Cloning, Expression and Purification of Gene Products involved in Peptidoglycan Synthesis for *Chlamydia trachomatis*

by

Joy LeBlang

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Approved:

Faculty mentor 4/12/00

Advising faculty 4/12/00

Director of Bioresource Research Program Apr 24, 2000

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Joy C. LeBlang 4/12/00

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ABSTRACT

Chlamydiae are obligate intracellular bacterial pathogens with a unique biphasic life cycle that consists of an infectious but non-dividing form, called elementary bodies (EB), and a noninfectious but metabolically active form, called reticulate bodies (RB). *Chlamydia* has an unusual process of cell division of which still remains uncharacterized subject matter; in particular, the paradox of peptidoglycan (PG) synthesis. A paradox is a principle or proposition contrary to received opinion (18). Chlamydial research has shown evidence in support of and against PG synthesis. The intention of this project incorporates clarification of the role of PG in *Chlamydia*, which would result in a greater understanding of chlamydial cell division. The primary objective of my research was to clone, express, and purify the three genes targeted for PG synthesis in *C. trachomatis*: pbp3, ftsW, and p60. This project incorporates many molecular biology techniques such as designing primers, PCR, restriction digests, transformation, PCR screening, cell culture, sequencing, column purification, and Western blot analysis. Unfortunately, this project was weighed down with much experimental difficulty, which produced subjective results.

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INTRODUCTION

Chlamydiae cause significant disease in both humans and many animal species. *Chlamydiae* are divided into four distinct species, *C. trachomatis*, *C. psittaci*, *C. pneumoniae*, and *C. pecorum*. *C. trachomatis* causes one of the most commonly reported sexually transmitted infections worldwide. It is estimated that 50 million new infections occur each year worldwide (20). Untreated infections of *C. trachomatis* lead to pelvic inflammatory disease, salpingitis, and ectopic pregnancy (5). Moreover, there is both laboratory and epidemiological evidence that chlamydial infection can facilitate the spread of HIV (4). It is estimated that 15 million people in sub-Saharan Africa acquire *Chlamydia trachomatis* infections each year. Syndromic treatment of STDs resulted in a 44% reduction in HIV incidence in Tanzania (6). Other serovars of *C. trachomatis* cause trachoma, the leading cause of preventable blindness worldwide (6). An estimated 500 million people are infected with the serovar trachoma, with 7 to 9 million blinded as a result (20). *C. psittace* and *C. pecorum* are primarily associated with animal diseases in many diverse phylogenetic groups, but humans can also serve as hosts of *C. psittace*. *C. pneumoniae* is the causative agent of pneumonia and other respiratory conditions.

While there are many differences in the disease caused by *chlamydiae* in these different species, the basic biology of the *chlamydiae* is conserved. *Chlamydiae* are obligate intracellular bacterial pathogens with a unique biphasic life cycle. Within the host, *chlamydiae* exist inside an inclusion, which is a nonacidified vacuole. *Chlamydiae* consist of two alternating developmental stages- an infectious but nondividing form (the elementary body; EB) and a noninfectious but metabolically active form (the reticulate body; RB).

