (30 μL) and minicell samples (20 μL) were loaded together on the SDS-PAGE gel. Radiolabel was loaded on one half of the gel, with unlabelled loaded

on the other half. The unlabelled half of the gel was subsequently transferred to nitrocellulose for immunoblot analysis. The radiolabelled half was dried for 1 h under vacuum and heat (in a Bio-Rad gel slab dryer) after Coomassie Blue staining to visualize marker bands and soaking in 1 M sodium salicylate for 1 h to enhance the radiolabel. The dried gel was then autofluorographed.

2.18 Autofluorography

All autofluorography was performed using Kodak X-OMAT AR X-ray film. Exposures were enhanced with Dupont Cronex Lightning-Plus intensifying screens, and exposures were done at -76°C for varying times. Films were developed in Kodak automatic X-ray film processors in the University of Alberta Hospitals.

3. Results

3.1 Surface exposure of the 18 kDa binding protein

Monospecific polyclonal antibodies directed towards the 18 kDa binding protein were purified from whole EB antisera. The specificity of the purified antibody was verified on Western blot against representatives of the B-complex (D, L_2), C-complex (J) and "bridge" (K) serovars (Fig.1A,B). This was aligned with a ligand electroblot (Fig. 1C) of serovar L_2 EB whole lysate to confirm the identity of the protein bound by the antibody as the 18 kDa binding protein.

Whole EBs of serovars D, J, K and L_2 were dot-blotted on nitrocellulose before and after a 5 min pre-treatment with 2% SDS, and reacted with anti-18 kDa and anti-40 kDa monospecific polyclonal antibodies. SDS pretreatment was included in order to control for any cross-reaction of proteins released by lysed EBs. Autoradiographic intensities were uniform for various serovars with or without SDS pretreatment using anti-18 kDa antibody. EBs labelled with antibody to MOMP, on the other hand, demonstrated increased autoradiographic intensities following SDS pretreatment (Fig. 2).

Purified whole EBs of serovars D, J, K, and L_2 were absorbed onto carbon-coated formvar coated copper grids, reacted with antibody and stained with protein A/gold conjugate. The immunogold labelling of whole EBs demonstrates the uniformity of surface labelling of the EB with MAb to MOMP (Fig.3A). This surface labelling is also seen, though to a lesser degree, using monospecific

Fig.1. Specificity of the 18 kDa monospecific polyclonal antibody. (A) SDS-PAGE analysis of whole EB lysates from serovars D, J, K and L_2. (B) Western blot of (A) reacted to the antibody. (C) Ligand electroblotting of whole L_2 EB lysate reacted to ^{125}I-labelled HeLa cell membranes.

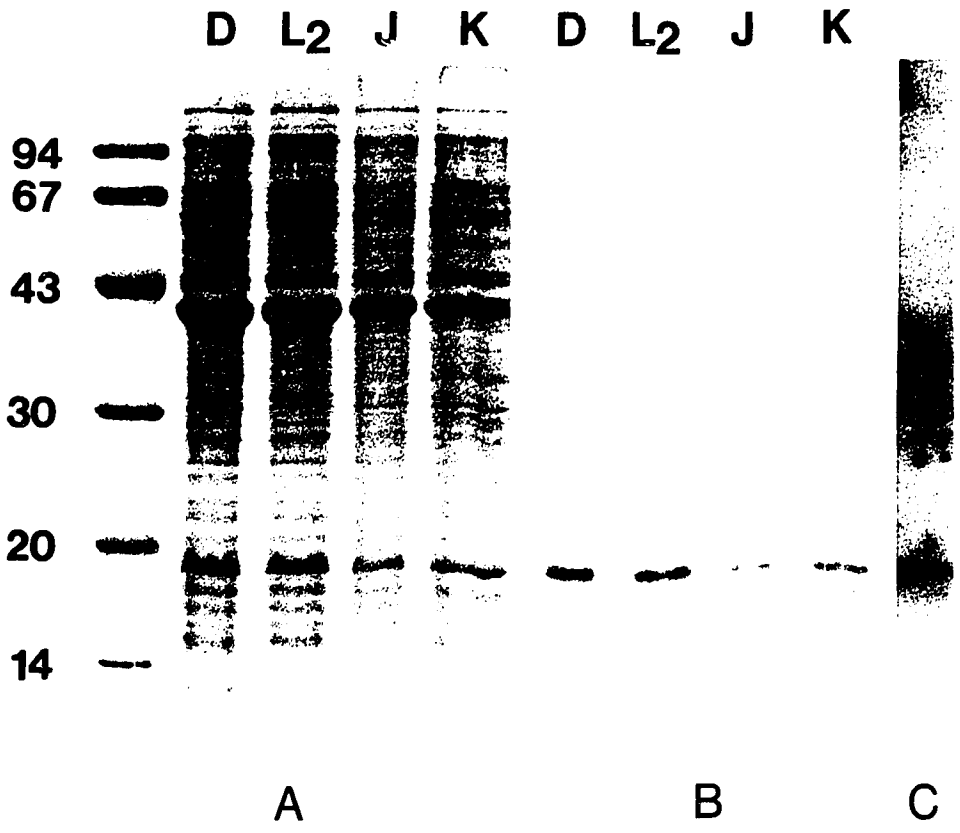

D
L2
J
K
D
L2
J
K
94
67
43
30
20
14
A
B
C

Fig.2. Dot-blot analysis of 18 kDa and 40 kDa (MOMP) protein surface exposure. Whole EBs of serovars L_2, D, J, and K were dot-blotted onto pre-soaked nitrocellulose before and after 5 min incubation with 2% SDS. These were then reacted with monospecific polyclonal antibodies to the 18kDa protein and the 40 kDa protein (MOMP).

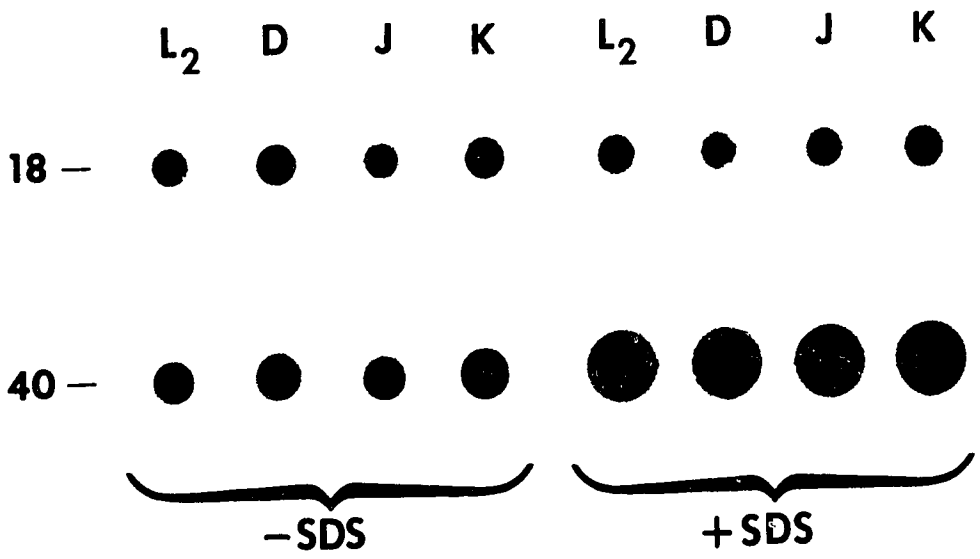
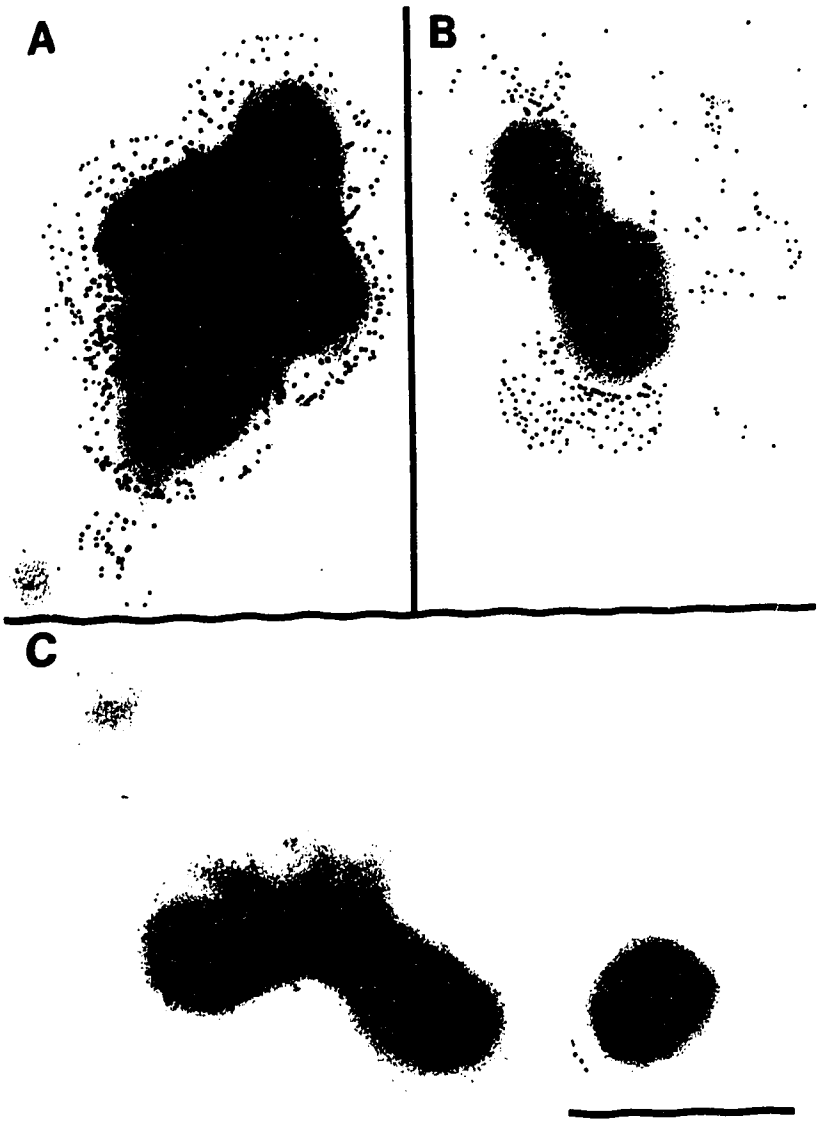

L2
D
J
K
L2
D
J
K
18 —
40 —
−SDS
+SDS

Fig.3. Whole L_2 EBs adsorbed to carbon-coated, formvar-coated copper grids were reacted with antibodies, then stained with protein A-colloidal gold. (A) EBs reacted with MOMP MAb, (B) EBs reacted with monospecific polyclonal antibodies to the 18 kDa protein, (C) EBs reacted with normal mouse serum as a negative control. Bar, 0.5 μm.


A
B
C

polyclonal antibodies to the 18 kDa binding protein (Fig.3B). No immunogold labelling occurred with the negative controls (Fig.3C). The total amount of immunogold label per EB appears to be similar with both MOMP MAb and 18 kDa monospecific polyclonal antibodies, with the only difference being that the 18 kDa label seems to be less strongly associated with the EB membrane.

For immunogold staining of serovar L_2 EBs and RBs in glutaraldehyde fixed sections, ultra-thin sections were picked up on nickel grids for processing and viewing. In fixed sections of L_2 EB inclusions in HeLa cells (48 h post-infection), both the MOMP MAb (Fig.4A) and the 18 kDa monospecific polyclonal antibodies (Fig.4B) bind the immunogold label on the periphery of the EB with very few gold particles labelling the interior. No colloidal gold was bound to the negative controls (Fig.4C). Total number of gold particles per EB is similar for both MOMP MAb and 18 kDa monospecific polyclonal antibody treated sections.

Immunogold labelling of RBs in section revealed intense staining of the RB periphery with MOMP MAbs (Fig.5A), while the 18 kDa monospecific polyclonal antibody (Fig.5B) and the normal mouse serum control (Fig.5C) labelled very weakly.

