Cloning and immunogenicity of a Chlamydia Trachomatis 36 kilodalton recombinant gene product in Escherichia Coli

Hector Rivera

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CLONING AND IMMUNOGENICITY OF A CHLAMYDIA TRACHOMATIS 36 KILODALTON RECOMBINANT GENE PRODUCT IN ESCHERICHIA COLI

A Thesis
Presented to the
Faculty of
California State University,
San Bernardino

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biology

by
Hector Rivera
September 1991
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Approved by:

[Signatures and dates]

Darlene Gamboa, Chair, Microbiology,
San Bernardino Valley College

Richard Fehn

Nicole Bournias

James Ito, M.D., Infectious Diseases,
City of Hope Medical Center.
Abstract

Chlamydia trachomatis (Ct.) is a Gram negative prokaryote responsible for the single largest cause of infectious human blindness, involving 500 million cases of trachoma worldwide, and strongly implicated in genital tract infections. Chlamydia trachomatis contains on its outer membrane surface a variety of antigens, some eliciting a protective, others a hypersensitive and detrimental immunological response. The cloning, isolation, and immunogenicity of antigens in the development of a chlamydial subunit vaccine is the subject of this study. I have identified a 36kd Ct protein in E.coli lysates, originally derived from a bacteriophage lambda library. Due to low expression yields in the bacteriophage vector, the Ct DNA fragment was sub-cloned into pBluescript plasmid and propagated in E.coli XL-1 Blue cells.

The cloning, isolation, and immunogenicity of the recombinant gene product were tested in 4 assays: 1) enzyme restriction analysis to determine the recombinant insert size, 2) dot immunoblots to screen for positive-expressing recombinant clones, 3) Western immunoblots to identify the recombinant chlamydial product and 4) enzyme immunoassays (EIA's) to determine the probable location of the 36kd protein in Chlamydia trachomatis.

The results of these serological studies demonstrated that: 1) the Ct recombinant protein is immunogenic for at least one epitope of the 36kd Ct protein (maybe more), 2) the expression of the recombinant Ct protein in pBluescript increased when compared to the product yield obtained in the lambda bacteriophage vector, and 3) the location of the 36kd protein in Ct. remained inconclusive. Based on the results obtained, further studies are necessary to clearly evaluate the protective capabilities of this potential vaccine candidate, and to determine its role during the growth cycle of chlamydial infections.
I would like to thank God for His many blessings and gifts in my life. I thank Him for giving me the pleasure to be surrounded by so many special friends!

To my family and friends: To my Mom and Dad and my brother Jorge, thank you for your constant love and faith in me, and for allowing me the freedom to make my own decisions in life. Thank you Tony for your support and constant "nagging" to get this project finished. Despite all the difficulties, you always found time to motivate me in all my endeavours.

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Introduction

*Chlamydia trachomatis* (Ct.) is a major human pathogen responsible for a wide spectrum of diseases in both industrialized and developing nations. Over 4 million infections of *C. trachomatis* in the United States, affect men, women, and infants at a cost exceeding $1.4 billion dollars per year in direct and indirect medical costs (21). Infections caused by *Chlamydia* include intestinal infections and diarrhea, pneumonia, urogenital infections, hepatitis, and conjunctivitis (14). *Chlamydiae* are Gram negative obligate intracellular prokaryotes which have evolved morphologically distinct infectious and reproductive forms. Two main structures in the chlamydial growth cycle are recognized: the elementary bodies (EB) which are the infectious stage of the growth cycle, and the reticulate bodies (RB) which are concerned solely with the multiplication of the chlamydial population within an infected eukaryotic cell (4). At present only one family (*Chlamydiaceae*) is recognized. The genus *Chlamydia* presently contains two species, *C. psittaci*, and *C. trachomatis*, each with an average genomic molecular weight of 6.0 x 10^8 daltons (8). The three biovars of *C. trachomatis* include mouse, lymphogranuloma venerum (LGV), and trachoma.

The current classification of *C. trachomatis* serovars is based on the antigenic differences observed using either the classic micro-immunofluorescence assay (MIF) (20), or the recently developed panels of enzyme-linked monoclonal antibodies (5). Most strains of *C. trachomatis* can be classified into 1 of 15 distinguishable serovars: chlamydial isolates from cases of hyperendemic trachoma generally belong to serotypes A, B, Ba, or C; isolates from ocular infection of sexually transmitted origin or genital tract infections to
serotypes D to K, while LGV isolates are of serotypes L1, L2, or L3.

Much of what is now known about the location of various epitopes on specific macromolecules has come from Western immunoblot analysis of serum from humans or animals with chlamydial disease, serum from immunized animals, and monoclonal antibodies (3). It has been possible to identify antigenic proteins of purified EB's, representing the 15 serovars of C. trachomatis (13). The most prominent membrane protein (60%) has a molecular weight of 38,000-42,000 daltons, depending on the serotype, and is designated the major outer-membrane protein (MOMP) of the chlamydial EB. In addition, many other species-specific reactions have been observed with a 60K outer-membrane protein, and proteins ranging in molecular masses from 115K to 12 K daltons. Little is known about these chlamydial proteins due to the difficulty in obtaining these proteins from intact C. trachomatis EB's, and the only recent applications of DNA technology. However, it appears that some surface antigens may regulate attachment, while others may induce phagocytosis, or inhibit phagolysosomal fusion, infectivity, and the host immune responses that contribute to immunity and pathogenesis (14).

It is now apparent that of these chlamydial proteins only a few are important in provoking protective immunity whereas others may frequently induce hypersensitive reactions which could be detrimental to the host. Thus, development of a highly specific vaccine, free from competing and non-essential components will require the identification and isolation of a single protective immunogenic determinant (23). Early trials with conventional vaccines against C. trachomatis consisting of inactivated and purified EB's in both nonhuman primates and human populations have resulted in both short-lived protective immune responses and, detrimental
hypersensitive reactions in the hosts (10). A murine model of *C. trachomatis* (Ct.) genital tract infection (GTI) has been developed by innoculating progesterone-treated female mice intravaginally with human oculogenital isolates of Ct. (9). Ito et al. has determined that homologous protection occurs against Ct GTI following systemic immunization of CF-1 mice with formalin-killed (FK) Ct. serovar H. Immunization with serovar D provides heterologous protection. Currently, there are no commercially available vaccines. However, recombinant DNA techniques have been employed to clone chlamydial DNA coding for MOMP and other *C. trachomatis* proteins into *Escherichia coli* expression vectors (16). The cloning and isolation of antigens as an alternative route in the development of a subunit vaccine against *C. trachomatis* is the subject of this study.

This study involves the identification of a bacteriophage-derived 36 KD chlamydial recombinant component from serovar D by Western immunoblots. An immunoreactive band of approximately 36KD has been identified in the recombinant clone and the Ct. serovar D antigen preparations, and is absent in the parent (non-insert containing) lambda vector; the prospect that this 36KD protein may be related to the 30KD Ct. protein involved in mediating attachment to host cells, emphasizes the significance of this recombinant gene product.

The biological relevance of this 36KD protein and other chlamydial antigens has prompted efforts to utilize molecular genetic approaches to prepare quantities of defined chlamydial antigens in an *Escherichia coli* expression system. The low yield of expression in initial experiments with EMBL-3 prompted the use of the pBluescript expression vector. Relatedness between this recombinant component and the analogous chlamydial component obtained from EB's was determined by serological assays, following the successful
sub-cloning of the Ct gene fragment into pBluescript. The specific aim was to determine the immunogenicity of an HPLC-purified recombinant chlamydial protein by Western immunoblots. In addition, DNA sequencing and enzyme immunoassays may provide insights into the structural and functional mechanisms of this recombinant gene. The likelihood of this recombinant Ct. protein becoming a candidate for vaccine development is discussed.

**Materials and Methods**

**Animals.** Outbred Cf-1 female mice > 8 weeks old (Charles River Breeding Lab.; Willmington, MA.) were used for immunogenicity studies.

**Bacteria.** A single strain of *E. coli* LE 392-P2 and *E. coli* XL-1 Blue (rec A<sup>-</sup>, lac<sup>-</sup>, end A1, gyr A96, thi, hsd R17, sup E44, rel A1, {F'proAB, lac I<sup>O</sup>, lac ZM15, Tn10 (Tet<sup>R</sup>)} (Strategene, San Diego, CA.) were used as hosts for recombinant cloning.

**Plasmid.** pBluescript KS (+/-) is a 2,964 basepair phagemid derived from pUC19. The vector possesses an f1 phage origin, a Col El origin, and T3 and T7 promoters flanking a polylinker region containing 21 unique restriction sites. pBluescript also contains a lac Z promoter for blue/white color selection or fusion protein induction with IPTG and provides ampicillin resistant colonies (Strategene, San Diego, CA.).

**Bacteriophage.** Lambda phage EMBL-3, a derivative of lambda 1059 (Promega Biotech., CA.), was used to clone and amplify recombinant Ct. lambda clones.
Antisera. Polyclonal antiserum to *C. trachomatis* was obtained from systemically (sub-cutaneous) immunized mice with purified Ct serotype D.