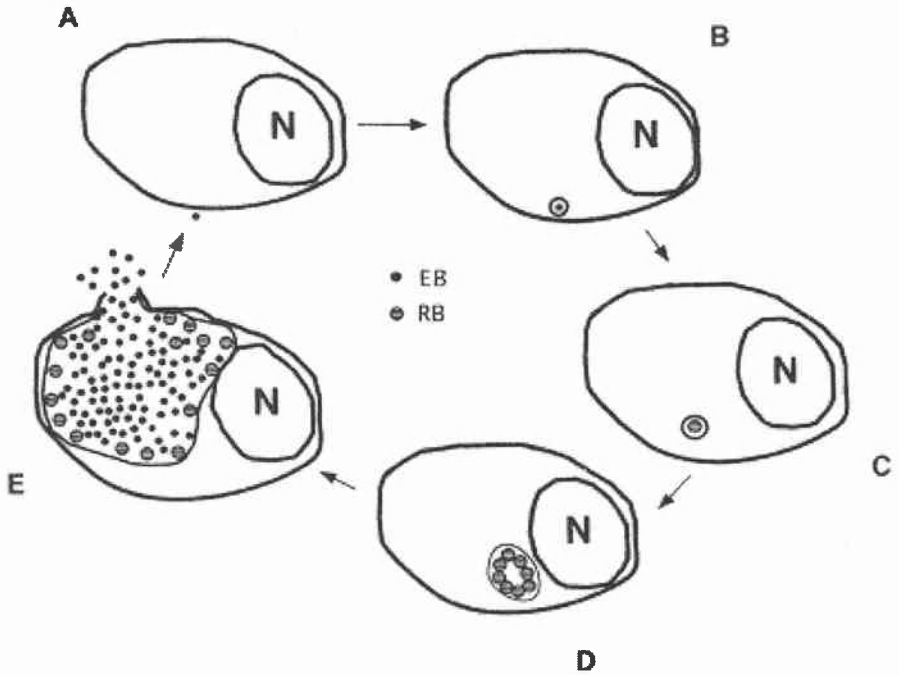

FIGURE 1. Reference 12 provides a line drawing of a generalized chlamydial developmental cycle. Infection begins when an infectious but metabolically inactive EB comes in contact with a host cell (A) and is endocytosed (B). The phagocytic vacuole (the inclusion) migrates toward the Golgi apparatus, and the EB differentiates into the noninfectious but metabolically active RB (C). RB division ensues, and the inclusion increases in size (D). Reticulate bodies then begin to reorganize back into EBs, and the inclusion grows until it occupies the entire cytoplasm of the infected cell (E). The inclusion lyses, the host cell lyses, and EBs are freed to infect another cell. While there are differences in this cycle among the different chlamydial strains and species, the general process is similar. N corresponds to the nucleus.

A major paradox of chlamydial biology involves peptidoglycan (PG) synthesis. PG is not found in purified infectious *chlamydiae*; however, other forms of evidence argue strongly that *chlamydiae* do in fact produce PG. PG is a thin sheet composed of two sugar derivatives, N-acetlyglucoamine and N-acetylmuramic acid, and a small group of amino acids, which connect the sugars by peptide cross-links (19). PG is found in virtually all bacteria; PG is a main component of the gram (+) cell wall structure and a minor constituent of the gram (-) cell wall structure. In addition to PG involvement in osmotic stability and cell wall rigidity, PG is involved in bacterial cell division by forming an invagination between separating daughter cells during cytokinesis (9). Bacteria that lack PG are not expected to be sensitive to penicillin. This is because penicillin and beta-lactam antibiotics inhibit the growth of PG-

containing bacteria by specific inhibition of penicillin-binding proteins (10). Yet, the production of EBs within infected cells is completely blocked by inhibitors of PG synthesis, such as beta-lactam antibiotics and d-cycloserine, which proves that *Chlamydia* is sensitive to penicillin (13). Treatment with these inhibitors of PG synthesis to *Chlamydia*-infected cells cause aberrant forms, which are formations of enlarged RBs that are unable to divide or differentiate back to EBs. Therefore, beta-lactam antibiotics and D-cycloserine influence cell division and discern development of intracellular *chlamydiae* (13). It can also be concluded from this evidence that PG is present in *Chlamydia* during cell division and proper differentiation. Another reason supporting PG synthesis comes from the recently completed *C. trachomatis* genome (14). The genome reveals virtually a complete set of genes that are homologous to predicted proteins involved in carrying out PG synthesis, assembly, and degradation in other bacteria with typical gram (-) cell wall structure (13). Although, contemporary research, such as colorimetry, chromatography and high-resolution mass spectrometry, have been unable to detect PG within developmental or structural forms of *chlamydiae* (13,10).

PG is thought to be associated with inflammatory and arthritogenic diseases (13). The *Chlamydia* genome has also revealed commonalities between homologous genes of *Chlamydia* and particular types of bacteria that collectively exhibit similar symptoms which cause arthritogenic and inflammation traits. Because these common genes of bacteria are all associated with the production of PG, it leads us to presume that these genes would contain PG for *Chlamydia* also. Thus, investigating the presence of this antigen that causes these traits is highly valuable. There is a strong correlation between many Chlamydial diseases, which is supported by evidence from both animal and human model systems that show similar responses to genus-common antigens associated with disease. The identification of antigens responsible for deleterious immune response and arthritic and inflammatory causes in *Chlamydia* are extremely important for Chlamydia research.

The wide-ranging practice of Rockey/Brown's research incorporates the identification of the SEP antigen. The SEP antigen was accidentally discovered using an adjuvant from Ribi Immunochem (Hamilton, MT.) containing three components: *Salmonella typhimurium* monophosphoryl Lipid A (MPL), synthtic corynebacterial trehalose dimycolate (TDM), and mycobacterial cell wall skeleton (MCWS). A unique antibody reagent specificity was produced, in cases where the immunizing protein was apparently a weak antigen (4). This antigen is presently theorized to be PG for the following reasons: when analyzed by Western blot analysis, the SEP antibody did not bind to the corresponding

band, suggesting the antigen is nonproteineous; the SEP antigen is evident in the septum, or plane of cell division, which is where PG is likely to reside; and, the antigen redistributes away from septum when exposed to penicillin (9).