3.2 Cloning, restriction mapping and DNA sequence determination

Following the strategy of Kaul *et al.* (93) in the cloning of the 18 kDa binding protein, recombinant DNA libraries of *Pst*I-digested genomic DNA from serovars D, J, and K were constructed using the expression vector pUC18. Thousands of recombinants were screened

Fig.4. Ultrathin sections of EBs in inclusions in serovar L2-infected HeLa cells (48 h post-infection) on nickel grids were reacted with antibodies and then stained with protein A-colloidal gold. (A) EB section reacted with MOMP MAb, (B) EB section reacted with monospecific polyclonal antibodies to the 18 kDa protein, (C) EB section reacted with normal mouse serum as a negative control. Bar, 1.0 μm.

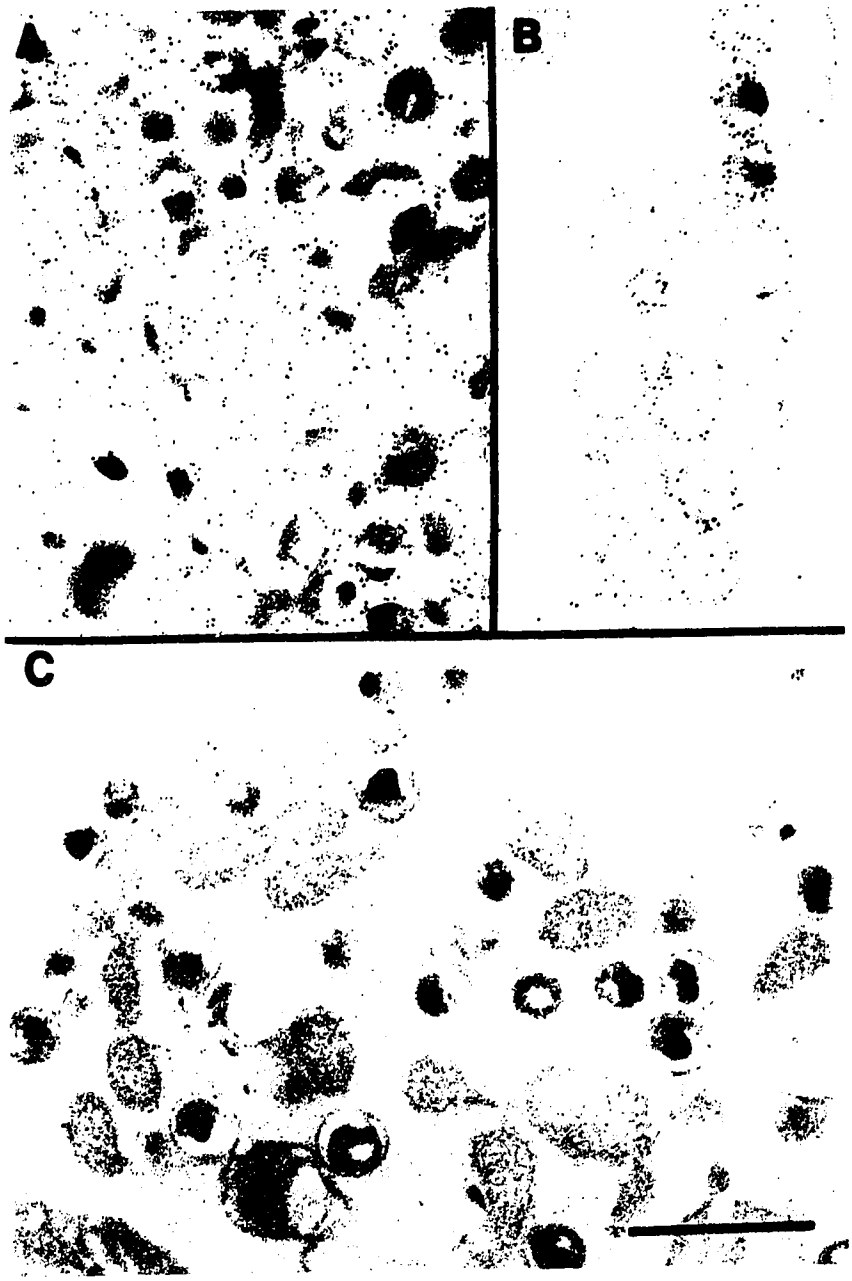
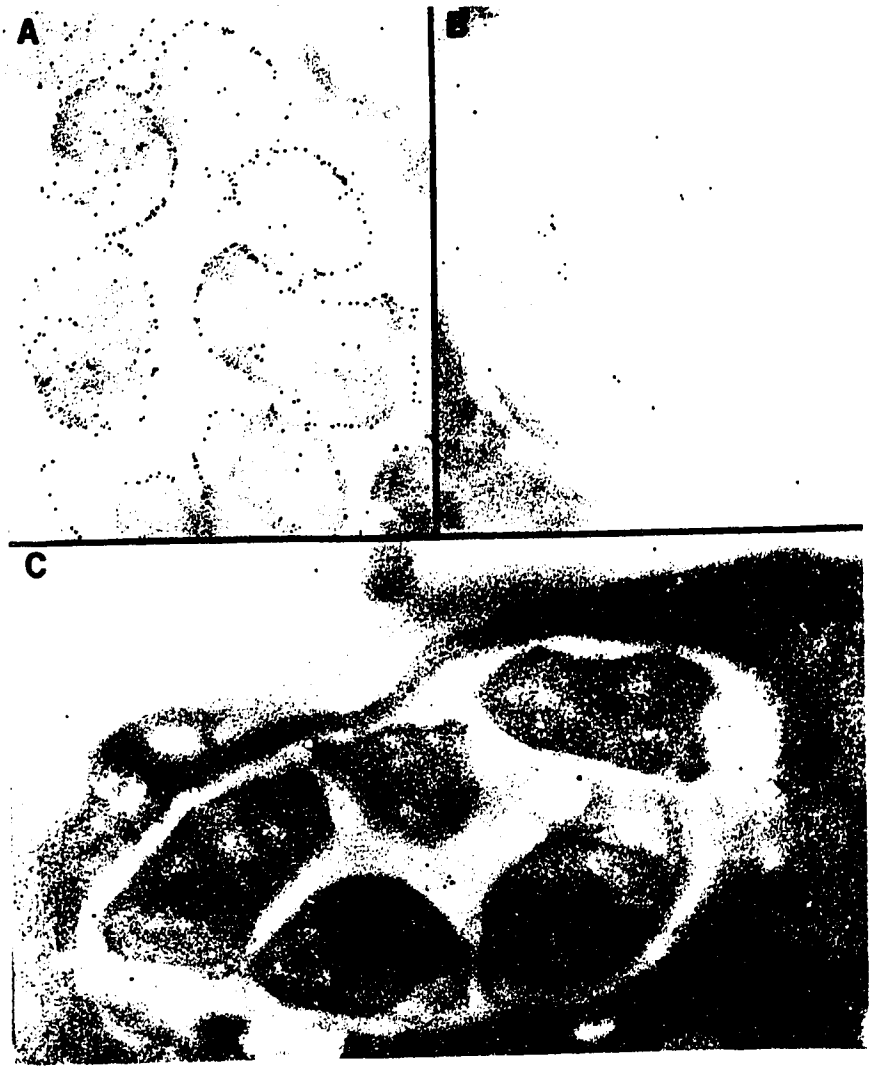

A
B
C

Fig.5. Ultrathin sections of RBs in inclusions in serovar L_2-infected HeLa cells (18 h post-infection) on nickel grids were reacted with antibodies and then stained with protein A-colloidal gold. (A) RB section reacted with MOMP MAb, (B) RB section reacted with monospecific polyclonal antibodies to the 18 kDa protein, (C) RB section reacted with normal mouse serum as a negative control. Bar, 1.0 μm.


A
B
C

by colony hybridization with the ^{32}P-labelled pCT161/18 insert. One clone from each serovar was isolated. These clones were designated pCTDP1 (plasmid *Chlamydia trachomatis*, serovar D, *Pst*I cut, the 1st), pCTJP1 (serovar J), and pCTKP1 (serovar K). These clones each bear a 440 basepair (b.p.) fragment of *C. trachomatis* DNA. Restriction mapping of these inserts, combined with determination of their complete nucleotide sequence on both coding and noncoding strands, revealed complete homology with the pCT161/18 insert to the *Sau*3A site at position 430. Beyond this point, the sequences and restriction maps of clones pCTDP1, pCTJP1 and pCTKP1 differ completely from that of pCT161/18 (Fig. 6).

Confirmation of this restriction fragment length polymorphism between serovars was attempted by probing a Southern transfer of genomic *Pst*I digests of serovars D, J, K, and L2 with the end-labelled *Pst*I-*Hin*dIII (458 b.p.) fragment of pCT161/18. For all four serovars tested, the probe hybridized to a single band ~440 b.p. in size (Fig.7). Serovar L2 genomic DNA (lane 4) hybridizes weakly to the probe because less DNA is present in this lane (as determined by EtBr staining).

All three 440 b.p. *Pst*I clones encode a portion of a putative open reading frame (ORF), that is they encode a transcriptional start codon, but no transcriptional stop codon in frame. In an attempt to elucidate the entire ORF, we identified the presence of this 440 b.p. *Pst*I fragment within an 800 b.p. *Eco*RI fragment and a ~3.0 kilobase (kb) *Sac*I fragment in all four serovars by Southern hybridization of genomic digests. Recombinant libraries of genomic DNA from serovars D, J and K cut with *Eco*RI and *Sac*I and sized on agarose gel

Fig. 6. Restriction endonuclease map of recombinant clones from serovars D, J, K and L_2. Mapping was done by generating single and double digests of recombinants and visualizing these on 0.75% agarose gels by ethidium bromide staining. End-labelled fragments were visualized by autoradiography. The restriction map for pCTJS1 was deduced from sequence information. Hatched bars indicate the putative open reading frame. The 458 b.p. Pst I- Hind III region used as a probe is indicated. For details of clone construction, see p.54 (pCTDP1, pCTKP1 and pCTJP1) and p.59 (pCTJE1 and pCTJS1) Size bar= 100 b.p. (P= *Pst*I, Xh= *Xho*I, Ha= *Hae*III, S= *Sau*3A, Hi= *Hin*dIII, Xb= *Xba*I, E= *Eco*RI, Sac= *Sac*I, A= *Alu*I)

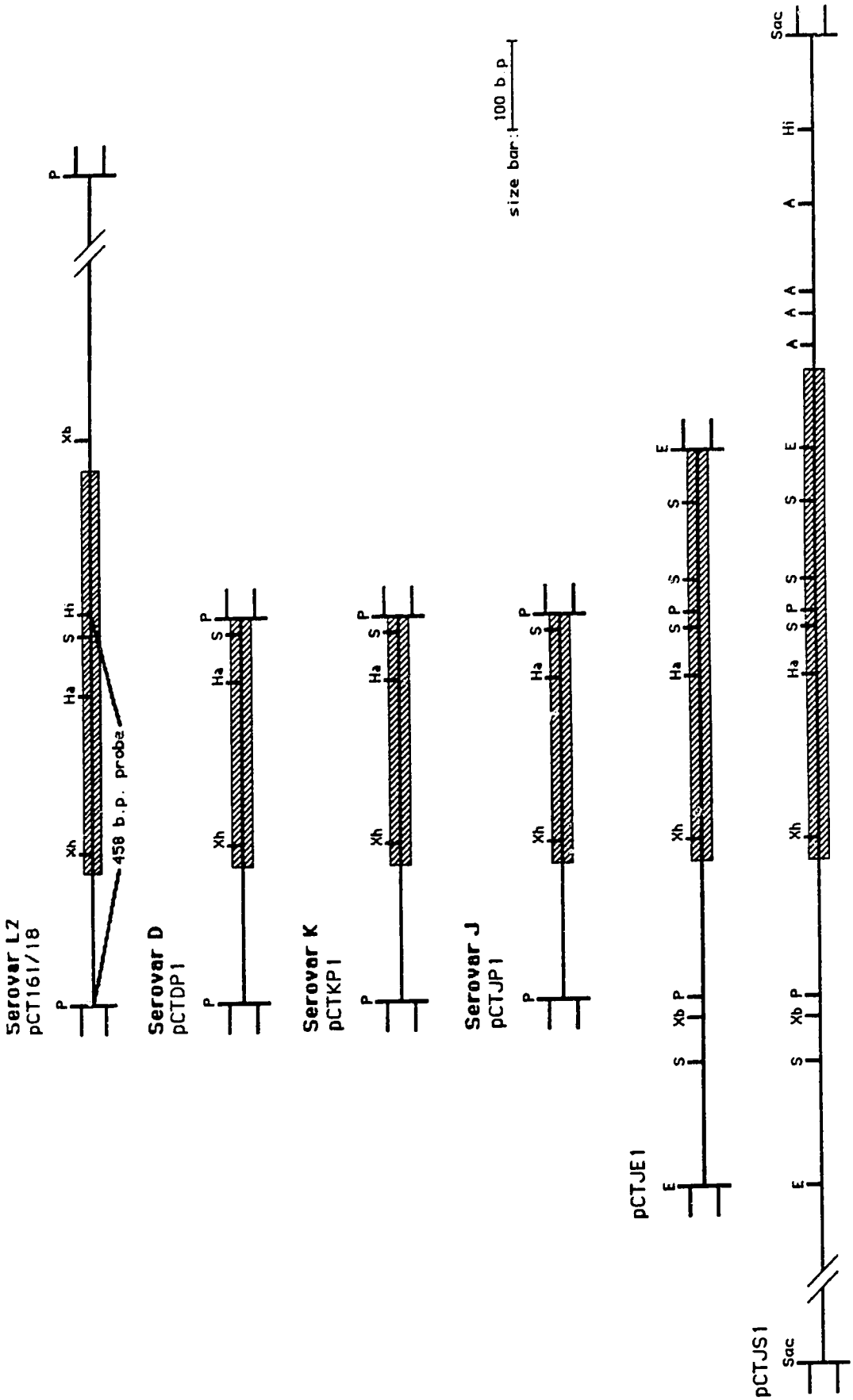

Serovar L2
pCT161/18
458 b.p. proba
Serovar D
pCTDP1
Serovar K
pCTKP1
Serovar J
pCTJP1
size bar: 100 b.p.
pCTJE1
pCTJS1
P
Xb
Hi
S
Ha
Xh
Sac
A
E