Preparation of *E. coli* LE 392-P2 and Recombinant Bacteriophage Lambda EMBL-3 stocks. *Escherichia coli* LE 392-P2 cultures were established in NCZ broth, consisting of NZ amine, NaCl, casamino acids, bactoyeast extract, MgSO₄ • 7H₂O, pH 7.5 (Gibco Laboratories; Madison, WI.), with 0.2% maltose, and incubated overnight at 37°C with moderate agitation. Following the incubation period, the cultures were centrifuged at 2,772.2 X g for 20 minutes at 4°C (Sorvall RT 6000 refrigerated centrifuge; DuPont Instruments) and the pellet fraction resuspended in half the original volume in SM media (NaCl, MgSO₄ • 7H₂O, 1M Tris-Cl, pH 7.5) (11).

Maximum yield of plate lysate stocks was assured by infecting 0.1 ml of plating bacteria with 10⁵ EMBL-3 lambda bacteriophage particles. Plating bacteria (0.1 ml) were pre-incubated with 10⁵ bacteriophages at 37°C for 30 minutes, and then combined with 3 ml of melted top agar (1.5%) and poured over bottom agar (2.0%). The plates (150 x 15 mm) were incubated overnight at 37°C. The soft agar of confluent lyzed plates was scraped into sterile centrifuge tubes; 5 ml of SM media and 0.1 ml of chloroform were added to the tubes, and the above mixture was incubated for 15 minutes at 37°C under continuous shaking. Following the incubation period, the mixture was centrifuged at 2,772 X g for 20 minutes at 4°C, and the supernatant (recombinant) recovered. The recombinant bacteriophage stock was titered to approximately a final concentration 1.0 x 10¹⁰/ml, and stored at 4°C after adding chloroform to 0.3%.

Screening of Recombinant Lambda EMBL-3 Bacteriophage. *Escherichia coli* LE 392-P2 overnight culture was infected
with recombinant phage preparation at a 10:1 multiplicity of infection (M.O.I.), and incubated at 37°C for 75 to 80 minutes with strong agitation. The *E. coli* lysate was collected and centrifuged for 20 minutes at 2772.2 x g. Whole lysate, pellet, supernatant fractions along with the appropriate controls (*E. coli LE-392-P2*, *Lambda EMBL-3* bacteriophage, and *Ct. serovar D* whole cell suspension) were dot blotted onto nitrocellulose membranes (Bio-Rad laboratories; Richmond, CA.). Membranes were immersed into a blocking solution (1% gelatin, 1% BSA in TTBS [Tween-20 Tris buffered saline, pH 7.5]) for 30 to 45 minutes and washed in TTBS twice at room temperature.

The nitrocellulose membranes were incubated with mouse anti-serovar D (*E. coli* absorbed) polyclonal antibody diluted 1:200 in Tris buffered saline containing 0.05% Tween-20, 1% BSA, 1% gelatin, and 0.01% Thimerosal overnight at room temperature. Membranes were washed for 10 minutes in TTBS washing solution and incubated with peroxidase-conjugated goat anti-mouse IgG antibody (1:3,000) for one hour at room temperature. The membranes were then washed twice for ten minutes, and a final wash with TBS was applied to remove Tween-20 residues which affect the color development reaction. The immune reactions were detected by adding a solution containing 0.5mg of 4-chloro-1-naphthol per ml and 0.015% H₂O₂ in TBS and agitated for 45 minutes. Double distilled water was added to stop the color reaction.

**Subcloning of Recombinant Ct gene fragment from bacteriophage Lambda EMBL-3 to pBluescript KS (+).**

**i. Purification and Extraction of EMBL-3 DNA.**

*Lambda EMBL-3* bacteriophage DNA was concentrated and purified by cesium chloride gradient centrifugation. An equal volume of chloroform was added to 25 ml of the recombinant EMBL-3 bacteriophage stock and vortexed for 30 seconds. The organic
and aqueous phases were separated by centrifugation at 1600g for 15 minutes at 4°C. The aqueous phase containing the recombinant bacteriophage was collected and 0.5g/ml of solid ultra-pure cesium chloride (Sigma, St. Louis, MO.) was added and mixed gently. After the cesium chloride was dissolved, the recombinant bacteriophage suspension was carefully layered onto cesium chloride step gradients in decreasing density (1.7 g/ml, 1.50 g/ml, 1.45 g/ml, and 1.15 g/ml) in cellulose nitrate centrifuge tubes. The mixture was centrifuged in a Beckman SW 41 rotor at approximately 80,000 x g for 2 hours at 4°C. The bacteriophage formed a visible band between the 1.45 g/ml and 1.50 g/ml cesium chloride layers. The recombinant bacteriophage particles were drawn from the cellulose nitrate tube by puncturing the side of the tube with a 21 gauge needle. Cesium chloride (1.5 g/ml in SM) was added to the recovered recombinant bacteriophage suspension to fill another cellulose nitrate tube and the suspension was centrifuged at 150,000 x g for 24 hours at 4°C in a Beckman Type-50Ti rotor. The band of bacteriophage particles was collected a second time as described above and stored in CsCl at 4°C.

The cesium chloride was removed from the purified recombinant bacteriophage preparation by dialysis at room temperature for 1 hour against a 1000:fold volume excess of dialysis buffer (10mM NaCl, 50mM Tris-Cl pH 8.0, and 10mM MgCl₂). After addition of EDTA (final concentration of 20mM), pronase (final concentration 0.50 mg/ml), and SDS (0.5% final concentration), the dialysis tube was inverted several times and incubated for an additional hour at 37°C. The bacteriophage DNA was extracted with an equal volume of phenol and centrifuged at 1600 x g for 5 minutes at room temperature. A second extraction of the aqueous phase was done with a 50:50 mixture of phenol and chloroform. A third and final extraction of the aqueous phase was performed with
an equal volume of chloroform. The final aqueous phase was
dialyzed once more in three 1000-fold volume of TE buffer
(10mM Tris-Cl pH 8.0 and 1mM EDTA) at 4°C overnight.

ii. Enzyme Restriction Analysis of EMBL-3 DNA.
Recombinant lambda EMBL-3 DNA (1 ug) was digested with 5U/ul
of ECO-RI (Bethesda Research Laboratories and Life
Technologies, Inc., Gaithersburg, MD.), and 5U/ul of SAL-I
(BRL, Inc., MD.) individually in the presence of 10X buffer
#3 (50mM NaCl, 10mM Tris-Cl pH 7.5, 10mM MgCl2, 1mM
dithiothreitol) and sterile double distilled water (ddH2O) at
37°C for 1 to 1.5 hours. One tenth of the digested DNA
mixture was analyzed by 0.8% agarose gel (Sigma, St. Louis,
MO.) electrophoresis, and stained with ethidium bromide
(10mg/ml stock; Sigma, St. Louis, MO.). The remaining
DNA mixture was phenol/chloroform extracted once, and the
aqueous layer was treated with 1/4 volume of NH4OAc (10M)
and two volumes of cold 95% ethanol. The DNA preparation was
precipitated overnight at -20°C, and centrifuged at 20,000
x g for 30 minutes at room temperature. The pellet was
washed in 70% ethanol and centrifuged for 5 minutes at 20,000
x g. The supernatant fraction was discarded and the pellet
was lyophilized.

iii. pBluescript plasmid DNA 5' end dephosphorylation
procedure. pBluescript plasmid (10 ug) previously digested
with respective restriction enzymes ECO-RI and SAL I was
extracted once with equal volumes of phenol/chloroform,
precipitated with ethanol, and treated with calf intestinal
alkaline phosphatase (CIP) (Boehringer Mannheim Biochemicals,
Indianapolis, ID.) to minimize self-ligation of the plasmid
vector.

The digested and purified pBluescript DNA mixture
obtained from the above procedure was dissolved in 10mM Tris-
C1 (pH 8.0). The amount of calf intestinal alkaline phosphatase (CIP) used to remove the terminal phosphates from the 5' ends of the digested 3Kb pBluescript vector took into account: 1) the number (pmoles) of 5' ends to be dephosphorylated, 2) the size of the DNA fragment (3Kbase), and 3) the amount of DNA available (10ug). Five microliters of 10X CIP buffer (0.5 M Tris-Cl pH 9.0, 10 mM MgCl₂, 1mM ZnCl₂, 10mM spermidine), sterile water, and 0.083 units of CIP (1 ul) were added to the dissolved plasmid DNA, and the 50 ul total reaction mixture was incubated at 37°C for 30 minutes, after which a second aliquot of CIP was added to the solution and incubated for an additional 30 minutes. Inactivation of CIP was accomplished by adding to the reaction mixture nitrilotriacetic acid to a 10mM concentration and incubating at 65°C for 45 minutes. The plasmid DNA was extracted twice with equal volume of phenol/chloroform and twice with chloroform, precipitated with 95% ethanol at -20°C, and lyophilized.

iv. **Ligation of Lambda-derived Ct DNA fragment to pBluescript plasmid, and Calcium Chloride transformation.** The EMBL-3 DNA lyophilized pellet obtained from the enzyme restriction procedure described above was resuspended in 10 ul of sterile dd H₂O, and an aliquot (2 ul) ligated to the CIP treated plasmid vector. The ligation reaction mixture consisted of sterile ddH₂O, complementary digested CIP-treated pBluescript vector, 10X ligase buffer (0.6M Tris-Cl pH 7.6, 50mM Mg Cl₂, 50mM dithiothreitol, ATP) and T4 DNA ligase (10 units/ul) (Strategene, San Diego, CA.) in a 10 ul volume. The reaction mixture was briefly centrifuged and incubated at 4°C overnight.