The proteins being targeted for peptidoglycan synthesis in *Chlamydia* are pbp3, ftsW and p60, which are associated with terminal PG synthesis and the formation of the septum (14). Pbp stands for penicillin binding protein and was chosen as a gene to study because of it association with peptidoglycan specifically in the cell envelope. FtsW, which stands for filamentation temperature sensitive (9), facilitates cell division and participates in septum formation. P60 is common to many prokaryotes in recycling especially involving peptidoglycan in the cell envelope. The goal for this project is to determine if these genes are expressed in *Chlamydia*, and if so, where the protein localizes in the chlamydial cell, by developing antisera against chlamydial peptidoglycan associated proteins. The bigger picture involves examining if these antisera colocalize with the antisera of SEP.

MATERIALS AND METHODS

General Methodology

The primary objective of this project was accompanied by a great amount of problems and undesirable results. This is mentioned in this section, Materials and Methods, so to understand why additional measures, such as two sets of primers, are included, and other procedures, such as developing antisera against PG associated proteins, are not included. The general methodology toward accomplishing the primary objective of this research is described as follows (FIGURE 2). Primers were designed to amplify the appropriate gene products from chlamydial genomic DNA. A restriction site(s) sequence was added to the end of these primers to directionally clone the gene products into the pMal vector, so that the coding sequence of interest is fused in frame to the 3' end of the *malE* gene that encodes the maltose binding protein (MBP). After digestion, the vector and insert were heat inactivated and cleaned using a Quiagen PCR Purification kit. Both the insert and vector are run out on a DNA agarose gel to determine their concentrations. Next, the insert and vector are ligated together using a DNA ligase enzyme. This gene product is sequenced by Central Services in ALS. Transcription begins when IPTG is added. After the gene is cloned and expressed, cells are lysed, and the extract is poured

over a column of amylose resin. The maltose binding protein (MBP) fusion protein binds to the column and the remaining proteins in the cell extract are washed through the column. The fusion protein is eluted with maltose and the pure product is then ready to be injected into an animal. After a waiting period of 45 days, the antibodies are collected and analyzed using fluorescent microscopy.

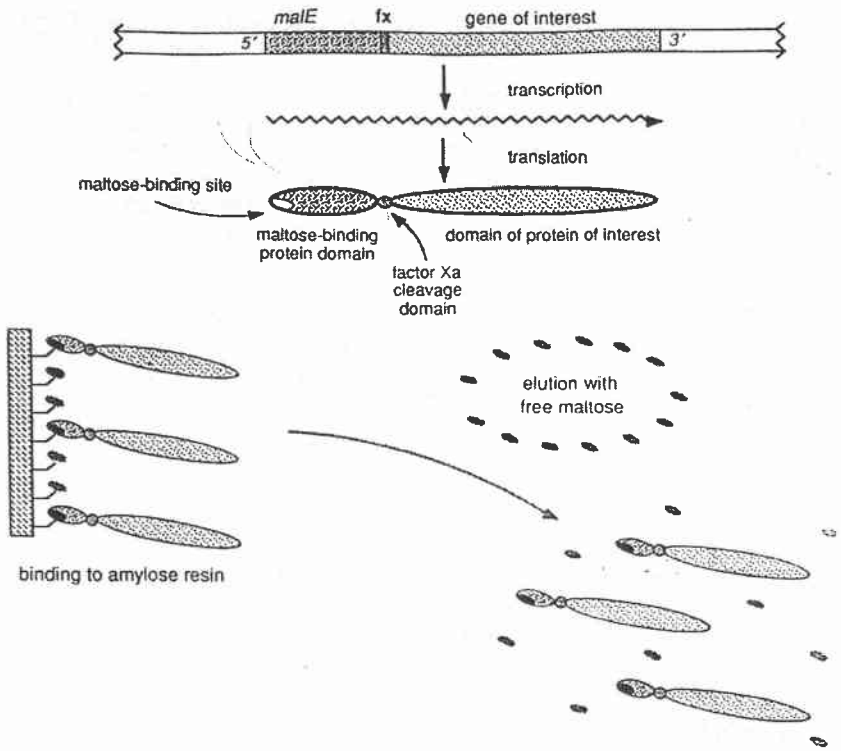

FIGURE 2. Schematic representation of expression and purification of a protein using the Maltose Binding Protein (MBP) vector. (16)

PMal-p2 was selected as a suitable vector because of the ease of purification, high copy number, selection of recombinants using the *lacZ* gene, and clustering of restriction sites within a short region of the *lacZ* gene. pMal is maltose-binding protein (MBP) vector which allow the purification and expression of a protein programmed by the cloned gene of interest by fusing it to MBP. MBP is encoded by the *malE* gene of *Escherichia coli*. The pMal vector contains the strong, inducible P-tac promoter and the *malE* translation initiation signals to give expression at high level of the cloned gene.