Fig. 7. Southern blot hybridization of serovars D (lane 1), J (lane 2), K (lane 3), and L_2 (lane 4). Genomic DNA was restricted with Pst I. End-labelled Pst I-Hind III (458 b.p.) fragment of pCT161/18 was used as a probe. Arrow indicates 440 b.p. size fragments, determined using DNA size markers.


1 2 3 4
440

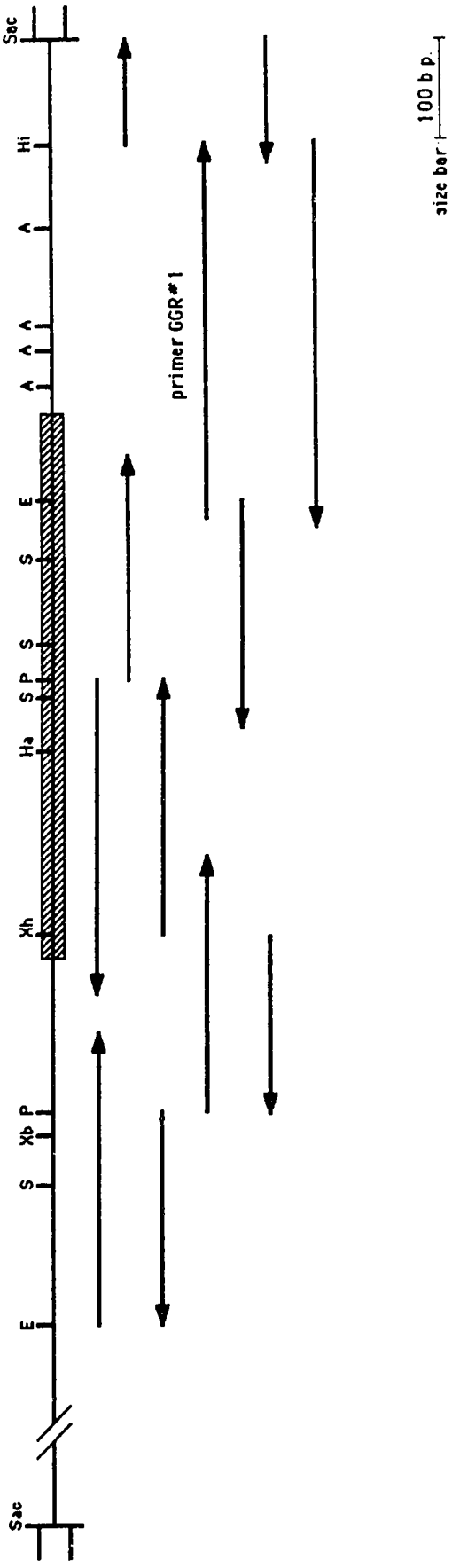
were constructed using the expression vector pUC18. Several hundred colonies were screened by colony hybridization using the ^{32}P-labelled 440 b.p. *Pst*I fragment. Two hybridizing clones were identified, one for each enzyme and both in serovar J libraries. These clones were designated pCTJE1 and pCTJS1 and contained an 800 b.p. *Eco*RI fragment and a ~3.0 kb *Sac*I fragment, respectively (see Fig. 6).

Complete sequencing of the pCTJE1 insert on both strands revealed the insert to be 804 b.p. in length. This clone allowed the extension of the restriction map (Fig.6) but did not contain the codon for termination of transcription of the putative ORF.

Extension of this sequence was accomplished using a specific synthetic oligonucleotide probe in sequencing the pCTJS1 insert. Subsequently, the known sequence was extended on both strands to a total of 1194 b.p. The sequencing strategy is shown in Fig.8, demonstrating the entire known sequence within the ~3.0 kb pCTJS1 insert. The complete sequence is shown in Fig.9. The entire putative ORF and the deduced primary structure of the protein encoded by this gene are identified.

Southern hybridization of *Xba*I-*Hin*dIII genomic digests of serovars D, J, K and L_2 (using the ^{32}P-labelled 440 b.p. *Pst*I fragment) revealed the existence of a single copy of this open reading frame in the genome (Fig. 10). Again, serovar L2 genomic DNA (lane 4) hybridizes weakly to the probe due to the small amount of DNA in this lane, as visualized by ethidium bromide staining.

Fig. 8. Diagram of the strategy used to sequence 1,194 b.p. of the ~3.0 kb Sac I fragment. For the entire 1,194 b.p. region, both strands were sequenced. Arrows indicate direction of 5'-3' primer extension in sequencing reactions. Primer GGR#1 (5'-TTTGCGGCTTGTGTTCGT-3') was used to extend the sequence past the EcoRI site. All other sequences were primed with universal primer. The hatched bar indicates the putative open reading frame. Size bar= 100 b.p. (P= *Pst*I, Xh= *Xho*I, Ha= *Hae*III, S= *Sau*3A, Hi= *Hin*dIII, Xb= *Xba*I, E= *Eco*RI, Sac= *Sac*I, A= *Alu*I).


Sac
Hi
A
A A A
E
S
S
S P
Ha
Xh
Xb P
S
E
Sac
primer GGR#1
size bar 100 b p.

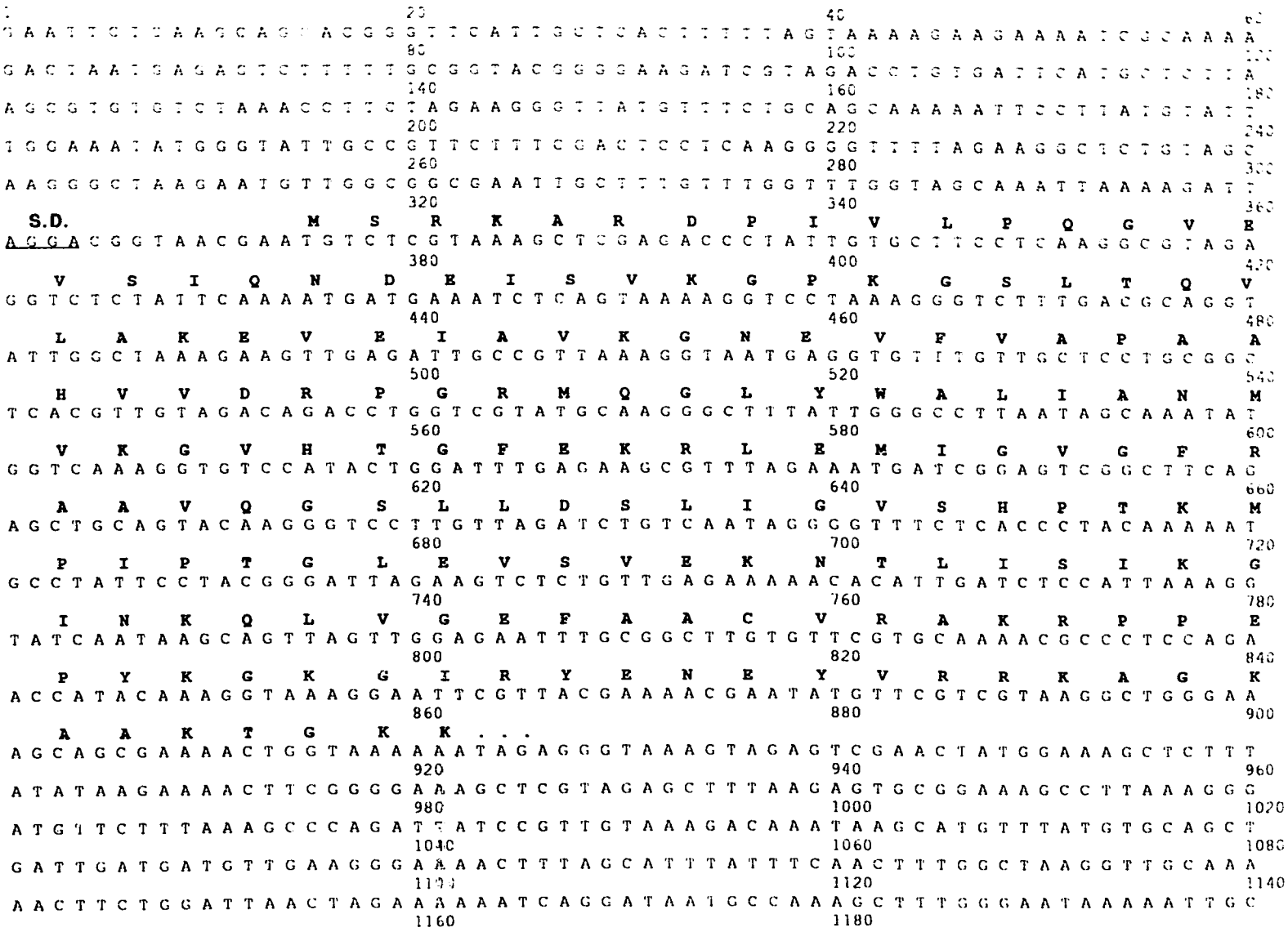

S.D.
M S R K A R D P I V L P Q G V E
V S I Q N D E I S V K G P K G S L T Q V
L A K E V E I A V K G N E V F V A P A A
H V V D R P G R M Q G L Y W A L I A N M
V K G V H T G F E K R L E M I G V G F R
A A V Q G S L L D S L I G V S H P T K M
P I P T G L E V S V E K N T L I S I K G
I N K Q L V G E F A A C V R A K R P P E
P Y K G K G I R Y E N E Y V R R K A G K
A A K T G K K . . .

Fig. 9. Complete nucleotide sequence of the 1,194 b.p. chlamydial DNA encoding the putative chlamydial ribosomal protein CL6. The open reading frame is translated to the single-letter amino acid code. The numbers above each line refer to nucleotide position. The translation initiation start site is the methionine codon at 314. The postulated Shine-Dalgarno region (ribosome binding site) is underlined.

Fig. 10. Southern blot hybridization of DNA from serovars D (lane 1), J (lane 2), K (lane 3), and L_2 (lane 4). Genomic DNA was digested with *Xba*I- *Hin*dIII. Random primer labelled *Pst*I (440 b.p.) fragment of pCTJP1 was used as a probe. Arrow indicates 980 b.p. size fragments, determined using DNA size markers.

1 2 3 4

3.3 Expression experiments

The 980 b.p. *Xba*I-*Hin*dIII fragment of pCTJS1 was subcloned into pUC18 and pUC19, generating plasmids pCTJS8 and pCTJS9, respectively, for use in plasmid-encoded protein expression experiments (see Fig. 11). These plasmids carried the putative ORF in direct (pCTJS8) and inverse (pCTJS9) orientation to the *lac*Z' promoter of the pUC vector.