*Escherichia coli* strain XL-1 Blue cell cultures were grown to log phase. The bacterial cell suspension was concentrated by centrifugation (2,272 x g, at 4°C) and
resuspended in half of the original culture volume of an ice-cold, sterile solution of 50mM CaCl₂ and 10mM Tris-Cl (pH 8.0). The cell cultures were chilled for 15 minutes and then centrifuged at 2,272 x g for 5 minutes at 4°C. The supernatant was discarded and, the pellet was resuspended in 1/15 of the original volume of an ice-cold solution of 50mM CaCl₂ and 10mM Tris-Cl (pH 8.0). Ten nanograms of ligated recombinant DNA in a volume of 10 to 25 ul were aliquoted into 15-ml sterile, round-bottom test tubes, and 100 ul of competent E. coli strain XL1-Blue cells were added to each test tube. The tubes were gently swirled to mix, then placed on ice for 30 minutes. The cells were heat shocked at 43°C for 3 minutes, 0.8 ml of 2x YT media added (16 g bactotryptone, 10 g NaCl, 10 g Yeast extract, 1 liter ddH₂O, pH 7.3), and incubated for 1 hour at 37°C. Transformed cells were spread onto plates containing ampicillin (100 ug/ml; BMB, Indianapolis, ID.), tetracycline (12.5 ug/ml; Sigma, MO.), 40ug/ml X-Gal (BMB), and 5mM Isopropyl-B-D-thiogalactopyranoside (IPTG; Five prime to three prime, Inc., Westchester, PA.), and incubated overnight at 37°C.

Plasmid Sizing and Screening of Recombinant Colonies. Each millimeter diameter white recombinant colony was transferred to a tube containing 5 ul of protoplast buffer solution (30 mM Tris pH 8.0, 5 mM EDTA, 50 mM NaCl, 20% sucrose, 50 ug/ml RNA'se A (BRL, MA.), and 50 ug/ml lysozyme (Sigma, MO.), and mixed rapidly by twirling a wire loop in the solution. Each suspension was streaked onto a master plate for recovery. Two microliters of lysis buffer (Tris-acetate electrophoresis buffer, 2% SDS, 5% sucrose, and 0.04% Bromophenol blue) were added into the wells of a 0.8% agarose gel. The samples were then loaded into the wells, electrophoresed (50-100V/1h.), stained with ethidium bromide, and photographed.
Prospective recombinant and non-insert containing colonies were selected and grown in 50 ml of LB media (pH 7.5) (Gibco; Madison, WI.) containing ampicillin (75 ug/ml) at 37°C overnight. Bacterial cultures were diluted to early log phase and then incubated for 2-3 hours to early log phase at 37°C. At this point in the growth curve of the cell cultures, IPTG to a final concentration of 5 mM was added and newly induced cells were incubated for an additional 3 hours or until cell cultures reached early stationary phase. IPTG induced cultures were concentrated 50X by centrifugation (3000 x g) and lysed by sonication at setting 3 (Biosonic III, Bronwill Scientific; Rochester, N.Y.). Samples were centrifuged at 12,400 x g (Beckman microfuge 12) for 15 minutes; supernatant and pellet fractions were collected, and screened by dot-immunoblots for product expression.

Recombinant pBluescript Isolation for DNA Sequencing.
A 50 ml recombinant cell culture was grown overnight in LB Media under ampicillin selective conditions at 37°C. The culture was centrifuged at 4000 rpm (~3000 x g) for 15 minutes in a J6B centrifuge. The pellet fraction was resuspended in 3.3 ml of lysis buffer (50 mM glucose, 25 mM Tris-Cl pH 8.0, 10 mM EDTA, 4 mg/ml lysozyme). The lysozyme was added to the buffer just prior to use and the cell suspension was incubated at room temperature for 5 minutes; 6.6 ml of 0.2 M NaOH, 1% SDS was added, mixed gently, and incubated on ice for another 5 minutes. The cells were incubated for an additional 5 minutes on ice following the addition of 5 ml of 5M potassium acetate. The lysate mixture was centrifuged for 15 minutes at 4000 rpm. The supernatant fraction was collected and placed in a 50 ml sterile centrifuge tube, and extracted with an equal volume of phenol for 5 minutes. The top aqueous phase (plasmid DNA) was recovered and placed into another sterile 50 ml centrifuge.
tube, two volumes of 95% ethanol were added and the suspension incubated for 3 minutes at room temperature. The plasmid DNA preparation was centrifuged for 20 minutes at 4000 rpm, rinsed with 70% ethanol and dried in a speed vac concentrator (Speed Vac SVC100H, Savant Instruments, Inc., Farmingdale, NY.). The dried DNA preparation was then dissolved in 1.6 ml TE buffer; 50 ul of RNase was (1 mg/ml pancreatic RNase) added and the suspension incubated for 30 minutes at 37°C. One milliliter of 20% polyethylene glycol (PEG) 8000, 2.5 M NaCl was added and the suspension incubated on ice for an additional 45 minutes. The plasmid preparation was then centrifuged for 20 minutes at 4000 rpm and the pellet was rinsed with 70% ethanol to remove PEG and submitted to a speed vac drying. A volume of 200 ul of TE buffer was added and the plasmid DNA concentration was determined at 260nm (Spectrophotometer 2000).

**Denaturation of Double Stranded pBluescript and Bsi Recombinant DNA for Sequencing.** Supercoiled plasmid DNA (3.5-5 ug) was dissolved in 18 ul of sterile ddH2O. A volume of 2 ul of 2M NaOH was added to the dissolved DNA and incubated for 5 minutes at room temperature. This was followed by the addition of 8 ul of 5M ammonium acetate (pH 7.4), and 100 ul of 95% ethanol, and an incubation for 15 minutes at -70°C. The denatured DNA sample was centrifuged for 15 to 30 minutes at 14,000 x g. After carefully removing and discarding the supernatant, the pellet was dried in a speed vac.

**DNA Sequencing of pBluescript DNA template with T7 DNA Polymerase.** A modification of the chain-termination DNA sequencing method originally described by Sanger, et al. (2) was utilized to sequence the recombinant plasmid DNA samples. Briefly, the chain-termination method involved the
synthesis of a DNA strand by a DNA polymerase in vitro using a single-stranded DNA template. Synthesis was initiated at only one site where an oligonucleotide primer annealed to the template. After denaturation of the pBluescript plasmid DNA (5 ug) with 2N NaOH, a synthetic oligonucleotide primer was annealed to the template DNA. The pBluescript vector contains 3 different primer-annealing sites on either side of the multiple cloning region of the double stranded plasmid DNA: M 13 -20 primer (5' GTAAAAACGACGGCCAGT 3' —>), T7 primer (5' AATACGACTCAGCTATAG 3' —>), SK primer (5' TCTAGA ACTAGTGATC 3' —>), KS primer (3' GCTATGGCAGCTGGAGC 5'), T3 primer (3' GAAATCACTCCCAATTA 5'), and Reverse primer (3' GTACCAGTATCGACAA 5') (Molecular Genetics Laboratories, City of Hope, CA.). The synthesis reaction was terminated by the incorporation of a nucleotide analog that will not support continued DNA elongation. A radioactively labeled nucleotide (35S) was also included in the synthesis, so that the labeled chains of various lengths could be visualized by autoradiography after separation by high-resolution electrophoresis.

The molar ratio of synthetic oligonucleotide primer to template was adjusted to 1:1. The concentration of the primer solution was adjusted so that 1 ul contained the mass of primer needed to achieve the desired primer:template ratio. The denatured template DNA (5 ug) was resuspended in 7 ul of sterile H2O, then 2 ul of annealing buffer (200mM Tris-HCl pH 7.5, 100 mM MgCl2, 250mM NaCl), and 1 ul of synthetic oligonucleotide primer solution (50 ng/ul) were added. The subsequent annealing reaction was incubated at 65°C for 2 minutes, and then allowed to cool slowly to room temperature over a period of about 30 minutes. The following were added to the annealed template-primer: 1 ul of dithiothreitol (DTT, 0.1M), 2 ul of labeling nucleotide mix (1.5uM dGTP, 1.5uM dCTP, 1.5uM dTTP), 5 uCi alpha-35S dATP
(>1000 mCi/mmol) (New England Nuclear, Wilmington, DE), and 3 units of T7 DNA polymerase to a total volume of approximately 15 ul. The labeling solution was mixed thoroughly and incubated for 5 minutes at room temperature.