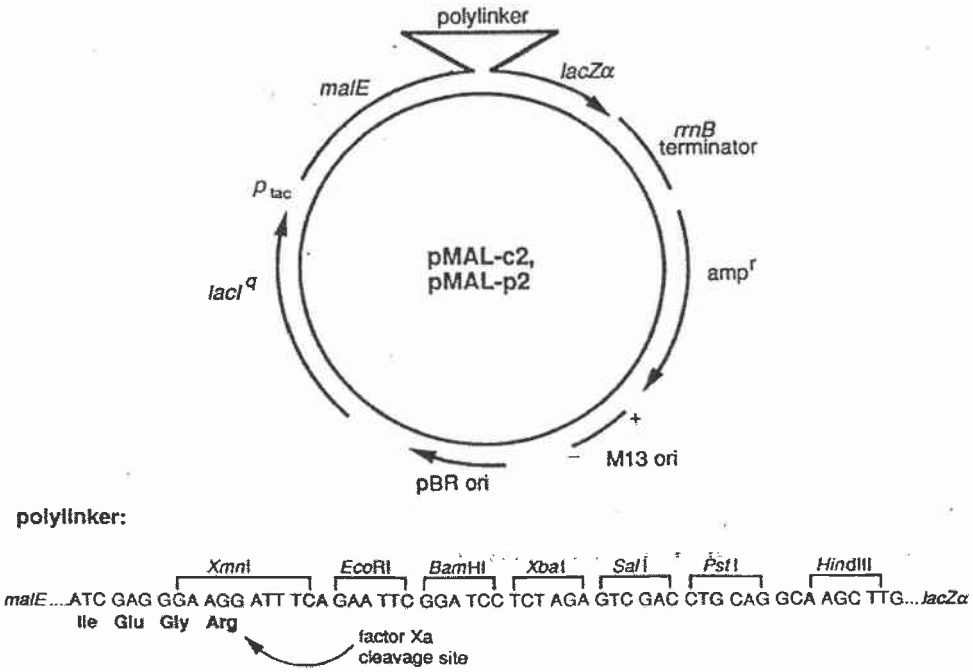

FIGURE 3. pMal vector. PMal-p2 (6721 base pairs) includes the *malE* signal sequence. Arrows indicate the direction of transcription. (16)

Primers

Two different sets of primers were designed for genes, pbp3, p60, and ftsW. Wasna Viratyosin and John Bassantine, fellow researchers in Rockey's laboratory, designed the initial primers, named Phase 1 primers, with different logic containing two incompatible digests. The initial primers contained digestion sites for restriction enzymes, *Xmn* I, to provide a "blunt end" on the 5'end, and *Bam* HI or *Hin* dIII, depending on the gene of interest, to provide "sticky ends". Wendy Brown, the graduate student I work with most closely, and I designed the second set of primers, named Phase 2 primers. These primers included restriction digest sites, *Eco* RI and *Bam* HI, which both create "sticky ends" for all three genes of interest.

The Phase 1 upstream primer is arranged as follows: 4-6 base pairs that attaches onto primer, GAATTC (*Xmn* I restriction enzyme), first 20 base pairs. The sequence itself is contained between the two primers. The Phase 1 downstream primer is arranged as follows: 4 – 6 base pairs that act as a 'fish hook' for primer, appropriate restriction enzyme (*Bam* HI for pbp3 and ftsW, *Hin* dIII for p60), reverse

compliment of last 20 base pairs. Phase 1 pbp3, ftsW, and p60 genes, are sizes 1022, 1155, and 486, respectively.

The phase 1 primers for pbp3 are as follows:

Pbp3 upstream: CCGCGAATTCATGGTTGGCGAAGGGGACTATCAG

Pbp3 downstream: CCGCGGATCCTTACCGAATGGTCTTTATCG

AGTATCCGTAGTGAATTTGATAGAAAATCGCGTTATCGCAAACTGTACGTTTCTCTAGATAT
TTCTATTCGTGATCGGATTTTAAGTTGGTGGAAACCTTACGCAGTTAAACATAAAATTCCGT
CAAATGCCTTATTCTTTATTAGTGATTATCAACGTTCTTATCCATTCGGGAAATTGTTAGGAC
AGGTTTTGCATACCCTTCGGGAGATAAAAGATGAAAAATCAGGAGAAGCATTCCCTACAGG
AGGTTTAGAGGCTTATTTTAATCGTTTGTTAGAAGGGGAAAATGGAGAACGCAAGTTGTTGC
GTTCTCCTTTAAACCGTTTAGACGTTGATAAGGTGACTAAAATCCCTAGGGATGGGAGCGAC
ATTTATCTAACAATAGATGCCAATGTACAGACGATTGCAGAACAAGAGATAGCCCTAGGCG
TGTTAGAAGCTAAAGCTCGTAGTGGGCGAGCTATTGTACTGAATTCACATACAGGAGAGATC
TTAGCTTTAGCGCAATATCCCTTTTTTAATCCGAGAGAGTATAGGGAATACTTCAACTGCAA
TGATCGCATAGAAGATACAAAAGTTAAGGCTGTTAGCGATGTGTTTGAGCCGGGCTCTATTA
TGAAACCCATAACTGTTGCTATTGCCTTATTAGCAAATGAAGAGATGCAAAAACGTGCTGGA
GAAAATCTTTTTGATCCTTATGAACCGTTAGATGTTAGTAGACGAGTATTTCCAGGACGGCA
AAAAATGCCTCTAAAAGACATTGTCAGTAATCGATACTTAAATATGTACATGGCGATTCAAA
AGTCATCCAATGTGTATATGGCTCAACTTGCAGATCGTATAGTACAAAAGTTAGGTGCTGAT
TGGTACGAACAGCGTTTGCAAGATTTTGGTTTTGGAAAAAGAACTGGAATTGAATTGCCGGC
AGAAGCTGTAGGATTGGTTCCTTCACGAAAACGTTTCCATAAAAACGGATCTCCGATAAAGA
CCATTCGG

FIGURE 4. Phase 1 pbp3 coding sequence.

The phase 1 primer designs for ftsW are as follows:

FtsW upstream: CGCGGAATCCAAATGGTTCCTGATTTCCTGT

FtsW downstream: CGCGGGATCCAGAGACTTTTATTCATGA

TTATTAGGAATTTTTTCTCTCGGGCTGATCATGGTGTTTGATACCTCATCAGCAGAGGTTTTG
GATCGAGCTTTGTCGTGTAGTACACACAAAGCTCTGATCCGGCAGATTACTTATCTTGGATT
GGGACTTGGTATCGCTTCATTTGTGTACATCTTAGGATGGAAGGATTTCTTGAAAATGAGCC
CTATGTTGCTGATTTTCGTGGGGATTACTCTTGTTTTGGTTCTTATTCCAGGTATTGGTGTTTG
TAGAAATGGAGCTAAGCGTTGGCTAGGAGTGGGGCAGTTAACTTTACAGCCTTCTGAATTTG
TTAAATATTTAGTTCCATGTGTTGCTATCGAATGTTTAACAACAAAACCTTCTATTCGTAGTA
GTTTTAAACGATTCGTAGCTTTCGTTGCTCTGTTGTTTATCCCCATTATGTTGATAGCGATTG
AACCTGACAATGGATCTGCGGCCGTGATCTCATTTTCCTTAATTCCAGTTTTTATCGTAACTG
CAGTGCGATTACGCTATTGGCTGCTTCCTTTGCTATGTATTCTGTGTATTGGAGGTACATTTG
CCTATCGGCTCCCTTATGTTCAGAATCGTTTGCAAGTTTACCTACATCCTGAATTAGATATTA
AAGGAAGAGGCCATCAACCTTACCAAGCTAAAATTGCAGCAGGCTCTGGAAGAGTGTTTGG
TAAAGGTCCAGGAAAAGGATTACAAAAATTAACTTATCTTCCAGAAGCTCAGAATGATTAC
ATTGCTGCTATTTACGCAGAAGAGTTTGGATTTATTGGGATGCTCCTATTGATTCTTCTCTAC
ATGGGATTTATTTATAGCGGGTATGTCATTGCAATGCGAGCCTCCCTTTTATCTGGAGCGGCT
CTTGCTATTTCAATCACTGTGATTATTGGGATGCAAGCTTTTATTAACTTGGGTGTTGTTTCT

GGGTTATTGCCTAGCAAGGGAGTGAACCTTCCATTTTTTAGTCAGGGAGGCTCTTCTCTAATT
GCTAATATGTGTGGCATGGGATTGCTATTAAGGATATGTGATGAAGAAAATCAACAAAATC
GTATTGGCAGTGGGGGGAACAGGAGGGCACATTATCCCTGCTCTAGCAGCAAGTCATGAAT
AAAAGTCTGT
FIGURE 5. Phase 1 ftsW coding sequence.