The product of this ORF was expressed in large quantities *in vivo* by *E. coli* harboring the pCTJS8 plasmid but not those harboring pCTJS9 or pUC18 plasmids, as is evidenced by Coomassie Blue-stained SDS-PAGE protein profiles of these cells (Fig.12A).

It has been noted in our laboratory that expression of plasmid encoded genes can be affected by growth media. The expression of this protein was examined in normal (YT) and enriched (2 x YT and L) broth culture, as well as on solid media (YT agar). The SDS-PAGE protein profile of cells under different growth conditions reveals the expression of the product in all media used (Fig. 13A). However, the greatest quantity of protein appears to be produced in enriched broth cultures.

The amino terminus of the protein encoded by the pCT161/18 plasmid has been shown to possess considerable homology to the ribosomal proteins E-L6 and B-L10. Whole cell lysates of cells harboring this plasmid and whole cell lysates of chlamydial EB's (serovar L_2) were prepared by SDS-PAGE and immunoblotted on nitrocellulose with a mixture of polyclonal antibodies to the ribosomal proteins EL3 and EL6 (Fig. 14). Both the 18 kDa recombinant protein and two EB proteins of apparent molecular

Fig. 11. Construction of plasmids for expression of the putative ribosomal protein. A restriction map of the constructs is shown. Hatched bars indicate the putative open reading frame. Arrows indicate direction of transcription from the *lac z* (vector) promoter. Expression was determined *in vivo* by SDS-PAGE analysis of recombinant whole cell lysates. (P= *Pst*I, Xh= *Xho*I, Ha= *Hae*III, S= *Sau*3A, Hi= *Hin*d *III*, Xb= *Xba*I, E= *Eco*RI, Sac= *Sac*I, A= *Alu*I).

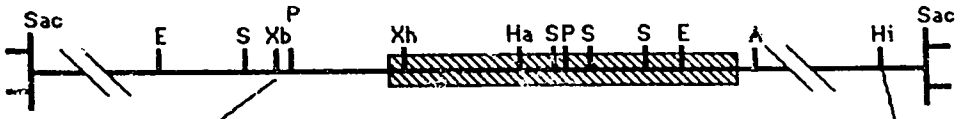
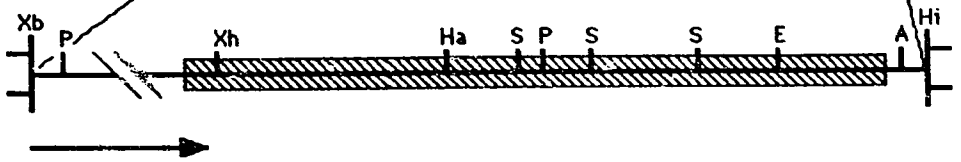
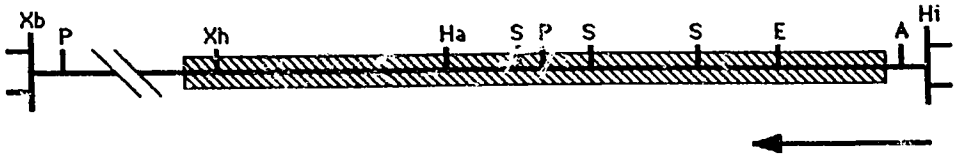
Vector Used	Host Strain	Recombinant Clones		R-Protein Expression
		Name	MAP	
pUC18	JM83	pCTJS1		N.D.
pUC18	JM83	pCTJS8		+
pUC19	JM83	pCTJS9		-

Fig. 12. Identification of the putative ribosomal protein gene product *in vivo*. (A) Whole cell lysates of (lane 1) pUC18- (lane 2) pCTJS9- and (lane 3) pCTJS8-containing *E. coli* strain JM83 and (lane 4) serovar L_2 EBs were resolved by SDS-PAGE and stained with Coomassie Blue. Low molecular weight markers (M) are expressed in kilodaltons. The arrowhead indicates the 23 kDa product of pCTJS8. (B) Western transfer of (A) immunoblotted with polyclonal antibodies to *E. coli* ribosomal proteins L3 and L6. The arrowhead indicates the 23 kDa product of pCTJS8.

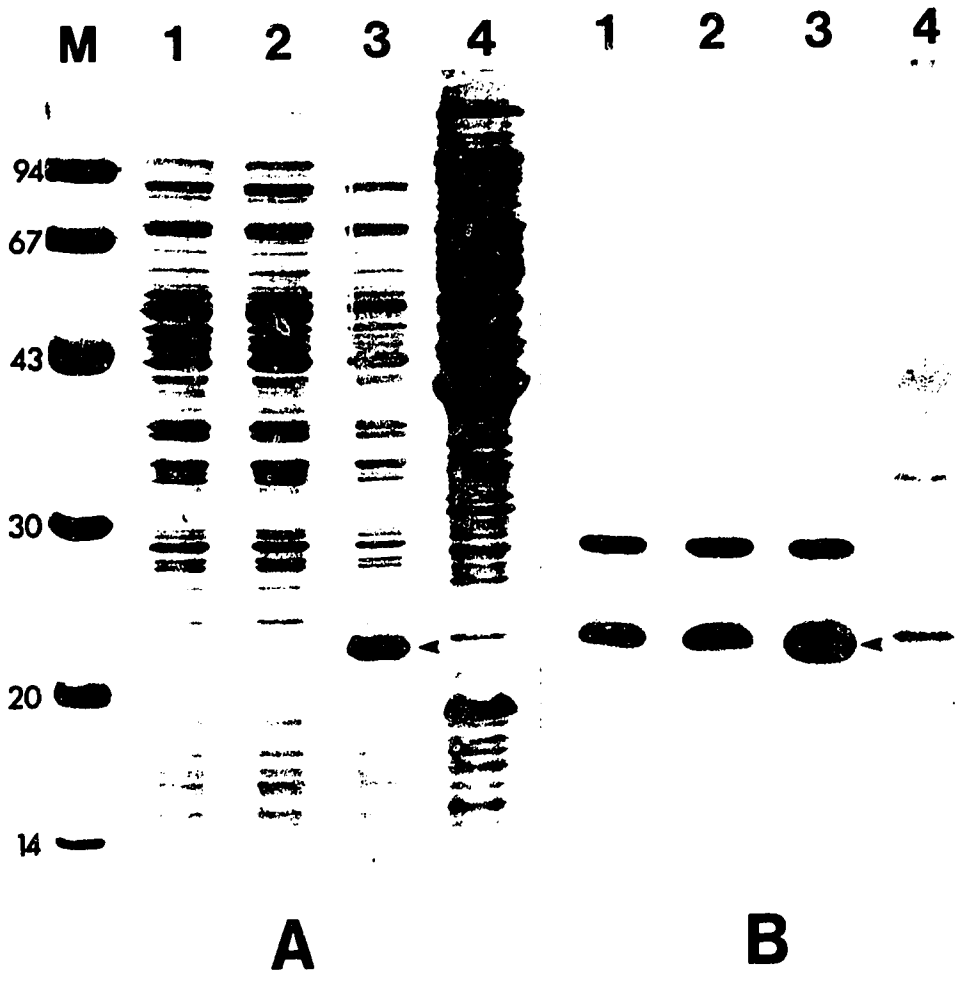

M
1
2
3
4
1
2
3
4
94
67
43
30
20
14
A
B

Fig. 13. Expression of the putative ribosomal protein gene product in various media. (A) SDS-PAGE analysis of protein products stained with Coomassie Blue. Lanes 1-4 represent whole cell lysates of pCTJS8 in *E. coli* strain JM83 grown in YT, 2 x YT, L-broth, and resuspended from YT agar, respectively. Lane 5 contains pUC18 in JM83 as a control. The marker lane (M) contains marker proteins of 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20 kDa, and 14 kDa. The arrowhead indicates the 23 kDa product of pCTJS8. (B) Western transfer of (A) immunoblotted with polyclonal antibodies to *E. coli* ribosomal proteins L3 and L6.

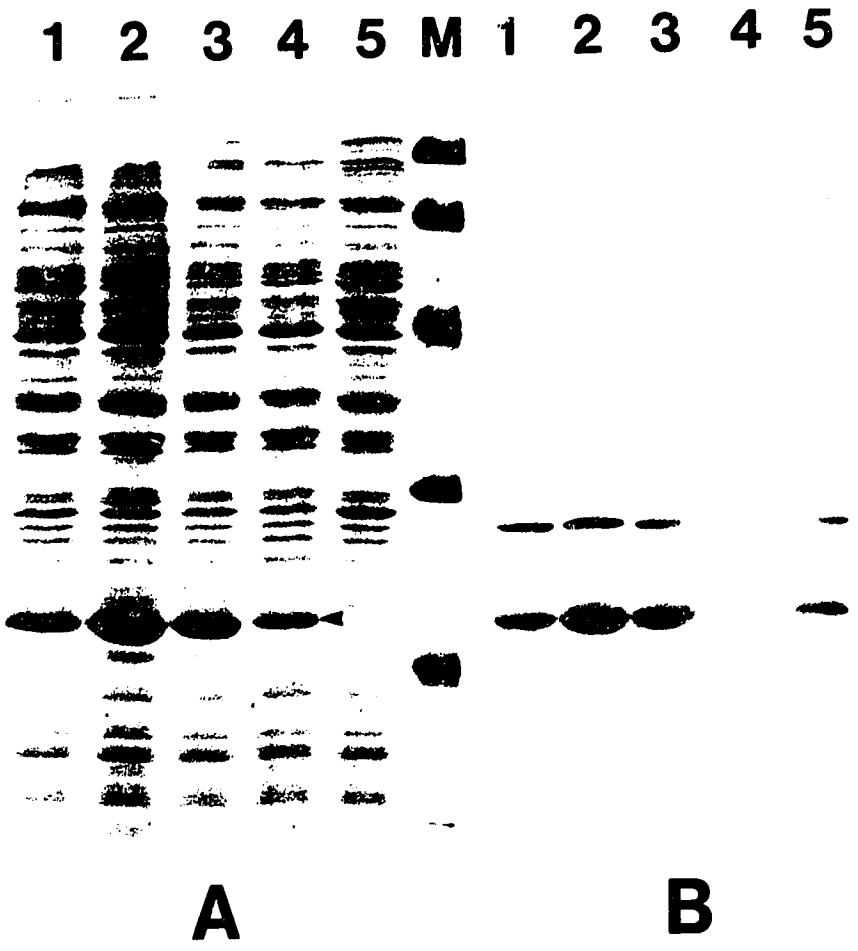
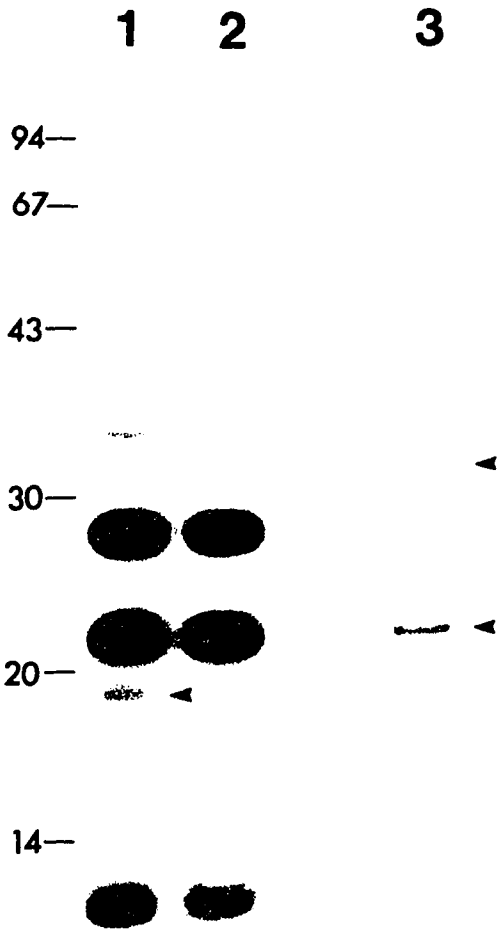

1 2 3 4 5 M 1 2 3 4 5
A
B

Fig. 14. Immunoblot of pCT161/18 and serovar L_2 EB protein products. Lanes 1-3 represent whole cell lysates of *E. coli* strain JM83 containing pCT161/18, JM83 containing pUC18, and serovar L_2 EBs, respectively, resolved by SDS-PAGE and transferred to nitrocellulose. The transfer was immunoblotted with polyclonal antibodies to *E. coli* ribosomal proteins L3 and L6. The arrows indicate (lane 1) the cross-reacting 18 kDa pCT161/18 product and (lane 3) two cross-reacting EB proteins. Low molecular weight markers are expressed in kilodaltons.