Four tubes labeled "G", "A", "T", "C", were filled each with 2.5 ul of the appropriate dideoxy termination mixture, and pre-warmed to 37°C. The dideoxy termination mix consisted of a mixture of 80uM dGTP, 80uM dATP, 80uM dTTP, and dCTP; in addition, the "G" mixture contained 8uM dideoxy-dGTP; the "A" mix, 8uM ddATP; the "T" mix, 8uM ddTTP; and the "C" 8uM ddCTP. Following the labeling reaction, 3.5 ul of labeling suspension was transferred to each corresponding dideoxy termination tube, labeled either "A", "C", "G", or "T". After a 2-5 minute incubation at 37°C, 4 ul of Stop solution (95% Formamide, 20mM EDTA, 0.05% bromophenol blue, and 0.05% Xylene Cyanol FF) were added to each termination reaction; and the suspension was mixed and stored at -20°C.

i. Sequencing Gel Electrophoresis

The glass plates (60cm long) were cleaned with soap, rinsed thoroughly with water, dried, and rinsed with ethanol and dried once again. The notched plate was treated with a 10% organo-silane (silicone) solution (Prosil-28, PCR Inc., Gainesville, Fl.), rinsed with distilled water and dried. The glass plates were arranged for assembly, with the silicone-treated plate facing inward. Two 0.4 mm side spacers (BRL, Gaithersburg, MA.) were placed between the long edges of the plates. An extra piece of the same material was inserted at the bottom of the plate sandwich, and the plates were clamped together.

The 6% acrylamide gel was prepared to a final volume of 50 ml by combining 20 ml of 20% acrylamide solution, 5 ml of 10X TBE buffer (108 g Tris base, 55 g Boric acid, 9.3 g EDTA/1 liter, pH. 8.3), and 25 ml of 46% Urea solution (Bio-Rad
Laboratories, Richmond, CA.). The acrylamide mixture was filtered and degased under low vacuum for 5 minutes. Gel formation was initiated by adding 250 ul of 10% ammonium persulfate and 50 ul of TEMED [N,N,N',N'-Tetramethyl-ethylenediamine] to the acrylamide mixture and swirled to mix. The final solution was immediately poured into a 50 ml syringe while blocking the tip. The plunger was inserted into the syringe, air ejected, and the solution was injected between the plates (set at a 45° angle), slowly to avoid introducing air bubbles.

After pouring the gel, the plates were laid in a horizontal position, the surface-former of a shark-tooth comb inserted, and the gel allowed to polymerize for ~ 2 hours. The comb was removed and the formed surface was rinsed with distilled water, to remove unpolymerised acrylamide. The gel was then placed in the electrophoresis apparatus (BRL, Bethesda, MD.), and 1X TBE buffer was added to the buffer chambers. The gel was pre-ran at 70 watts (constant) for approximately 30 minutes. Prior to loading the samples, the power supply was switched off and the shark-tooth comb was inserted, so that the points just touch the surface of the gel. The samples were then heated to 75°C for 2 minutes and 1.5 ul were loaded in each lane. The power supply was reconnected and the samples were electrophoresed at 55-60W for approximately 90-120 minutes.

**ii. Autoradiography**

After removing the gel/plate assembly from the electrophoresis apparatus, the assembly was laid in a horizontal position with the notched plate uppermost and the plates were carefully separated so that the gel remained attached to one of them. The gel was then transferred to a supporting sheet of filter paper (Whatman #1), covered with Saran plastic wrap, and dried using a vacuum gel dryer. When the gel was dried, the plastic wrap was removed and the gel
was transferred to a film cassette. A sheet of X-ray film (Kodak Diagnostic Film, 35 x 43 cm, Eastman Kodak, Rochester, N.Y.) was placed on the gel, and exposed in the dark overnight at room temperature. The film was developed according to the manufacturer's instructions.

Analysis of Recombinant Gene Product by SDS-PAGE Electrophoresis. A Bicinchoninic acid protein assay (BCA) (Pierce, Rockford, Il.) was first performed to determine the protein concentration of the lysate samples. A series of calibration protein standards was prepared by 2-fold serial dilutions of 1.0 mg/ml Bovine Serum Albumin (BSA) stock standard to a final standard concentration of 63 ug/ml in PBS diluent buffer. An aliquot of 0.1 ml of each standard dilution and unknown protein sample were placed into appropriately labeled tubes; blanks obtained 0.1 ml of diluent buffer. To each tube, 2.0 ml of "working reagent" (50 parts of Reagent A with 1 part of Reagent B) were added and mixed well.

The tubes were incubated at 60°C for 30 minutes. After allowing the tubes to cool to room temperature, the absorbance of each tube was measured at 562nm. A standard curve was prepared by plotting absorbance at 562nm against protein concentration. From this standard curve, the protein concentration for each unknown lysate protein sample was determined. Based on the protein concentration of each lysate sample, dilutions were performed to standardize all samples to a final gel loading concentration of approximately 1.0 mg/ml.

i. Gel Preparation

The plates were soaked in 1:1 HNO₃/ H₂O for 3 hours, rinsed with distilled water, and allowed to air dry. The running gel (12.5% acrylamide:0.011% Bis-acrylamide, 0.375M Tris-Cl pH 8.8, 0.1% SDS, ddH₂O) and the stacking gel (5%
acrylamide: 0.001% Bis-acrylamide, 0.125 M Tris Cl pH 6.8, 0.1% SDS, ddH<sub>2</sub>O) were prepared and degassed with a moderate vacuum. The plates were aligned and assembled in a casting stand (Protean II Slab cell, Bio-Rad, Richmond, CA.) according to the manufacturer's instructions. Prior to pouring the running gel, 15 ul TEMED and 150 ul of freshly made 10% ammonium persulfate (AP) were added to the acrylamide solution. An anaerobic environment was produced for gel polymerization by an overlay of a 0.1% SDS solution. The gel was allowed to polymerize for approximately 30 minutes. The overlay solution was poured off and the gel was rinsed three times with the 0.1% SDS solution.

The stacking gel was then poured, adding the TEMED and 10% AP just prior to pouring. The well-forming comb was appropriately placed, and the gel was allowed to polymerize for 20 minutes. The comb was removed and the wells were washed 3 times with running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS pH 8.3). The plate-sandwiched gel was assembled in the cooling core unit. The sample specimens were diluted 1:1 in 2X sample buffer (0.1 mM Tris-Cl pH 6.8, 2.5% SDS, 5.0% B-Mercaptoethanol, 20% glycerol, ddH<sub>2</sub>O), and boiled for 5 minutes. Approximately 50 ul of each protein sample were loaded into each well. The cooling-core unit with the glass plate sandwiched gel was placed in the electrophoresis buffer chamber. Running buffer was placed in the lower and top chamber. To the running buffer in the top chamber, 500 ul of 0.1% bromophenol blue dye were added and the gel was electrophoresed at a constant power of 3 watts for 4 to 5 hours.

ii. Western Blot

Once the electrophoretic run was completed, the power supply was turned off, and the plates were removed from the electrophoresis chamber. The plates were carefully pried open, and the gel was appropriately marked, and stabilized by
immersing it in transfer buffer (25mM Tris, 192mM glycine, 20% methanol) for 10 minutes. One sheet of nitrocellulose membrane and four sheets of absorbent filter paper (Whatman 3MM) were cut to the size of the gel, and soaked in transfer buffer. The gel, nitrocellulose, filter papers, and support pads, immersed in transfer buffer, were assembled to form a gel/membrane sandwich, keeping all the components wet and making sure the sandwich was tightly assembled, to exclude air bubbles. The complete sandwich was placed in the transfer tank with the membrane closest to the positive electrode. The electro-transfer was carried out at 100V for 2 hours. After transfer, the power supply was disconnected, and the gel/membrane sandwich was disassembled. The gel was then stained with Coomassie brilliant blue R-250 (0.1% Coomassie blue R-250, 46% methanol, 8% acetic acid) for 20 minutes, destained overnight in 20% methanol, 8% glacial acetic acid, at room temperature, and finally dried using a vacuum gel drier. The nitrocellulose sheet was treated according to Bio-Rad's Immuno-Blot™ enzyme immunoassay for the detection of reactive proteins (Bio-Rad, Richmond, CA.). Bovine serum albumin (1.0%) and gelatin (1.0%) were used as blockers. Bound antigen was then incubated with polyclonal CT serovar D antibodies. The membrane was washed to remove unbound antibody, and then incubated with GAM-HRP and washed again. Horseradish peroxidase substrate color development completed the procedure.

**Protein Purification by Size Exclusion HPLC.** The liquid chromatograph was assembled from a Waters Millipore M-45 solvent delivery system (Waters Instruments, Milford, MA.), a Waters Associates Model 441 absorbance detector (Waters Instruments, MA.), and a Model U6K universal liquid chromatograph injector (Waters Instruments, MA.). A Bio-sil TSK-250 (Bio-Rad, CA.) HPLC gel filtration column, having a
mean particle diameter of 10um and 300 x 7.5mm I.D. was used. The supernatant fraction from positive IPTG induced recombinant lysates was collected, and 0.3 ml of the protein solution were injected into the HPLC system. The mobile phase, consisting of 1X phosphate buffer saline (PBS), was set to a flow rate of 0.300 ml/min., and the ultraviolet absorbance detection was adjusted to a wavelength of 280 nm. The fractions were collected and screened for immunoreactive proteins.