The phase 1 primer designs for p60 are as follows:

P60 upsteam: GCGCGCGAANNNNTTCCAATTTGCAGACAAAAATCCTAAG

P60 downstream: GCGCGCAAGCTTTAGATTACGAATTCGAT

ACAGAGAAGGAATCTAGCGGGGGCTCTAAAGAGATTTCATCTACCCCTGTAGAATCGACGA
CTCCTGTCGCTCCAGAAATTTCTGTTGTGAACGCTAAGGTAGTAGAGAAAACTCCTGAAAAA
GAGGAATTCTCTACTGTTATTGTTAAGAAAGGAGACTTTTTAGAACGTATAGCTAGATCCAA
TCACACTACAGTTTCTGCATTGATGCAGTTGAATGACTTATCTTCGACACAGTTACAGATAG
GACAAGTGTTACGAGTTCCTAAAACGAATAAGACAGAGAAGGATCTTCAAGTGAAGACTCC
AAATCTGGAAGATTACTATGTAGTCAAGGAAGGAGATAGTCCTTGGGCCATTGCATTGAGTA
ATGGTATTCGTTTGGATGAGCTGTTGAAGTTAAATGGATTAGATGAGCAGAAAGCTCGTAGA
TTACGTCCAGGGGAATCGAATTCGTAATCTA
FIGURE 6. Phase 1 p60 coding sequence.

The second set of primers, named Phase 2 primers, were designed with the intention to contain a single double digest that incorporates two compatible restriction enzymes in performing two digests in one reaction tube. The primers were selected from a region of the gene of interest that was relatively in the beginning of the gene sequence, at least 85 base pairs long, good predicted protein hydrophilicity, highly conserved, and without applied restriction sites within the sequence of interest. The upstream primer is arranged as follows: GGCG, GAATTC (*Eco* RI restriction enzyme), first 20 base pairs. The sequence itself is contained between the two primers. The downstream primer is arranged as follows: GGCG, GGATCC (*Bam* HI restriction enzyme), reverse compliment of last 20 base pairs. Phase 2 pbp3, ftsW, and p60 genes, are 280, 340, and 346 base pairs in length, respectively.

The primer designs for pbp are as follows:

pbp3 upstream: GGCGGAATTCATGGTTGGCGAAGGGGACTA

pbp3 downstream: GGCGGGATCCTCCATTTTCCCCTTCTAACA

```
  1 GGCGGAATTC ATGGTTGGCG AAGGGGACTA TCAGAGTATC CGTAGTGAAT TTGATAGAAA
 61 ATCGCGTTAT CGCAAACTGT ACGTTTCTCT AGATATTTCT ATTCGTGATC GGATTTTAAG
121 TTGGTGGAAA CCTTACGCAG TTAAACATAA AATTCCGTCA AATGCCTTAT TCTTTATTAG
181 TGATTATCAA CGTTCTTATC CATTCGGGAA ATTGTTAGGA CAGGTTTTGC ATACCCTTCG
241 GGAGATAAAA GATGAAAAAT CAGGAGAAGC ATTCCCTACA GGAGGTTTAG AGGCTTATTT
301 TAATCGTTTG TTAGAAGGGG AAAATGGAGG CGGGATCCTC CATTTTCCCC TTCTAACA
```

Figure 7. Phase 2 pbp3 sequence including the unhighlighted upsteam and downstream primers.

The primer designs for ftsW are as follows:

ftsW upstream: GGCGGAATTCCGCTATTGGCTGCTTCCTTT

ftsW downstream: GGCGGGATCCTTCTGCGTAAATAGCAGCAA

```
  1 GGCGGAATTC CGCTATTGGC TGCTTCCTTT GCTATGTATT CTGTGTATTG GAGGTACATT
 61 TGCCTATCGG CTCCCTTATG TTCAGAATCG TTTGCAAGTT TACCTACATC CTGAATTAGA
121 TATTAAAGGA AGAGGCCATC AACCTTACCA AGCTAAAATT GCAGCAGGCT CTGGAAGAGT
181 GTTTGGTAAA GGTCCAGGAA AAGGATTACA AAAATTAACT TATCTTCCAG AAGCTCAGAA
241 TGATTACATT GCTGCTATTT ACGCAGAAGG CGGGATCCTT CTGCGTAAAT AGCAGCAA
```

FIGURE 8. Phase 2 fts W sequence including the unhighlighted upstream and downstream primers.

The primer designs for p60 are as follows:

p60 upstream: GGCGGAATTCCGCCATTCAGAGCAAGAAAT

p60 downstream: GGCGGGATCCCTCTTTTTCAGGAGTTTTCT

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  1 GGCGGAATTC CGCCATTCAG AGCAAGAAAT AGAGTATTCT CAGAAAATAG CTCCTATTAA
 61 AATCTTAGAG CCCGTTCCGG TTGTTGATAA GGCTCCAGAG AAGTTAGAGA AAAAGCCTGA
121 GGTGATTGCG AAGCCTTCTC AGGTCGTTAG AAATCCTGTC GTTTCTAAAG CTGAACTTGC
181 TGCGCAATTT GCAGACAAAA ATCCTAAGAC AGAGAAGGAA TCTAGCGGGG GCTCTAAAGA
241 GATTTCATCT ACCCCTGTAG AATCGACGAC TCCTGTCGCT CCAGAAATTT CTGTTGTGAA
301 CGCTAAGGTA GTAGAGAAAA CTCCTGAAAA AGAGGGCGGG ATCCCTCTTT TTCAGGAGTT
361 TTCT
```

FIGURE 9. Phase 2 p60 sequence including unhighlighted upstream and downstream primers.

Amplify inserts using PCR

The Polymerase Chain Reaction (PCR) offers a rapid and simple approach for the production of many copies of an identical sequence of DNA from minute quantities of template. Initiation of the PCR

occurs when primers are allowed to hybridize (anneal to the component strands of the target DNA); this is followed by enzymatic extension of the primers (from the 3' ends) using a thermostable DNA polymerase. A single PCR cycle consists of three distinct steps carried out at different temperatures, (FIGURE 10).

1. Denaturation of dsDNA by heating to 94° C separating the individual strands of the target DNA.
2. Annealing of the primers, which occurs when the temperature is reduced to 47° C.
3. Extension of the primers by a thermostable DNA polymerase at 72° C.

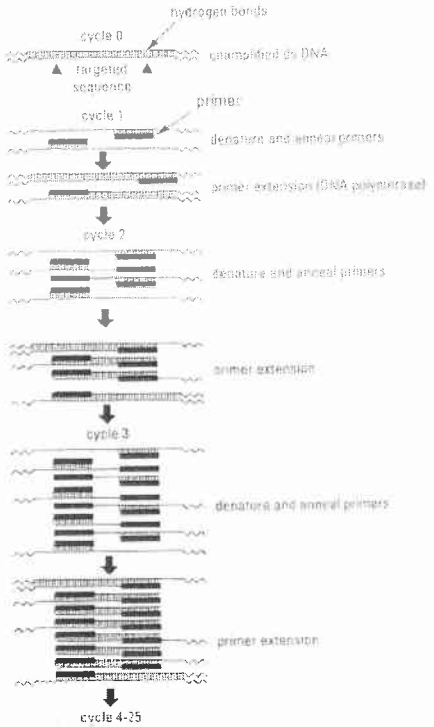

FIGURE 10. The polymerase chain reaction (PCR). In the first cycle, the product from one primer is extended beyond the region of complementarity of the other primer, so each newly synthesized strand can be used as a template for the primers in the second cycle. Successive cycles will thus generate an exponentially increasing number of DNA fragments. (17)

Phase 1 and Phase 2 primers were used using PCR to amplify the inserts, pbp3, ftsW, and p60, with *PWO* polymerase and *Taq* polymerase, respectively. *PWO* polymerase is used to achieve a 'blunt end' while *Taq* polymerase is used to achieve a 'sticky end', which is because Taq polymerase contains an extra Adenine. The amount used for both reactions are: 10 μl (PWO or Taq) buffer, 2 μl upstream

primer, 2 μl downstream primer, 2 μl dNTPs, 1 μl template (*C. trachomatis* seravar D), 0.5 μl (PWO or Taq) polymerase, and 82.5 μl water, to total a 100 μl reaction for each insert. Then, add 1-2 drops of mineral oil to each tube and run PCR program (12), which incorporates the cycle at the desired temperatures that is described above.

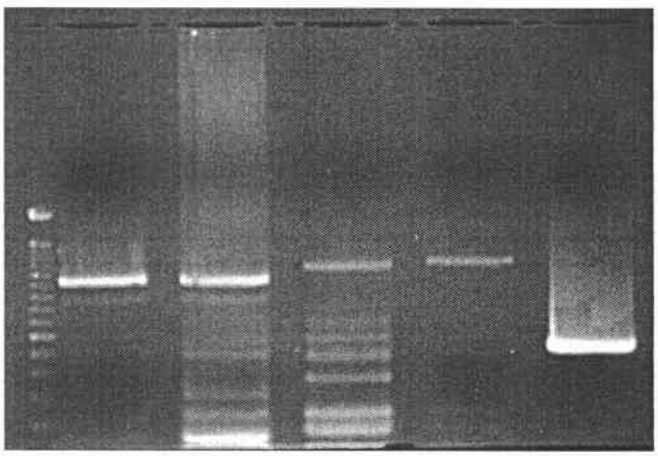

FIGURE 11: An agarose gel run after amplication by PCR. Lane 1= 100 bp ladder, lane 2=pbp3, lane 3=pbp3, lane 4=ftsW, lane 5= ftsW, lane 6= p60