1
2
3
94—
67—
43—
30—
20—
14—

weight 23 kDa and 32 kDa cross-react with these antibodies. Immunoblots of the protein encoded by pCTJS8 (Figs. 12B, 13B) also reveal cross-reactivity with these same polyclonal antibodies (seen as increased autoradiographic intensity of the lower [23 kDa] band with respect to pUC18- and pCTJS9-bearing cells). In addition, the pCTJS8-encoded protein appears to be of similar size to the 23 kDa cross-reacting EB protein (Fig. 12B).

Plasmids pCTJS8 and pUC18 were transformed into the minicell-producing *E. coli* strain P678-54 to identify plasmid encoded proteins. The recombinant plasmid pCTJS8 encodes a single protein of apparent molecular weight 23,000 as resolved by SDS-PAGE. This protein is not encoded by pUC18 (Fig.15A).

The recombinant gene product was further identified using an *E. coli*- derived coupled *in vitro* transcription-translation system with plasmids pCTJS8 and pUC18. Again a single protein of apparent molecular weight 23,000, not encoded by pUC18, is produced from the recombinant plasmid pCTJS8 (Fig. 15A).

In a parallel experiment, both of these experiments (minicells and *in vitro* transcription-translation) were carried out without radiolabel and the products immunoblotted on nitrocellulose with the polyclonal antibodies to ribosomal proteins EL3 and EL6 (Fig. 15B). The pCTJS8-derived product is not distinguishable on immunoblot when expressed in these systems.

3.4 Analysis of the ORF product

All analyses of the ORF product were carried out using assorted computer software for protein and DNA sequence analysis. Table 1 is

Fig. 15. Identification of the putative ribosomal gene product *in vitro* and *in vivo*. (A) Gel electrophoresis pattern of ^{35}S-labelled gene products synthesized in minicells (lanes 1 and 2) and the *in vitro* transcription-translation system (lanes 3 and 4). Lanes 1 and 3 represent pCTJS8 and lanes 2 and 4 represent pUC18 directed protein synthesis. Arrowheads indicate the 23 kDa pCTJS8 product. Low molecular weight markers are expressed in kDa. (B) Western transfer of (A) (unlabelled products) immunoblotted with polyclonal antibodies to *E. coli* ribosomal proteins L3 and L6.

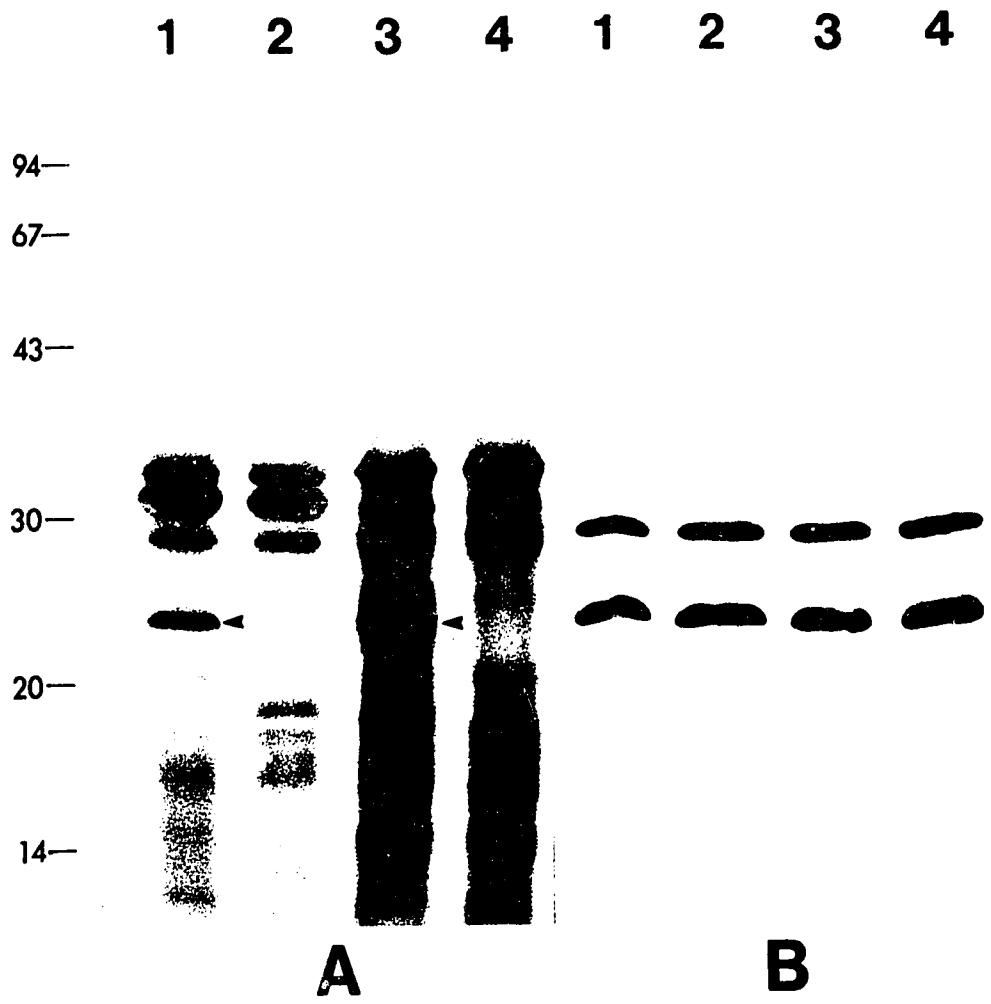

1 2 3 4 1 2 3 4
94—
67—
43—
30—
20—
14—
A
B

Table 1. Comparison of Ribosomal Proteins

Protein	*C. trachomatis* "L6"	*E. coli* L6	*B. stearothermophilus* L10
No. Amino Acids	183	176	177
Estimated pI	9.88	9.47	9.50
Deduced Molecular Weight	19,838	18,830	19,166
Apparent Molecular Weight	23,000	22,500	24,000

a comparison of three proteins (the pCTJS8 product, and ribosomal proteins EL6 and BL10) with respect to number of amino acid residues, isoelectric point (pI), deduced molecular weight, and apparent molecular weight. Table 2 presents a comparative tabulation of codon usage for the pCTJS8 ORF product and the codon usage of *E. coli* in the production of the ribosomal protein L6. Table 3 is a comparison of nucleotide distribution within each codon for these two proteins. No comparison of codon usage or nucleotide distribution was possible with ribosomal protein L10 of *B. stearothermophilus* as this DNA sequence is not published.

Homologies in primary protein structure are revealed by the peptide alignment in Fig. 16. The *C. trachomatis* pCTJS8 product primary structure is 60.2% homologous (counting 79 perfect matches and 27 conservative substitutions) to that of the r-protein E-L6, and 66.1% homologous to that of the r-protein B-L10 (90 perfect matches and 27 conservative substitutions). These proteins also reveal considerable homology in their secondary structure. An alignment of probability plots for secondary structure and antigenicity is shown in Fig. 17. Secondary stucture predictions were made using the algorithm of Garnier et al. (132), while hydrophobicity plots were made using the algorithm of Hopp and Woods (133).

Table 2. Comparison of Codon Usage in L6 r-proteins

Codon	No. of Times Used		Codon	No. of Times Used		Codon	No. of Times Used		Codon	No. of Times Used	
	C. trachomatis	*E. coli*		*C. trachomatis*	*E. coli*		*achomatis*	*E. coli*		*C. trachomatis*	*E. coli*
TTT- Phe	3	1	TCT- Ser	5	3	TAT- Tyr	2	1	TGT- Cys	1	1
TTC- Phe	1	2	TCC- Ser	2	-	TAC- Tyr	2	4	TGC- Cys	-	-
TTA- Leu	5	-	TCA- Ser	2	1	TAA	1	1	TGG- Trp	1	1
TTG- Leu	4	-	TCG- Ser	-	-	TAG	-	-	CGT- Arg	7	8
CTT- Leu	2	-	CCT- Pro	9	5	CAT- His	1	3	CGC- Arg	1	2
CTC- Leu	-	1	CCC- Pro	-	-	CAC- His	2	-	CGA- Arg	1	-
CTA-	-	-	CCA- Pro	2	-	CAA- Gln	4	-	CGG- Arg	-	-
CTG		11	CCG- Pro	-	3	CAG- Gln	2	7	AGT- Ser	-	-
ATT- Ile		2	ACT -Thr	2	6	AAT- Asn	4	2	AGC- Ser	-	-
ATC- Ile	4	6	ACC- Thr	-	5	AAC- Asn	2	5	AGA- Arg	2	-
ATA- Ile	2	-	ACA- Thr	2	-	AAA- Lys	16	9	AGG- Arg	-	-
ATG- Met	5	2	ACG- Thr	2	1	AAG- Lys	3	7	GGT- Gly	7	11
GTT- Val	9	14	GCT- Ala	7	6	GAT- Asp	2	5	GGC- Gly	2	5
GTC- Val	5	3	GCC- Ala	2	4	GAC- Asp	2	5	GGA- Gly	5	-
GTA- Val	5	2	GCA- Ala	4	6	GAA- Glu	8	3	GGG- Gly	5	1
GTG- Val	2	3	GCG- Ala	3	4	GAG- Glu	5	5			

Table 3. Nucleotide composition at three different positions of codons in L6 protein genes

	Number of Nucleotides at Position:					
	1		2		3	
Nucleotide	*C. trachomatis*	*E. coli*	*C. trachomatis*	*E. coli*	*C. trachomatis*	*E. coli*
T	29	15	54	47	67	68
C	32	40	42	44	25	42
A	50	45	56	57	59	22
G	73	77	32	29	33	45
A+T(%)	79(43%)	60(34%)	107(58%)	104(59%)	126(69%)	90(51%)

Average A+T%: *C. trachomatis* =56.7 %
E. coli =48.0 %

Fig. 16. Alignment of ribosomal protein amino acid sequences. Sequences for ribosomal proteins L10 from *B. stearothermophilus* (BL10) and L6 from *E. coli* (EL6) are aligned with the deduced amino acid sequence for the putative *C. trachomatis* ribosomal protein (CL6). The single-letter amino acid code is used. Wide bars indicate complete homology, narrow bars indicate a conservative substitution.