**Immunizations.** Twenty-one CF-1 female mice, separated into 7 different groups, were immunized with the following antigen preparations: (1) Pellet fraction from recombinant lambda EMBL-3 bacteriophage-derived lysate (containing the Ct gene), (2) pellet fraction from parent bacteriophage strain lysate (EMBL-3), (3) Supernatant fraction from subcloned recombinant pBluescript KS derived lysate (pBS-1), (4) supernatant fraction from parent plasmid strain lysate (pBluescript), (5) HPLC-purified pBS-1 lysate preparation, (6) formalin-killed *Chlamydia trachomatis* Serovar D, and (7) control group (non-immunized). Each group of animals was injected subcutaneously on each of two occasions (14 days apart) with suspensions (100 ul in PBS) containing 200 ug of total protein.

Two weeks after the second inoculation, the mice were CO2 euthanized and exsanguinated. The blood samples were allowed to clot for 30 to 60 minutes at 37°C. The clot was then separated from the sides of the collection vessel; and the serum was removed from the clot by centrifuging at 10,000 X g for 15 minutes at 4°C. The serum was stored at -20°C.

**Enzyme Immunoassay (EIA).** Renografin purified, SDS solubilized and whole cell chlamydial antigen (serovar D) were diluted 1:500 and 1:1000 with 0.05M carbonate buffer
(pH 9.6). In microtiter plates, 70 ul of each diluted antigen were dispensed per well, allowing the antigen to adsorb onto the bottom of each well for 3 days at 4°C. After allowing the microtiter plates to come to room temperature, the antigen excess was washed off 3 times with washing solution (1X PBS/ 0.05% Tween 20), and blotted dry.

An aliquot of 0.2 ml of buffer G (10X PBS, 0.01M MgCl₂, 1% BSA, 5% gelatin, ddH₂O) was dispensed into each well and incubated for 30 minutes at 37°C in a 10% CO₂, humidified incubator. The microtiter plates were washed again 3 times with washing solution and blotted dry. The serum against each group of antigen preparation from the immunization procedure described above, was diluted 1:100 and 1:500 with buffer G. Each dilution was dispensed into separate wells. The serum was incubated for 1 hour at 37°C in a CO₂, humidified incubator. Plates were washed 3 times to remove serum and blotted dry. Seventy microliters of a 1:2000 dilution of HRP-GAM IgG antibodies were aliquoted into each well and incubated for 1 hour at 37°C. Excess conjugate was removed with 3 rinses of 1X PBS /0.05% Tween 20, blotted dry, and 70 ul of 0.4 mg/ml ortho-phenylenediamine in phosphate citrate buffer (0.2M Na₂HPO₄, 0.1 M citrate, ddH₂O, 30% H₂O₂, pH 5.0) were added per well. The plates were incubated for 30 minutes in the dark at room temperature. The color development reaction was stopped by adding 50ul of 2.5M H₂SO₄ per well, and the absorbance read at 490nm.

**Results**

The original low yield of recombinant product (CT-9) obtained from the bacteriophage lambda EMBL-3 lysates necessitated an examination of all the factors influencing bacterial cell growth and bacteriophage infection. Parameters tested included MOI, bacterial concentration,
media, cell toxicity, and aeration. The following results identify the combination that supported maximum product yield.

Bacterial cultures of $8.0 \times 10^8$ cells/ml were infected at an M.O.I of 10:1 of a bacteriophage to bacterial cell, where the efficiency of phage attachment ranged between 80 to 90%. Thirty minutes after phage adsorption, the cell cultures were vigorously agitated, and visible lysis was apparent 75-90 minutes after initial infection. The cell lysate was collected, centrifuged, and the pellet and supernatant fractions were tested for a Ct product signal by dot immunoblots; most of the Ct product signal was found to be associated with the pellet fraction. Figure 1 compares the level of immunoreactive signal obtained from the supernatant and pellet fractions. Two-fold serial dilutions from each fraction confirmed the association of the recombinant product with the pellet fraction, despite some remaining product in the supernatant fraction. Aeration was examined as a means of influencing the virulent nature of EMBL-3's bacteriophage lytic cycle, thus providing a means to increase the yield of recombinant product. The effect of aeration during bacteriophage infection was determined by establishing cell cultures, infecting them with equal M.O.I.'s, and incubating either statically or with moderate aeration. Only a slight increase in Ct product signal was evident in the statically incubated cultures when compared to aerated cultures, despite a 50-fold pellet concentration (Figure 2).

Low expression yields in the EMBL-3 bacteriophage vector prompted efforts to subclone the recombinant chlamydial insert into a high expression vector (pBluescript) featuring better selection, and induction control.

Subcloning of Recombinant Bacteriophage Lambda EMBL-3 to pBluescript Plasmid. Isolated and purified
recombinant EMBL-3 DNA samples (CT-9) were cleaved with Eco RI and Sal I restriction endonucleases independently, and each separated on a 0.8% Tris-acetate (TAE) agarose gel (figure 3). Following phenol extraction and ethanol precipitation, the pool of fragments from each enzymatic reaction were randomly ligated to an alkaline phosphatase-treated pBluescript vector KS+, containing compatible Eco RI or Sal I staggered 3' ends. The two remaining open 5' phosphodiester bonds were closed in vivo after transformation into the bacterial cell. White colonies were selected, and screened for plasmid sizing (Figure 4). Based on differences in recombinant plasmid sizes, seven recombinant clones were selected and subsequently amplified. Enzymatic cleavage of each selected recombinant clone with Eco RI revealed potential insert sizes as well as false-positive recombinant clones (Figure 5). False positive clones may result from the loss of the episome ( F', pro AB, lac qZ^ M15) in XL-1 blue cells, coding for the B-gal omega fragment, necessary for Alpha-complementation, and/or the transformation of bacterial cells with concatenate configurations of the plasmid vector. Recombinant clones pBS 11, 14, and 3 contain inserts of approximately 1.5 kb, 2.3kb, and 5.5kb, respectively; while recombinant clone pBS 7 contains no apparent chlamydial insert. All seven recombinant clones (pBS-1, -3, -7, -11, -12, -14, and -24) were grown to log phase in rich media under selective conditions and IPTG-induced for 3 to 4 hours. Following cell lysis by sonication and one freeze-thaw cycle, the crude supernatant and pellet fractions from each clone were dot blotted and screened for chlamydial immunoreactive product (Figure 6). One clone (pBS1) expressed a recombinant chlamydial antigen. Complete enzymatic cleavage of the pBS1 DNA with Eco RI originally generated two fragments (3.0 kb and 2.0 kb), indicating a potential insert size of two kilobases.
Figure 1. Comparison of immunoreactive signal level between the supernatant and pellet fractions of the recombinant product Ct-9. The cell lysate from bacterial cultures infected with the recombinant CT-9 lambda bacteriophage was collected, centrifuged, and the pellet and supernatant fractions were dot blotted onto a nitrocellulose membrane. The membrane was exposed to whole cell Ct. Serovar D polyclonal antisera. Positive and negative controls include immunoblots A-1 (Ct. Serovar D), and A-2 (recombinant Ct-9 lambda bacteriophage). Immunoblots A-3 thru A-6 contain pellet fractions of the Ct-9 recombinant product, followed by two-fold serial dilutions of the same preparation. Immunoblots A-7 thru A-10 contain supernatant fractions of the Ct-9 recombinant product, followed by two-fold serial dilutions of the same preparation. Immunoblots A-11 and A-12 contain the recombinant Ct-9 cell suspension and a 1:2 dilution, respectively.
Figure 2. Comparison yield of recombinant gene product following static or aerated incubation. Cell cultures infected with Ct-9 lambda recombinant bacteriophage were incubated either statically or with aeration. Despite a 50-fold pellet concentration, only a slight increase in immunoreactive signal was obtained. Immunoblots A-1 and A-2 are positive and negative controls respectively. Immunoblots A-5 thru A-9 contain 50-fold concentrated pellet suspensions from aerated culture preparation, followed by two-fold serial dilutions. Immunoblots C-10 thru D-3 contain 50-fold concentrated pellet fractions from statically grown infected cultures, followed by two-fold serial dilutions.
Figure 3. DNA digestion of lambda recombinant Ct-9 clone with EcoRI and SalI. The purified recombinant EMBL-3 DNA sample (Ct-9 clone) was cleaved with EcoRI and SalI restriction endonucleases independently, and the DNA fragments generated from each reaction were separated on a 0.8% Tris-acetate (TAE) agarose gel. Cleavage with EcoRI yielded five distinct fragments of various sizes, while the SalI reaction mix generated three fragments.
Figure 4. Plasmid sizing of recombinant pBluescript clones. Potential white recombinant colonies and non-recombinant blue colony controls were screened by the rapid plasmid screen assay (see Materials and Methods). Lanes 1-14 contain supercoiled DNA from white recombinant colonies with inserts at the EcoRI site. Lanes 15 and 30 contain DNA from blue colonies (negative controls). Lanes 16-29 contain DNA from recombinant colonies with inserts at the SalI site.
Figure 5. Enzymatic cleavage of recombinant DNA clone with EcoRI. Supercoiled DNA templates from potential pBluescript recombinant clones were cleaved with EcoRI to reveal various Ct. DNA insert sizes. Lane 1 contains uncut DNA pBluescript control vector. Lane 2 contains uncut DNA from recombinant clone 1 (renamed, pBS1). Lanes 3-8 contain uncut pBluescript recombinant DNA clones: pBS3, pBS7, pBS11, pBS12, pBS14, and pBS24 respectively. Lane 9 contains the pBluescript control vector digested with EcoRI. Lane 10 displays the pBS24 recombinant DNA clone, containing the SalI site after cleavage with EcoRI. Lanes 11-14 contain recombinant pBluescript DNA clones digested with EcoRI: pBS14, pBS-11, pBS-7, and pBS-3, respectively. Lane 15 contains recombinant DNA (Ct-9 clone) cleaved with EcoRI. Lane 16 contains 100 ng of Lambda DNA mix markers.
Figure 6. Expression of the Recombinant Ct. product (clone immunoscreening). All seven potential recombinant clones were screened for immuno-reactive product. Only one clone, renamed pBS1 expressed a recombinant Ct. product. Immunodot A-1 is a positive control containing a whole cell suspension of Ct. Serovar D. A-4 is the pBS-1 clone whole cell reaction mix. B-8 and B-9 contain the 50X concentrated whole cell reaction mix of clone pBS1, followed by a 1:10 dilution of that suspension. C-12 and D-1 contain the concentrated suspension after sonication, followed by a 1:10 dilution. Immunodots F-4 and F-5 represent the pBS1 50X lysate suspension after sonication and one freeze-thaw cycle. Samples F-8 and F-9 represent the pBS-1 supernatant fraction followed by a 1:10 dilution. Finally, samples G-12 and H-1 show the immunoreactive signal obtained from the pBS-1 pellet fraction and its 1:10 dilution.
However, further restriction analysis of the pBS1 clone DNA with Hind III, and Sac I, and partial digestion with Eco RI revealed the inserted Ct DNA to be approximately 5.0 Kb long (Figure 7a, 7b and Figure 8), suggesting the existence of an Eco RI site internal to the pBS1 insert DNA.

pBluescript Recombinant Ct. Product. The pBluescript vector provided higher Ct product yield and demonstrated the product association with the supernatant fraction of cell lysates (Figure 9). The crude supernatant was analyzed by size exclusion HPLC (SEC-HPLC), where the stationary phase is silica based and possesses properties suitable for use with aqueous buffers. The crude supernatant from clone pBS1 was injected into the HPLC system, and each fraction was collected and tested for Ct product signal (Figure 10). It was found that: 1) recovery of the recombinant product was generally in excess of 90%, 2) the profile was highly reproducible, and 3) the elution order was predictable. The retention time (Tr) of the pBS1 sample, which is the time from injection to the time of maximum concentration in the eluted peak, was approximately 20 minutes. Dot immunoblots revealed that all the recombinant product was found in the void volume (Vo). The void volume represents the sum of the interstitial volume between the particles of the stationary phase and the accessible volume within the particle pores. Attempts to use denaturing and reducing agents such as SDS and B-mercaptoethanol and/or organic solvents (trifluoroacetic acid) to inhibit any intermolecular interactions of solutes proved unsuccessful. However, a significant number of molecules ranging from 10,000 - 150,000 daltons were successfully removed, thus semi-purifying the Ct recombinant protein mixture.
Identification and Comparison of the Recombinant Ct. protein product derived from EMBL-3 Lambda and pBluescript. The relationship between the recombinant protein mixture derived from the EMBL-3 lambda and pBluescript vector, and the Ct serovar D whole cell suspension was established by SDS-PAGE and Western immunoblots. The protein concentration for each antigen preparation was determined by the BCA protein assay (See Materials and Methods). Each protein sample was diluted in sample buffer to standardize the total amount of loading material to 30 μg/mm². The molecular weight range of the Ct serovar D solubilized whole cell suspensions was estimated between 17,000 to 130,000 daltons. Figure 11 shows a Western immunoblot of the recombinant clones and their corresponding parent vectors. An immunoreactive band of approximately 36 kd was observed in both the bacteriophage (Ct-9) and the plasmid derived (BS1) recombinant clones and in the Ct serovar D antigen preparations. However, a 36kd band is absent in both parent (non-insert containing) vectors, EMBL-3 and pBluescript.

Murine Immunogenic Response to the Ct. Recombinant 36kd Protein. Identification of the Ct recombinant 36kd protein was followed by the preparation of the recombinant lysate samples and controls for the immunization of mice to test their immunogenicity. Twenty one CF-1 female mice were divided into seven groups and immunized subcutaneously with suspensions (0.1 ml in PBS) containing 200 ug of total protein. Due to a lower protein concentration obtained from the HPLC-purified BS-1 lysate preparation, 50 ug (total protein) was administered to this particular group. An analysis of the sera collected from each group demonstrated that all recombinant groups presented an immunogenic response to the Ct. recombinant 36kd protein.
A solubilized Ct serovar D whole cell suspension was separated on a 12.5% SDS polyacrylamide gel, and electrophoretically transferred onto a nitrocellulose membrane. The nitrocellulose paper was then cut into strips of equal length, and each strip was exposed to individual serum collected from each immunized group of CF-1 mice. No immunoreactive bands at the 36kd location were found in any of the control groups (non-immunized mice, EMBL-3, and pBluescript) (Figure 12). However, the sera from all recombinant preparations (lambda Ct-9, pBluescript BS-1, and HPLC-purified BS-1) demonstrated an immunoreactive band of approximately 36kd. The group of mice inoculated with the Ct serovar D whole cell suspension failed to present the 36kd immunoreactive band, which may be due to the variable frequencies of response in mice to the Ct. 36kd protein, and/or an immune response below detectable levels; thus, minimizing or apparently eliminating the 36kd band signal from the immunoblot. Finally, to determine the putative cellular location of the 36kd protein in Ct serovar D, an antibody-capture enzyme immunoassay was designed; however, no conclusive data was found to clearly support a putative cellular location of the 36kd Ct protein by this assay.

Partial DNA sequencing of the Ct. Recombinant gene. The Bluescript plasmid-derived Ct. recombinant gene (BS-1) was partially sequenced by the dideoxy-chain termination sequencing method. The T7 and T3 primers, found at either side of the target insert in the multiple cloning site of the pBluescript KS\(^+\) vector, were hybridized to a single stranded template, and extended by T7 DNA polymerase to approximately 350 bases on either priming site. Figure 13 illustrates the partial DNA sequence obtained from the Ct.recombinant gene. The KS and T3 sequencing reactions extended the Ct recombinant DNA coding strand.
Figure 7a and 7b. Digestion of pBluescript and Recombinant clone DNA pBS1 with HindIII, SacI, and EcoRI. In Figure 7a, lanes 1, 3, and 6 contain pBluescript control DNA digested with HindIII, SacI, and EcoRI, respectively. Lanes 2, 5, and 7 display the separation profile of DNA fragments obtained from the enzymatic cleavage of the recombinant pBS1 DNA clone with HindIII, SacI, and EcoRI, respectively. Lane 4 contains 500 ng of lambda DNA molecular mix markers cut with HindIII and EcoRI.