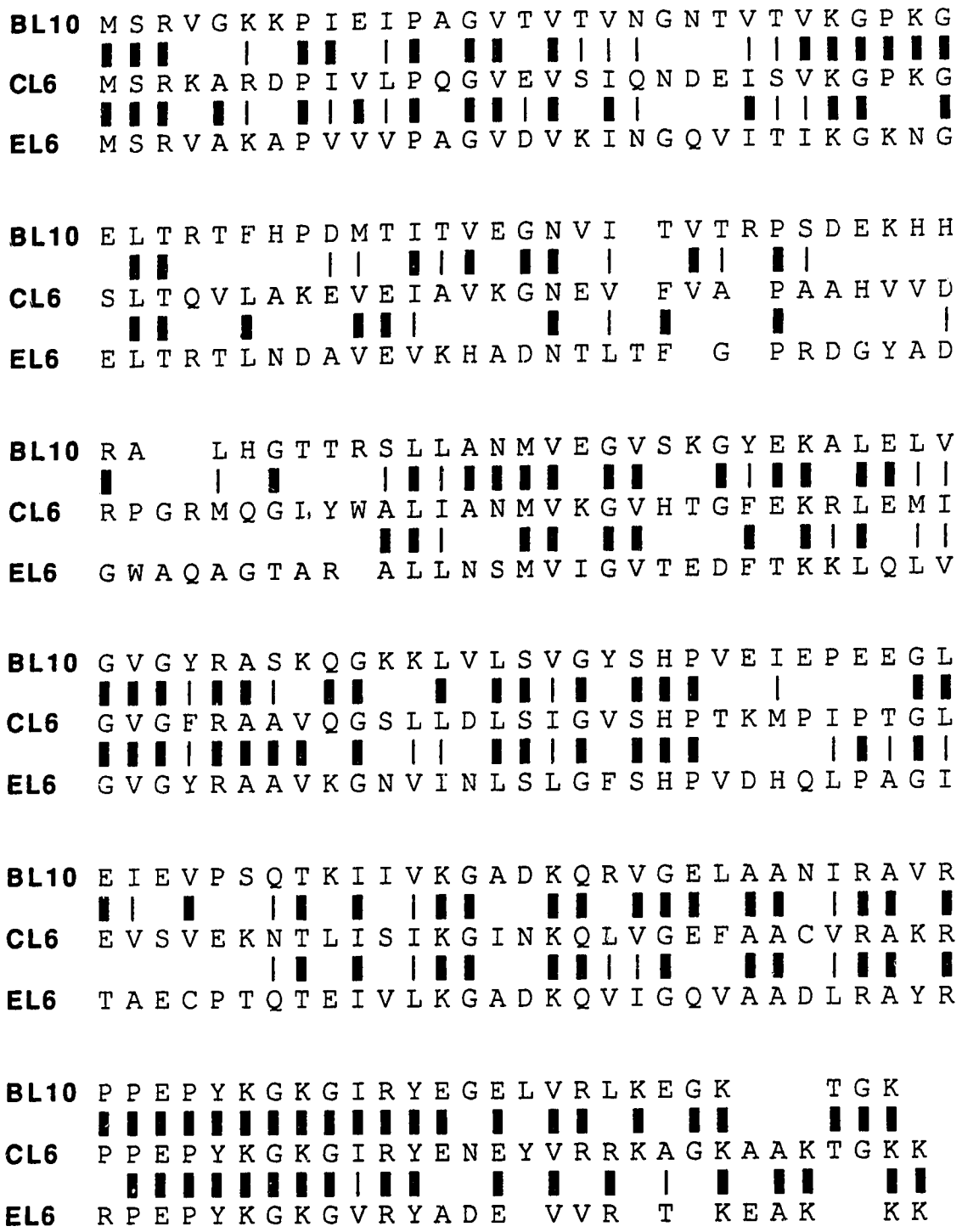
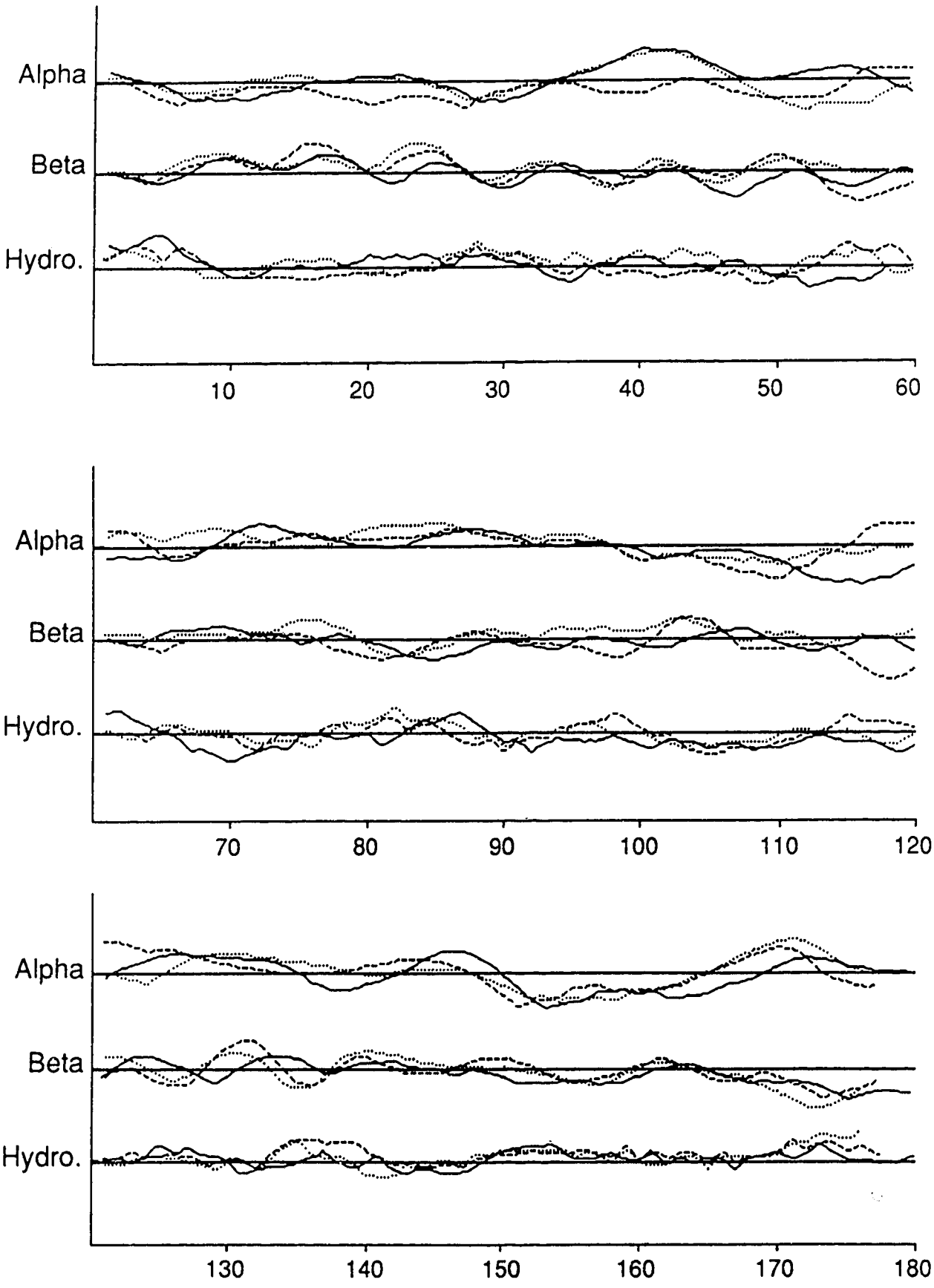

BL10 MSRVGKKPIEIPAGVTVTVNGNTVTVKGPKG
CL6 MSRKARDPIVLPQGVEVSIQNDEISVKGPKG
EL6 MSRVAKAPVVVPAGVDVKINGQVITIKGKNG
BL10 ELTRTFHPDMTITVEGNVI TVTRPSDEKHH
CL6 SLTQVLAKEVEIAVKGNEV FVA PAAHVVD
EL6 ELTRTLNDAVEVKHADNTLTF G PRDGYAD
BL10 RA LHGTTRSLLANMVEGVSKGYEKALELV
CL6 RPGRMQGLYWALIANMVKGVHTGFEKRLEMI
EL6 GWAQAGTAR ALLNSMVIGVTEDFTKKLQLV
BL10 GVGYRASKQGKKLVLSVGYSHPVEIEPEEGL
CL6 GVGFRAAVQGSLLDLSIGVSHPTKMPIPTGL
EL6 GVGYRAAVKGNVINLSLGFSHPVDHQLPAGI
BL10 EIEVPSQTKIIVKGADKQRVGELAANIRAVR
CL6 EVSVEKNTLISIKGINKQLVGEFAACVRAKR
EL6 TAECPTQTEIVLKGADKQVIGQVAADLRAYR
BL10 PPEPYKGKGIRYEGELVRLKEGK TGK
CL6 PPEPYKGKGIRYENEYVRRKAGKAAKTGKK
EL6 RPEPYKGKGVRYADE VVR T KEAK KK

Fig. 17. Probable ribosomal protein secondary structure and antigenicity. Probability plots for secondary structure (Garnier *et al.*, 131) and antigenicity (Hopp and Woods,132) of ribosomal proteins BL10 (---), EL6 (·····) and CL6 (——) are overlayed for comparison. Alpha= alpha helix, Beta= beta pleated sheet, Hydro.= antigenicity. Numbers on abscissa indicate amino acid residue position. Probability of all parameters increases above the line of normality and decreases below it.


Alpha
Beta
Hydro.
10
20
30
40
50
60
Alpha
Beta
Hydro.
70
80
90
100
110
120
Alpha
Beta
Hydro.
130
140
150
160
170
180

4. Discussion

4.1 Surface exposure of the 18 kDa binding protein

The 18 kDa HeLa cell membrane binding protein of *C. trachomatis* has been implicated in the pathogenesis of chlamydial infection. Elucidation of the precise role of this protein has been hampered by lack of knowledge of its surface exposure. In order for this protein to be involved in the attachment of the organism to the host cell it must be located on the outer membrane of the EB, as is indicated by the fact that EB membranes are taken up into host cells nearly as efficiently as whole EBs (34). While lactoperoxidase-mediated ^{125}I-labelling of EB surfaces seems to demonstrate the surface accessibility of this protein (25,26), interference by host cell contaminants has not been ruled out (16).

Evidence that the 18 kDa binding protein is surface-exposed was derived by dot-blot analysis of chlamydial EBs with or without SDS pre-treatment. The 40 kDa MOMP of *Chlamydia* spp. had been shown to be surface exposed (19,20) and was chosen as a positive control for surface exposure in the dot-blot and immunogold studies presented here. The specificity of the monospecific polyclonal antibodies used to label the 18 kDa protein was determined by Western blot and comparison with a ligand electroblot (Fig. 1). These antibodies were specific to an 18 kDa protein migrating at exactly the same position on SDS-PAGE as did the smaller of the two HeLa cell membrane binding proteins.

Using the anti-18 kDa antibody, dot-blotted whole EBs of various serovars were labelled with homogeneous autoradiographic intensities, independent of SDS pretreatment. EBs labelled with

antibody to MOMP, on the other hand, demonstrated increased autoradiographic intensities following SDS pre-treatment (Fig. 2). These data demonstrate that the 18 kDa binding protein possesses species-specific, surface-exposed, antigenic epitopes. Since SDS pre-treatment seems to have enhanced MOMP antibody binding, these results may also indicate that MOMP epitopes are exposed on both sides of the outer membrane (a transmembrane protein) to form a partially inaccessible loop as suggested by Baehr *et al.* (134). Conversely, lack of enhancement of 18 kDa antibody binding by SDS pre-treatment would indicate that the 18 kDa protein is only associated with the outer surface of the outer membrane.

Additional evidence for the surface exposure of the 18 kDa binding protein on chlamydial EBs was obtained by the use of immunogold labelling. The immunogold labelling of whole EBs with MAb to MOMP (Fig. 3A) demonstrates relatively even labelling of all aspects of the EB surface. This surface labelling is also demonstrated, though to a lesser degree, using monospecific polyclonal antibodies to the 18 kDa binding protein (Fig.3B). Negative controls did not label (Fig.3C). Representative counts of the gold particles indicate that the total amount of immunogold label per EB is similar with both MOMP MAb and 18 kDa monospecific polyclonal antibodies, the only difference being that the 18 kDa label seems to associate in highest density with what appears to be EB membrane debris. This weak surface association supports the indication by dot-blot analysis of the 18 kDa protein as a peripherally associated outer membrane protein.

In fixed sections of L_2 EB inclusions in HeLa cells (48 h post-infection), both the MOMP MAb (Fig.4A) and the 18 kDa monospecific

polyclonal antibodies (Fig.4B) bind the immunogold label on the periphery of the EB with very few gold particles labelling the interior. Negative controls were not labelled (Fig.4C). Again, representative counts indicated the total number of gold particles per EB was similar using either MOMP MAb or 18 kDa monospecific polyclonal antibody to label sections. This feature, also seen in Fig. 3, may be a factor of the polyclonal nature of the 18 kDa antibody. MOMP constitutes nearly 60% of the outer membrane protein (19) and would be expected to label more strongly than the 18 kDa protein. The MOMP MAb, however, was monoclonal and, therefore, would bind only a single epitope. The binding of the polyclonal 18 kDa antibody is not limited to a single epitope and therefore each protein molecule may bind more than one antibody. Kuo and Chi (135) observed low numbers of colloidal gold particles associated with chlamydial EBs in ultrathin sections using monoclonal antibody to the species-specific epitope of MOMP. These differences may reflect sample preparation for electron microscopy (e.g. different embedding resins were used).

MOMP is present in equal amounts in the outer membranes of both EBs and RBs, the only difference being the amount of cross-linking between the two forms (16). In earlier work, the 18 kDa binding protein was identified in the EB, but not in the RB (25,26). Our immunogold labelling experiments using fixed sections of L_2 RB inclusions in HeLa cells (Fig.5) strongly support these earlier conclusions. The MOMP MAb labels the RB periphery intensely (Fig.5A) while the 18 kDa monospecific polyclonal antibody (Fig.5B) and the normal mouse serum control (Fig.5C) label very weakly.