In figure 7b, lane 1 contains supercoiled pBS1 DNA. Lane 2 contains lambda molecular mix markers, and lanes 3 and 4 contain the pBS1 DNA cleaved with HindIII and SacI, respectively.
Figure 8. Partial Digestion of pBluescript vector and pBS1 recombinant DNA with EcoRI. Lane 1 contains undigested pBluescript vector DNA; and lane 2 contains pBluescript DNA partially digested with EcoRI. Lane 3 contains 400 ng of lambda molecular mix markers. Lane 4 and lane 5 each contain DNA from clone pBS1 partially digested with EcoRI and in supercoiled configuration, respectively.
Figure 9. Comparison of Recombinant product yield obtained between the lambda Ct-9 and the pBS-1 clones. Immunodots A-1 and C-1 contain the Ct Serovar D whole cell suspension (positive controls). Negative controls are represented by immunodots A-2 (lambda bacteriophage particle suspension), and C-2 (pBluescript whole cell lysate suspension). Immunodots A-3 thru A-10 represent a two-fold serial dilution of the pellet fraction extracted from Ct-9 recombinant cell lysates. Rows B-1 thru B-8 represent serially diluted pellet fractions from the parent lambda vector EMBL-3. Immunodot C-3 contains the pBS1 whole cell lysate suspension. Immunodots C-4 thru D-1 contain a 2-fold serial dilution of the supernatant fraction extracted from the pBS1 recombinant cell lysates. Finally, D-2 thru D-9 contain serially diluted supernatant fractions derived from the parent pBluescript cell lysates.
Figure 10. Separation Profile of Crude pBS1 lysate supernatant by Size-Exclusion HPLC. The supernatant fraction (in 1X PBS) from the pBS1 lysate was injected into an HPLC unit. Six chromatographic peaks were resolved; and aliquots from all the peaks were taken at 1 minute intervals, dot blotted onto nitrocellulose membrane and exposed to Ct. Serovar D whole cell antibodies. Immunodot A-1 (Ct Serovar D whole cell suspension) and A-2 (pBluescript whole cell suspension) represent positive and negative controls, respectively. Immunodots A-3 thru A-9 correspond to the void volume peak. Immunodots A-10 thru B-6 represent aliquots obtained under peak# 1; B-7 thru B-11 contain HPLC fractions from peak# 2; and B-12 thru C-12 correspond to peaks #3, #4, and #5.
**Figure 11.** Immunoblot of Recombinant clones and parent vectors. Lane 1 contains standard molecular weight markers. Lane 2 contains the lambda recombinant clone Ct-9. Lane 3 contains the pBS1 plasmid Ct recombinant clone. Lane 4 contains the Ct. Serovar D whole cell suspension. Lane 5 contains the parent lambda bacteriophage (EMBL-3) or non-insert containing vector, and lane 6 contains the pBluescript parent plasmid or non-insert containing vector. Ct. serovar D polyclonal antisera was used for all the antigens.
Figure 12. Western Immunoblot of Ct. Serovar D exposed to antisera from different immunized murine groups. Strips of nitrocellulose membrane, containing electrophoretically transferred Ct. Serovar D antigens were exposed to antisera obtained from immunized, non-immunized, and control groups. Group 1 represents antisera obtained from non-immunized mice. The other groups included mice immunized with: a pellet suspension from the EMBL-3 lambda vector (group 2), the Ct-9 lambda recombinant pellet product (group 3), Ct Serovar D whole cell suspension (group 4), the pBS1 recombinant supernatant product (group 5), the supernatant fraction from the parent pBluescript vector (group 6), and the HPLC-purified pBS1 recombinant product (group 7). An immunoreactive band of approximately 36kd was observed in all the recombinant groups. A control strip of nitrocellulose membrane ("C") was exposed to Ct. Serovar D (E. coli absorbed) polyclonal antisera.
Figure 13. Partial DNA sequence of the Recombinant clone pBS1 with primers T3 and T7.

Denatured double stranded DNA clone pBS1 was hybridized with primers T3 or T7, and extended by T7 DNA polymerase to approximately 350 base pairs on either direction. At the present time, no open reading frames or regulatory sequences have been located in the pBS1 clone.
### PBS1 /T3 Primer

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52
pBS1/ T7 Primer

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10  20  30  40  50
GAATTCTATA GTGTTTCTAG CTTTTATCGC AATCTCATTT TAGAGATTTT
CTTAAGATAT CACAAAAATC GAAATAGCG TTAGAGTAAA ATCTCTAAAA

60  70  80  90  100
TTTGATTTGG ACAAAAGAAA ATAAAGTACT TGAGATTGTT TTCTAAGTTT
AAACTAAACC TGTGGGCATT TATTTCCATA AGTCTAAACAA AAGATTCCTAA

110  120  130  140  150
GTTTGCAATAA ACAGCCATCA TTATGCAGG TTAGTAGCAG AAATCTGTTT
CAAACGTATT TGTCGGTAGT AAATACGTCC AAATACGTCC TTTAGACAAA

160  170  180  190  200
GGAAACAATTT TACTTTCTTT CAGACTTTTT ATAGTTGTTT GCTTAGAAAA
CCTTGTAAAT ATGAAAAAGA GTCTGAAAAA TATCAACAAA CGAATCTTTTT

210  220  230  240  250
CACGTATTAG TATGCATTTT TCTTAGTTGT TAAAATTATT TAGAATCGTC
GTGCATAATC ATACGTAATA AGAATCAACA ATTTTAATAA ATCTTAGCAG

260  270  280  290  300
TCTTGCAGAT GAGGAACGCG CTTAAGATTTC CTTTGCAGTTG AAGGATATAA
AGAAGCTCTA ATCCCTTGCC GCATCTAAAA GAAAGCCCAA TCCTATATT

310  320  330  340
TTTATCTATG TAAAGATACC TTTGCCGATAA GCGAGGTTT
AAGTAAGACAA ATTTTCTATAG AAACGCTATT CGCTCCAAAG

53
(transcriptional orientation), while T7 and SK oligonucleotides were employed to sequence the opposite or non-coding DNA strand. So far, no chlamydial promoter regions and/or open reading frames have been found in pBS-1.

Discussion

The essence of DNA cloning is joining a stretch of DNA of interest to a vector molecule which serves to propagate that DNA segment in either bacterial or eukaryotic cells. Vectors for Escherichia coli are derived from the natural phages and plasmids of this organism. The use of lambda bacteriophage as cloning vectors is desirable because of its ability to accommodate large fragments of DNA (~25 kb) in its genome, and its packaging into capsids in vitro (7). Depending on the investigator's needs, lambda vectors may be useful either as a primary or intermediate cloning step. To clone a specific gene from a particular species, the usual approach is to make a "library" containing some or all of the genes from that species. This library consists of a population of clones, each containing small segments of genetic information from the species in question. Once the library has been made, the next step - and usually the biggest challenge - is to screen it to select the one or few clones which contain the gene of interest (6). Lambda gene libraries may be efficiently screened with either an antibody against the protein encoded by the gene of interest (which requires some part of that protein to be expressed, provided an efficient promoter region is found close to the gene of interest), or a labelled nucleic acid probe (which implies some knowledge of, or assumption concerning the sequence being screened for). In this study, however, due to low recombinant product yield
in lambda EMBL-3, the pBluescript plasmid vector became an alternative to obtain a greater yield of recombinant product needed to conduct further studies.

The approach taken to sub-clone the lambda clone CT-9 expressing a 36Kd chlamydial protein into the plasmid vector pBluescript is called the shot-gun method; the lambda clone CT-9 (with chlamydial DNA insert) was enzymatically cleaved and the pool of fragments were ligated to corresponding sites on a pBluescript plasmid vector. This method requires careful screening techniques to insure proper DNA orientation. The first screening after bacterial transformation was a rapid selection among blue and white colonies to check for size variations of supercoiled plasmid DNA. The DNA from both putative positive clones, and from some negative clones was then isolated and cleaved with restrictions enzymes to reveal respective insert sizes (refer to figure 5). A second screening entailed the DNA sequencing of prospective positive clones to confirm correct orientation with respect to existing promoters in the pBluescript vector (refer to figures 17a and 17b). A third and final screening involved a dot-immunoblot and/or Western immunoblot to determine the presence of the desired recombinant product.

False positive white colonies obtained during cell transformation may result from the loss of host episomal DNA, preventing the synthesis of the B-gal-omega fragment necessary for alpha complementation, or the transformation of bacterial cells with concatemer configurations of the plasmid vector. Some white recombinant colonies apparently containing DNA inserts did not necessarily express a recombinant product when screened with Ct. serovar D polyclonal antibodies. Such an occurrence may be attributed to several factors including: (1) the lack of an immunoreactive epitope in the recombinant Ct. protein, (2) the splicing of the recombinant gene coding for the 36kd
protein, (3) the improper orientation of the recombinant gene with respect to the promoter sequence, resulting in either an early transcriptional stop or wrong transcript synthesis, and (4) immunoassay conditions including the existence of epitope sites hidden from reactive antibody. From all the recombinant clones screened, one clone (pBS1) expressed a recombinant chlamydial antigen.

The insert size of the pBS1 clone was initially determined to be 2.0 kilobases long by EcoRI cleavage. However, further analysis led to the conclusion that the recombinant fragment was approximately 5.0 kb long. Co-migration of same size fragments on an agarose gel may explain the visual absence of the 3.0kb chlamydial insert fragment (refer to figures 8 and 9). At the protein level, the pBS1 recombinant product was found in the supernatant fraction after the screening of plasmid-derived cell lysates.

The Ct product signal in dot immunodots obtained from the recombinant Ct-9 clone was found to be mostly associated with pellet fraction, whereas in the pBS1 plasmid-derived clone the recombinant product was found in the supernatant fraction. A difference in location of the recombinant chlamydial product relative to the membrane pellet and the supernatant fraction of bacterial cell lysates in different cloning vectors may be the result of the intrinsic nature of each cloning system, i.e. cell lysis efficiency. Although lytic infection in the parent bacteriophage lambda EMBL-3 was usually a rapid process (<1 hr.) (7), leading to destruction of the host cell, the recombinant bacteriophage lambda clone CT-9 presented a longer incubation period (>1.5 hr.) before visible lysis. Upon lysis, an infected cell culture may contain free bacteriophage particles, cell membrane debris, partially lysed cells, and intact cells. In effect, depending on the actual rate of infection and/or lytic efficiency of the recombinant bacteriophage clone, a certain
percentage of intact cells, containing recombinant product may be found in the pellet fraction, thus explaining the appearance of higher signal levels in the pellet fractions of dot immunoblots. A different situation is presented in a plasmid vector system, where bacterial cell lysis may be manipulated in a variety of ways to insure maximum cell lysis efficiency.

The supernatant fractions from cell lysates were then resolved by size exclusion HPLC. A mixture of molecules of different sizes is applied to a column packed with porous material of a specific pore size. The separation is based on the physical parameters of the molecules themselves; only small molecules will enter the pores and slowly be passed thru the column. Larger molecules will pass through the column more rapidly, and thus be excluded first. Following calibration of the TSK-250 size exclusion column with molecular weight standards, the crude supernatant fraction was injected into the system and fractions from each corresponding peak were tested by dot immunoblots for the presence of the chlamydial recombinant product.

The BS1 recombinant product was found present in the void volume. This indicated the possibility of either chemical interactions occurring between molecules or experimental parameters affecting the resolution of the solute molecules. The former possibility was further tested by adding SDS, B-mercaptoethanol, or trifluoroacetic acid to the supernatant fraction to effect the separation and/or denaturation of molecules before chromatographic separation. However, no apparent shift in the location of the eluted product was obtained. A different size exclusion column, having a greater pore size, was also tried, but only a small shift in the elution pattern was achieved. Despite the apparent failure to effectively purify the recombinant product, a variety of other molecular weight species were removed from
the original supernatant fraction, thus yielding a semi-purified BS1 product. Relative to the Ct serovar D whole cell suspension, a 36Kd recombinant protein was identified by Western immunoblots from both the lambda EMBL-3 and pBluescript vectors. The immunoreactive bands thus confirmed the intact transfer of the entire Ct.recombinant gene from the lambda bacteriophage vector to the pBluescript plasmid vector. Further, no translational fusion protein was obtained from the plasmid-derived recombinant gene (17). The constitutive expression of the Ct. recombinant gene in E.coli, in fact, suggests the presence of native regulatory sequences. It was found that no apparent difference in the amount of recombinant product yield was obtained from either IPTG-induced or non-induced recombinant E.coli cultures (data not shown), suggesting the existence of a chlamydial promoter region upstream from the ribosomal binding site. Alternatively, other regulative sequences compatible with the E. coli (XL-1 Blue strain) enzymatic machinery may be in place.

It has been shown (18) that a good immunogen must have three chemical features: (1) it must have an epitope that can be recognized by the cell-surface antibody found on B-cells, (2) it must have at least one site that can be recognized simultaneously by a class II protein and by a T-cell receptor, and (3) usually, it must be degradable. Therefore, an animal may fail to promote a good immunogenic response to a given molecule if the appropriate B and/or T cells have been eliminated during the development of self tolerance (15, 18), or if class II proteins fail to bind to the antigen fragments. The lack of an antibody response due to this may be overcome by modifying the antigen to add new sites for class II protein binding.

The immunogenicity of the BS1 product was evaluated, following the identification of the Ct. recombinant 36 kd
protein. Immunogenicity, the ability of a molecule to induce an immune response, was determined both by the intrinsic chemical structure of the injected molecule and by whether or not the antigen was recognized by T and B cells (10, 18). Recognition is mediated by T and B lymphocytes. B cells can recognize antigens in their native conformation either free in solution, on membranes, or on the surface of cells, using surface immunoglobulin (Ig) as their specific antigen receptor. The T cell antigen receptor (TCR) is structurally different from antibody, it is generated by different sets of genes and most T cells can only recognize antigen on the surface of other cells (15). Specifically, T cells usually recognize processed or degraded antigen, and only when it is physically associated with molecules encoded by the major histocompatibility complex (MHC). In effect, MHC molecules act as a guidance system for T cells, allowing them to recognize antigens from within cells. These three groups of molecules, antibody, the TCR and MHC molecules, control the process of immune recognition (15). Seven groups of CF-1 female mice were immunized with protein suspensions from either recombinant CT-9 lambda, BS1 plasmid clones, HPLC-purified BS1, whole cell Ct. serovar D, or appropriate controls. The antisera from each group of mice were collected, and reacted with strips of nitrocellulose membrane containing electrophoretically transferred Ct. serovar D whole cell proteins. All Ct. recombinant preparations presented an immunoreactive band of approximately 36kd, while all negative controls showed no immunoreactive bands at this location, concluding that the recombinant product has met the requirements for a good immunogen, and may fill the needs for a future sub-unit vaccine.

An antibody capture immunoassay was used to determine the cellular location of the 36kd recombinant protein (1). The purified antigen was attached to a solid support (polystyrene
plates), and antisera from each group of mice immunized with the recombinant Ct protein preparation was allowed to bind (1). The antisera against an epitope (or more) of the Ct. 36kd recombinant protein was used to "probe" Ct Serovar D whole cell and SDS-solubilized ocugenital isolates. After washing, the amount of antibody retained on the solid support was measured. No conclusive data was found to confirm the cellular location of the 36 kd recombinant protein in Ct. serovar D. Three factors may have affected the sensitivity of the assay: (1) the amount of antigen that was bound to the solid phase, (2) the avidity of the antibody for the antigen, and (3) the type and number of labeled moieties used to label the antibody. First, the amount of antigen that was immobilized on microtiter plates was adjusted by preparing dilutions 1:500 and 1:1000 which was adequate to saturate the capacity of the chosen matrix. Higher levels of antigen binding may have been achieved by changing the matrix to nitrocellulose, which binds approximately 1000-fold more protein than polystyrene plates (19). The second factor that may have affected the sensitivity of the assay was the avidity of the antibody for the immobilized antigen. Polyclonal antisera are rich in non-specific antibodies which could physically obstruct interaction of specific antibodies with bound antigen. Finally, the secondary enzyme-linked anti-immunoglobulin antibody was diluted 1:2000, which was adequate according to standard procedure, to adjust the sensitivity of detection. In summary, despite its rapid, quantitative use, an antibody capture immunoassay offered a sensitivity level dependent on specific activity of antigen and avidity of antisera. The use of polyclonal antisera may have provided a fair signal strength and specificity, but high background levels. A secondary technique (Western immunoblot) confirmed immunogenicity. One of the major factors that will determine the success of an enzyme
immunoassay is the nature and amount of antigen bound to the microtiter plate surface. In effect, when presented in a mix pool of antigens, it is possible that the sensitivity of the immunoassay may be compromised by the amount and affinity of the Ct. reactive epitope in the 36kd protein to the surface of the microtiter plate. All immunochemical techniques have a certain background, and as the abundance of a particular antigen decreases relative to the other proteins, the ability of the antibodies to distinguish the correct antigen from the background is reduced. This is particularly true for polyclonal antibodies. In general, minor antigens will always need a secondary technique to differentiate the signal from the noise. Thus, any technique that allows the antigen to be identified specifically among the background can be used as a secondary technique. In this study, antibody detection to determine immunogenicity was supplemented with a secondary technique, immunoblotting (19). Western immunoblotting combines the resolution of gel electrophoresis with the specificity of immunochemical detection. It is a particularly powerful technique for assaying the presence, quantity and specificity of antibodies from different samples of polyclonal antisera.

The likelihood of this recombinant Ct. protein becoming a candidate for vaccine development depends on future protection studies, where mice immunized with the Ct. recombinant protein will be challenged with competent Ct. serovar D suspensions. If these studies demonstrate a significant level of protection, then a sub-unit vaccine may be developed to study its effect on a broader range of Ct. serovars. The DNA sequence analysis of the recombinant gene can provide the primary amino acid sequence. Sequence information can then be used to develop testable hypotheses concerning the potential structure and function of this molecule. The BSl recombinant gene was partially sequenced
in this study; however, at the present time, no regulatory sequences or open reading frames have been located. Future sequencing strategies will include the use of PCR-gene walking techniques, and perhaps further enzymatic cleavage and subcloning of recombinant fragments (12). The presence and sequence of a Ct. promoter compatible with the E. coli host's transcriptional machinery would be highly desirable given the possibility of cloning this Ct. promoter sequence into a high expression vector. The likelihood of cloning other low expression Ct. genes into E. coli may subsequently be increased. However, other factors such as transcriptional levels, and gene expression and regulation need to be considered.

The potential applications of molecular genetic technology toward chlamydial studies are numerous. The expression of unlimited quantities of chlamydial components in heterologous hosts such as E. coli, provides a method of obtaining sufficient quantities of these components for molecular research (12). The use of recombinant DNA technology plays an important role in identifying components essential for pathogenesis and for induction of important immune pathways. The ability to express antigenic determinants that are recognized by B-cells, and others that are recognized by T-cells, can be investigated in animal model systems (9, 10). Molecular biological approaches provide a means for construction of multiple determinants in one polypeptide (22, 23). A mosaic construction that can induce immunity to multiple serovars is both theoretically feasible and highly desirable. With these tools, essential questions may be addressed regarding the genetics of unique elements of chlamydial biology, immune response stimulation, pathogenesis, and virulence.
Bibliography