Dot-blot analysis and immunogold labelling techniques have been used here to demonstrate the surface localization of the 18 kDa chlamydial adhesin. Binding is very similar for the 18 kDa monospecific polyclonal antibodies and the positive control, the MOMP MAb. These antibodies bind the protein A/colloidal gold complex to the EB surface on whole and sectioned EBs, and therefore recognize surface epitopes. Non-specific binding of the immunogold label does not readily occur, as was shown with negative controls.

These results are consistent with earlier observations suggesting the surface exposure of the 18 kDa binding protein using radioiodination of purified EB (25,26). Both dot-blotting and immunogold labelling are powerful tools for studying antigenic localization and surface accessibility. Further work on characterization of both antigenic and neutralizing epitopes for the 18 kDa chlamydial adhesin is currently in progress.

4.2 Cloning and sequencing of a putative ribosomal protein from *C. trachomatis*

The cloning, sequencing and characterization of a putative 18 kDa chlamydial binding protein by Kaul *et al.* (93) offered the chance to achieve in-depth understanding of one mechanism of chlamydial pathogenesis. Unpublished primary sequence comparisons using the SWISS-PROT protein sequence database via BIONET revealed considerable homology of the N-terminus of the putative chlamydial binding protein to the homologous ribosomal proteins L6 and L10 (of *E. coli* and *B. stearothermophilus* respectively). Work presented here was performed to elucidate the role of this protein and the

meaning of its apparently close relationship to these ribosomal proteins.

The plasmid pCT161/18, derived by Kaul *et al.* (93) and encoding the putative 18 kDa binding protein, consists of a 1,658 b.p. *Pst*I fragment of serovar L_2 genomic DNA inserted in the polylinker region of the vector pUC8. No internal *Pst*I sites are present in this fragment (Fig. 6). Repeated attempts to select similar *Pst*I clones from recombinant libraries constructed from genomic DNA of serovars D, J and K met with failure. Positive clones were isolated (pCTDP1, pCTJP1 and pCTKP1); however, their inserts were only 440 b.p. in size. Determination of the complete nucleotide sequence of these clones, as well as restriction mapping (Fig. 6), revealed complete homology with the pCT161/18 sequence and restriction map up to position 430, the *Sau*3A site. Past this point the sequence differs completely between pCT161/18 and the three serovars cloned. A *Pst*I site at position 440 in the serovar D, J and K clones completes the fragment sequence and restriction map. In order to establish conclusively the different *Pst*I fragment size in serovar L_2, the DNAs of all four serovars were subjected to digestion by *Pst*I followed by Southern hybridization with the radiolabelled 458 b.p. *Pst*I- *Hin*dIII fragment of pCT161/18 (Fig. 7). The hybridization results indicate that all four serovars possess the same 440 b.p. *Pst*I fragment, and that no 1,658 b.p. *Pst*I genomic DNA fragment exists which hybridizes to the probe used. These data indicate that pCT161/18 represents a recombination event. At least two unrelated fragments of DNA from the *C. trachomatis* genome appear to have re-ligated in the *Sau*3A site at position 430. Fortuitously,

this recombination has provided an open reading frame of sufficient length to encode a protein of 18 kDa which possesses HeLa cell membrane binding ability, apparently by coincidence.

Since the N-terminus of the pCT161/18-encoded protein bears considerable homology to ribosomal proteins and appears unrelated to the C-terminus of the 18 kDa pCT161/18-directed fusion product, it was decided to complete the sequence of this open reading frame for further comparison with ribosomal proteins EL6 and BL10. Clones were selected from *Eco*RI (pCTJE1) and *Sac*I (pCTJS1) serovar J genomic DNA libraries by hybridization with the 440 b.p. *Pst*I fragment. The 804 b.p. *Eco*RI insert of pCTJE1 was mapped (Fig. 6) and completely sequenced, but did not contain the entire open reading frame. With knowledge of this sequence, an 18 base synthetic oligonucleotide primer, specific for a stretch of DNA 30 bases from the *Eco*RI site, was made. This primer, GGR#1 (Fig. 8), was used to help extend the known sequence in the ~3.0 kb *Sac*I insert of pCTJS1 (Figs. 8,9). The incomplete restriction map of pCTJS1 was derived from this sequence information (Fig. 6).

The ORF translation initiation site, the methionine codon ATG, begins at nucleotide 315. The ORF is 552 nucleotides long, with the translation termination codon positioned at nucleotide 866. The probable ribosome binding site for translation of this ORF is the AGGA sequence ten bases upstream of the start codon (93,136). No rho-independent termination site for transcription is seen in the sequence: the ORF terminates with a stretch of 6 adenine nucleotides, but no G-C-rich palindrome is found immediately upstream to stall the RNA polymerase (137). The lack of a strong transcriptional

terminator suggests the existence of a rho-dependent termination system for this gene, or this gene may be part of a polycistronic message.

The latter explanation may in fact be the case. The genes coding for all 52 r-proteins constitute 12 operons in the *E. coli* genome (138). The L6 gene, named rplF, is located in the *spc* operon, mapping at 72 minutes on the chromosome (139). The *spc* operon consists of 12 co-transcribed genes. Ten of these genes are ribosomal proteins (7 are constituents of the 50S ribosomal subunit), and L6 is sixth in order of transcription. Twelve nucleotides upstream of the L6 gene translation initiation codon the S8 r-protein terminates, while the L18 r-protein gene translation initiation codon is 9 nucleotides downstream of the L6 gene termination codon (139). Further analysis of the 1194 b.p. *C. trachomatis* sequence reveals portions of two open reading frames flanking the 551 nucleotide "L6" ORF (all in the same frame). The upstream open reading frame terminates 27 nucleotides from the putative chlamydial "L6" ORF, while the downstream ORF initiation codon is 21 nucleotides from the "L6" termination codon.

Southern hybridization analysis of serovar D, J, K and L_2 genomic DNA cut with *Xba*I and *Hin*dIII (Fig. 10) reveals a single band 980 b.p. in size for all serovars. This indicates that the "L6" gene is present in a single copy in the *C. trachomatis* genome, and that the restriction map of this region is similar for all serovars tested. It has been shown that genes for the 52 r-proteins in *E. coli* are also present in a single copy per haploid genome (140). Preliminary comparison of the deduced primary structures from the

upstream and downstream open reading frames flanking "L6" indicate some homology with r-proteins S8 and L18. respectively, flanking L6 in *E. coli*. The precise degree of homology cannot be determined using these partial products for comparison.

The 552 b.p. *C. trachomatis* L6 (CL6) open reading frame encodes a protein product 183 amino acids in length. From this deduced primary structure, the molecular weight of the CL6 protein is calculated to be 19,838 daltons. This value agrees closely with molecular weights of 18,830 and 19,166 calculated for the 176 amino acid EL6 (141) and the 177 amino acid BL10 (142), respectively based on amino acid sequencing data.

The amino acid compositions of these three proteins are also very similar. The ratios of non-polar amino acids (%) to polar amino acids (%) are: CL6- 45 : 28, EL6- 43 : 30, and BL10- 37 : 32. A comparison of acidic and basic amino acid content reveals further similarities. The ratios of acidic amino acids (%) to basic amino acids (%) are: CL6- 9 : 18, EL6- 10 : 17, and BL10- 12 : 19. From this data it is easily inferred that these are all basic proteins. The isoelectric points (pI) derived for these proteins from their primary structures (CL6 pI= 9.88, EL6 pI= 9.47, and BL10 pI= 9.50) support this contention. Indeed, these data and the literature values for EL6 (pI= 10) and BL10 (pI= 10) (141,143) fit well with the observation (144) that most ribosomal proteins are basic in nature.

In order to study the expression of the plasmid-encoded CL6 ORF, the 980 b.p. ORF-containing *Xba*I-*Hin*dIII fragment was subcloned into the vectors pUC18 and pUC19. The resulting subclones were designated pCTJS8 and pCTJS9 respectively (see Fig.

11). *In vivo* expression of the CL6 ORF was investigated by transformation of these subclones and non-recombinant pUC18 (as a negative control) into competent *E. coli* strain JM83. Only pCTJS8 was capable of directing the expression of a 23 kDa product, as resolved by SDS-PAGE (Fig. 12A). Since pCTJS8 and pCTJS9 differ only in the orientation of the CL6 ORF with respect to the *lacZ'* promoter, we conclude that the product of pCTJS8 expression originates from the *lacZ'* promoter and possesses no functional promoter on the 980 b.p. *Xba*I-*Hin*d III subcloned fragment. This finding further supports the nomination of this gene as a member of an operon. Expression of the pCTJS8-encoded CL6 ORF was examined under nutrient-rich and nutrient-poor conditions (Fig. 13A) and was found to be positively correlated with level of nutrition.

Plasmid pCTJS8 was introduced into *E. coli* minicells and an *in vitro* transcription-translation system to examine further plasmid-directed protein synthesis. Polypeptides of similar molecular size (23 kDa) were expressed in both systems (Fig. 15) from plasmid pCTJS8. No expression of this polypeptide was visible in pUC18 negative controls.

In all expression studies, the size of the pCTJS8-directed product, as measured by SDS-PAGE, was consistent: 23,000 daltons. This value is 16% larger than the value for molecular weight estimated by sequence analysis (19,838 daltons). This observation is mirrored by proteins EL6 and BL10. The *E. coli* protein EL6, with a calculated molecular weight from sequence data of 18,830, yields an apparent molecular weight of 22,500 by SDS-PAGE measurement (a difference of 19.5%; see ref. 94). The *B. stearothermophilus* protein

BL10, whose amino acid sequence suggests a molecular weight of 19,166 (142), migrates with an apparent molecular weight of 24,000 on SDS-PAGE (a difference of 25.2%).

A possible explanation for these discrepancies may be post-translational modification of these proteins. Prokaryotic ribosomal proteins can be post-translationally modified by methylation (145), acetylation (146) and amino acid addition (147). Phosphorylation of ribosomal proteins has not been proven in prokaryotes (148), although it appears to be ubiquitous in eukaryotes (149). No post-translational modification of proteins EL6 or BL10 has been demonstrated to date.

Analysis of the molecular weight of ribosomal protein EL6 by more accurate methods (150) such as sedimentation equilibrium and chemical cleavage, a calculation based on the number of tryptic peptides and the content of arginine and lysine, agrees more closely with the sequence data. Sedimentation equilibrium measurements place the EL6 molecular weight at 17,300 (a difference of 8.8%) while chemical methods give a range of 17,700-24,100. Clearly, the source of error is in the accuracy of the method. SDS-PAGE has been demonstrated to possess on average an accuracy for molecular weight measurements of only 10% (151). It is possible for certain proteins to exhibit deviations of 15% or more from known molecular weight values (152), possibly due to differences in SDS-binding capacity with respect to molecular weight marker proteins. This percentage of error could account for the variations seen when sizing ribosomal proteins EL6, BL10 and CL6 on SDS-PAGE (with respect to the molecular weights of these proteins calculated from sequence

information; Table 1). These are highly basic proteins and are therefore strongly positively charged. Migration of these proteins on SDS-PAGE may be either directly affected by this strong positive charge, or indirectly affected by resulting variations in SDS-binding capacity, or both.

Alignment of the deduced CL6 primary structure with those of EL6 and BL10 (Fig. 16) reveals considerable homology. In comparison with BL10, the CL6 sequence matches at 90 positions (48.9%) over the length of the BL10 sequence. In addition, there are 27 conservative substitutions, amino acid substitutions not expected to have an effect on secondary structure, raising the amount of shared homology to 66.1% between these sequences. CL6 has 79 matches (42.7%) over the length of the peptide EL6, and an additional 27 conservative substitutions. The resultant shared homology with EL6 is therefore 60.2% over the length of the sequence. These values compare favorably with homology comparisons between EL6 and BL10. EL6 and BL10 share complete matches in sequence at 88 positions (50%) and conservative substitutions at another 28 positions over the length of their sequences. This results in a shared homology between EL6 and BL10 of 66%.

Alignment of nucleotide sequences for these proteins was not performed. The BL10 nucleotide sequence is unpublished and cannot be compared. Comparison of codon usage (Table 2) between *C. trachomatis* and *E. coli* L6 ribosomal proteins reveals a striking dissimilarity in codon choice for the same amino acids. When the nucleotide composition of the chosen codons is examined (Table 3), a

strong tendency to the use of A and T nucleotides is seen for *C. trachomatis*. There is a nearly 70% bias towards A or T in the third position of codons in the CL6 gene, compared with 51% for the EL6 gene. The overall calculated A+T% content of CL6 and EL6 genes is 56.7 and 48.0, respectively. This is consistent with calculated averages for whole chlamydial and *E. coli* genomes, 70% and 50% A+T% respectively (92,152). These dissimilarities suggest that DNA sequence homology would be poor, and therefore a comparison of little use.

Regions of primary sequence homology among these three proteins are relatively general. However, there are three areas of considerable homology to note: residues 1-34 (24 matches/34 residues= 71% homology), residues 76-118 (~32 matches/42 residues= 76% homology), and residues 134-183 (~32 matches /49 residues= 65% homology). With this generalized homology (a high percentage of homology throughout the length of the sequence), one would expect equivalent homology in the predicted secondary structures. By superimposing probability plots for alpha helix and beta-pleated sheet formation (Fig. 17) for all three proteins, secondary structure can be directly compared. Visually, the homologies between the secondary structures of these three proteins are obvious. Even more interesting is the correlation of secondary structure homology with primary structure homology. The algorithm of Garnier *et al.* (132) predicts consensus beta-pleated sheets in the residue regions 7-35, 100-110, and 155-165. Alpha helices are predicted for all three proteins in residue regions 70-96, 118-130, 140-150, and 165-183. All of these regions of consensus secondary

structure are at least partially included in the previously mentioned 3 regions of concentrated primary structure homology. The algorithm used for secondary structure prediction appears to be only 60 % accurate on a given protein (132). However, the predictive value of this algorithm is of less importance as it is used here since we wished only to establish homologies and possible functional relationships. It is clear by treatment of all three of these proteins with this predictive algorithm that strong similarities in secondary structure are present, suggesting the functional homology of these proteins.

Ribosomal proteins EL6 and BL10 have been established as homologous using immunochemical evidence (94). By using polyclonal antibodies to ribosomal proteins EL3 and EL6, cross-reactivities with both the pCT161/18 product and two native chlamydial proteins have been established (Fig. 14). Immunoblotting of pCTJS8 (Figs. 12B,13B) reveals cross-reactivity of this plasmid-directed product also. The cloned product appears to be nearly identical in size to the smaller cross-reacting EB protein on SDS-PAGE (Fig. 12B). Any differences in mobility on SDS-PAGE may be due to variations in processing between the recombinant and native proteins.

From the work presented here it is clear that pCT161/18, derived by Kaul *et al.* (93), is in fact a cloning artifact and does not represent the cloning of the 18 kDa binding protein of *C. trachomatis.* The apparent homology and immunochemical cross-reactivity seen between the pCT161/18-derived product and *E. coli* ribosomal protein L6 appears to be due to the ligation of a portion of the *C.*

trachomatis L6 gene to an unrelated fragment of *C. trachomatis* DNA. The result was a fused open reading frame directing the expression of an 18 kDa protein binding HeLa cell membranes, the N-terminus of which represented the N-terminus of the *C. trachomatis* L6 ribosomal protein.

The clone-derived CL6 protein can only be distinguished by its overproduction in the recombinant, resulting in increased autoradiographic intensity after immunoblotting. This is because the *E. coli* L6 ribosomal protein is similar in size and also reacts to the antisera. Immunoblotting of expression products of *in vitro* transcription-translation and minicell experiments was unsuccessful in distinguishing CL6 from ribosomal proteins present in these systems (Fig. 15B). The likely explanation for this difficulty is that the product was expressed in amounts too low in these systems for its antigenic cross-reactivity to affect relative autoradiographic intensities.

Protein antigenic regions are defined by surface presentation and local charge. The algorithm of Hopp and Woods (133) has been used to plot the antigenic probability of the proteins CL6, EL6, and BL10. By superimposing the plots (Fig. 17), it is possible to visualize the basis for the immunochemical cross-reaction of these proteins with each other. The similarities in antigenicity are strong, with a low level of antigenicity generalized over the length of these sequences and peaks in regions of residues 1-10, 78-88 and 135-140. Again, these regions are completely included in the three major regions of primary structural homology discussed previously.

Homologies between the ribosomal proteins of various organisms have been well studied, both immunochemically and by molecular biological techniques (94, 154-163). In comparisons involving closely related organisms, it has been shown that conservation of homology rests in the DNA sequence (159). For less closely related organisms, such as archaebacteria, eubacteria and eukaryotes, homologies reside largely in the primary and secondary protein structure, as established by immunochemical and primary sequence comparison methods (155, 160-162). Especially among eubacteria codon usage varies so that DNA sequence homology may be poor, but primary sequence and immunochemical homologies remain (153,154,157).

Functional homologies are largely assumed from sequence (DNA or protein) and immunochemical homologies. In the elucidation of r-protein function, one of the most useful techniques to be developed has been the reconstitution of functional ribosomal subunits from purified r-proteins (164,165). By omitting certain proteins of interest during these reconstitution experiments and evaluating the ability of these ribosomal subunits to function normally, it is possible to establish the function of these proteins. In this manner, it has been determined that ribosomal protein L6 from *E. coli* is required for efficient synthesis of polyphenylalanine (164). An intriguing application of this reconstitution method has been the reconstitution of ribosomal subunits using purified proteins of varying origin (94,163). In this way, functional homology can be quantitated by the ability of the "homologous" protein to function correctly in replacement of the native protein. Ribosomal proteins

from sources as diverse as chloroplast ribosomes from the alga *Chlamydomonas reinhardtii* (163) and ribosomes of *B. stearothermophilus* (94) have been shown to function in reconstituted *E. coli* ribosomes in replacement of their homologues.

Topographical studies of ribosome structure have also served to confirm homologies. Techniques of immunoelectron microscopy (158,166) and distance measurements by energy transfer (167) have enabled EL6 and its homologue BL10 to be localized to the same areas in their native ribosomes.

Homologies in r-protein gene arrangement have also been noted between unrelated organisms. In *Mycoplasma capricolum*, the r-protein genes for homologues of the products of r-protein operons *S10* and *spc* in *E. coli* are arranged in an identical manner and order of transcription (153,154,157).

The nucleotide sequence, primary structure and predicted secondary structure of a putative ribosomal protein from *C. trachomatis* have been presented here. These data, along with demonstrated immunochemical cross-reactivity of this protein with antibodies directed towards *E. coli* ribosomal protein L6, support the nomination of this protein CL6 as a structural and therefore functional homologue of ribosomal proteins EL6 and BL10. Further support for this contention is derived from the apparent position of this gene within an operon resembling the *spc* operon of *E. coli*. The CL6 ORF appears to be flanked by ORFs encoding proteins homologous to r-proteins S8 and L18, which flank the L6 ORF in the *E. coli spc* operon (139).

The cloning and sequencing of a ribosomal protein from *C. trachomatis* has not been previously reported. If this gene is indeed co-transcribed as part of an operon, this will also be the first operon described in the chlamydial system. This work is an important step towards a more complete understanding of the metabolism of this organism. As this knowledge is achieved, we will approach the goal of extracellular cultivation of *C. trachomatis*. The benefits of this development include the ability to perform genetic studies involving mutagenesis, and the ability to harvest the organism in large quantities at lower cost. In addition, the characterization of chlamydial ribosomal proteins may assist the development of new and powerful antibiotics for fighting chlamydial infections.

The significance of similarities discussed here in determining the relatedness of organisms is obvious. Based on the importance of an efficiently functioning ribosome for cell growth, it is predictable that homologues will be found in unrelated organisms. As mentioned earlier, this assumption forms the basis of the rRNA sequence comparison method for determining phylogeny (103). It may be possible to establish paths of evolutionary change by comparing parameters such as gene arrangement, codon usage and similarities in promoter consensus sequences. The arrangement of homologous ribosomal protein genes appears to be identical in the closely-related organisms *Yersinia pseudotuberculosis* and *E. coli* (159). Similarity but not identity exists between less closely related organisms. Genes are in the same order in the *spc* operons of *M. capricolum* and *E. coli*, but intergenic spacing and codon usage differ (153,154,157). In apparently unrelated genomes such as those of chloroplasts and *E.*

coli, the arrangement of homologous ribosomal protein genes is completely different (163). Comparisons of ribosomal protein gene promoter sequences have yet to be performed.

The sequence of the ~3.0 kb *Sac*I clone (pCTJS1) is currently being completed to establish homologies with the *spc* operon of *E. coli*. Further cloning will need to be performed since the *spc* operon of *E. coli* is 5.9 kb in length (139) and we have so far cloned only 3.0 kb of this. Promotion and regulation of this putative *C. trachomatis* ribosomal protein operon will also be examined.

The functional homology of the CL6 protein is currently being investigated using one and two dimensional gel electrophoresis techniques. We are attempting to identify the presence of the CL6 protein in isolated ribosomes from *E. coli* strain JM83 carrying the expressing subclone pCTJS8 by immunochemical labelling. Size and pI differences between CL6 and EL6 should allow resolution of these two proteins on the same gel.

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6. Appendix

YT broth (and agar)

0.8% bacto-tryptone
0.5%bacto-yeast extract
0.5% NaCl
(±1.1% agar)

L broth

1.0% bacto-tryptone
0.5%bacto-yeast extract
1.0% NaCl

Brain heart infusion broth (BHIB)

Difco; 52g/L

REB buffer

40 mM Tris-acetate pH 7.8
20 mM sodium acetate
2 mM EDTA

SDS-PAGE running buffer

27 mM Tris
19.2 mM glycine
0.1% SDS
pH 8.3

Final sample buffer (FSB)

6.25 mM Tris-HCl pH 6.8
2.5% SDS
12.5% glycerol
0.001% bromophenol blue
6.5% B-mercaptoethanol

DNA sequencing polyacrylamide gels:

4%- 50% urea
60 mM TEB
10% stock acrylamide

6%- 50% urea
60 mM TEB
15% stock acrylamide

1M TEB

1 M Tris
20 mM EDTA
1 M Boric acid

10 x SSC

1.5 M NaCl
1.7 M sodium citrate, pH 7.0

Western transfer buffer

0.025 M Tris base
0.2 M glycine
20% methanol

Coomassie Blue stain

0.25% Coomassie Brilliant Blue R-250
45% methanol
9% acetic acid

Amido black stain

45% methanol
10% acetic acid
0.1% amido black

Nick translation buffer (5 x)

0.25 M Tris-HCl pH 7.2
0.05 M $MgSO_4$
0.5 mM dithiothreitol [DTT]
0.025% bovine serum albumin (BSA)

Fill-in labelling reaction buffer (10 x)

0.33 M Tris-acetate pH 7.9
0.66 M potassium acetate
0.1 M magnesium acetate
5 mM DTT
0.1% BSA

Random primer labelling reaction buffer (5 x)

250 mM Tris-HCl pH 8.0
25 mM $MgCl_2$
10 mM DTT
100 mM HEPES pH 6.6
0.2% BSA

TSA

50 mM Tris-HCl pH 7.4
150 mM NaCl

TSN

TSA + 0.1% nonidet P-40

DNA buffer

10 mM Tris-HCl pH 7.8
5 mM NaCl
0.1 mM EDTA

Plasmid extraction:

"Solution 1"- 50 mM glucose
25 mM Tris-HCl pH 8.0
10 mM EDTA
5 mg/mL lysozyme

"Solution 2"- 0.2 N NaOH
1% SDS

TE

10 mM Tris-HCl pH 8.0
1 mM EDTA

TES

20 mM Tris-HCl pH 7.5
10 mM NaCl
0.1 mM EDTA

DNA elution buffer

0.5 M ammonium acetate
10 mM $MgCl_2$
1 mM EDTA

BSG

0.15 M NaCl
2.2 mM KH_2PO_4
2.2 mM Na_2HPO_4
100 ug/mL gelatin

2x M9 salts

45 mM $Na_2HPO_4 \cdot 7H_20$
45 mM KH_2PO_4
17 mM NaCl
37 mM NH_4Cl

Minicell labelling medium

10 mL 2x M9 salts
160 mg glucose
0.2 mL 0.1 M $MgSO_4$
0.02 mL 0.1 M $CaCl_2$
0.02 mL 10 mg/mL threonine
0.02 mL 10 mg/mL leucine
0.02 mL 10 mg/ml biotin
9.6 mL water