Next, clean the PCR reaction products (inserts) with QIA quick Gel Extraction Kit protocol using a microcentrifuge. This step removes excess components of the agarose gel and accompanying fragments of vector, so to only retain the amplified gene of interest.

QIAquick Gel Extraction Kit Protocol using a microcentifuge

1.) Excise the DNA fragment from the agarose gel with a clean, sharp scalpel. Minimize the size of the gel slice by removing extra agarose horizontally and vertically.
2.) Weigh the gel slice in a colorless tube (Wt. of Phase 1 genes: pbp=0.244g, p60=0.420g, fts=0.214g. Wt. of phase 2 genes: pbp=0.221g, p60 =0.365g, fts= 0.302g). Add 3 volumes of Buffer QG to 1 volume of gel.
3.) Incubate at 50° C for 10 minutes (or until the gel slice has completely dissolved). Vortex, if necessary, the tube every 2-3 minutes during the incubation.
4.) After the gel slice has dissolved completely, check that the color of the mixture is yellow. (If color of mixture is orange or violet, add 10 μl of 3M Sodium acetate, pH 5.0, and mix. The color of the

mixture will turn to yellow.)

5.) Add 1 gel volume of isopropanol to the sample and mix.

6.) Place a QIAquick spin column in a provided 2-ml collection tube.

7.) To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 minute.

8.) Discard flow-through and place QIAquick column back in the same collection tube.

9.) Add 0.5 ml of Buffer QG to QIAquick column and centifuge for 1 minute.

10.) To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 minute.

11.) Discard the flow-through and centrifuge the QIAquick column for an additional 1-minute at 13,000 rpm. Let air dry for 5 minutes, so to let the ethanol evaporate.

12.) Place QIA quick column into a clean 1.5 ml microfuge tube.

13.) To elute DNA, add 50 μl deionized water to the center of the QIAquick column, let stand for 1 minute, and centrifuge for 1 minute at maximun speed.

Restriction digests of vector and insert

Restriction enzymes, *Eco* RI, *Bam* H1, and *Hin* dIII, are used to produce linear fragments of DNA with single stranded 'sticky ends', whereas, restriction enzyme, *Xmn* I, cleaves DNA to give blunt-ended fragments. Two restriction fragments, the vector and insert, cut with the same enzyme will anneal (base pair), due to the formation of hydrogen bonds between individual bases, allowing them to be joined together (ligated).

Examples of 'sticky ends' by *Hin* dIII, and 'blunt ends' by *Xmn* I, are as follows:

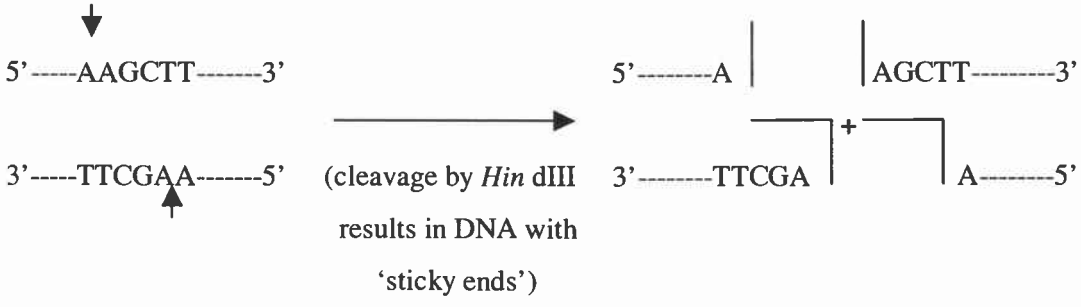

FIGURE 12. Recognition base sequence and cleavage site for the restriction enzyme *Hin* dIII. This is the conventional representation of double-stranded DNA, showing the individual bases, where Adenine is represented by the letter (A), cytosine (C), guanine (G), and thymine (T). An arrow shows the cleavage

site on each strand.

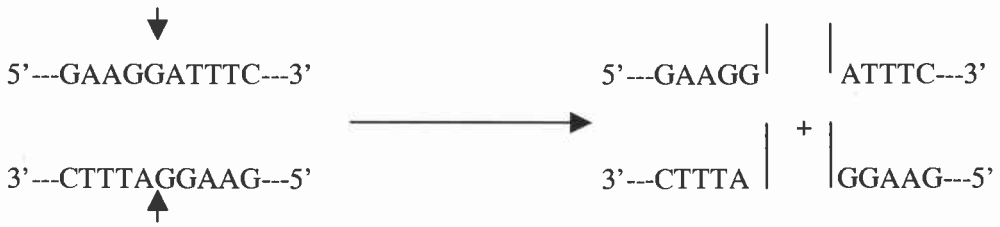

FIGURE 13. A 'blunt end' is showed by recognition base sequence and cleavage site for the restriction enzyme *Xmn* I.

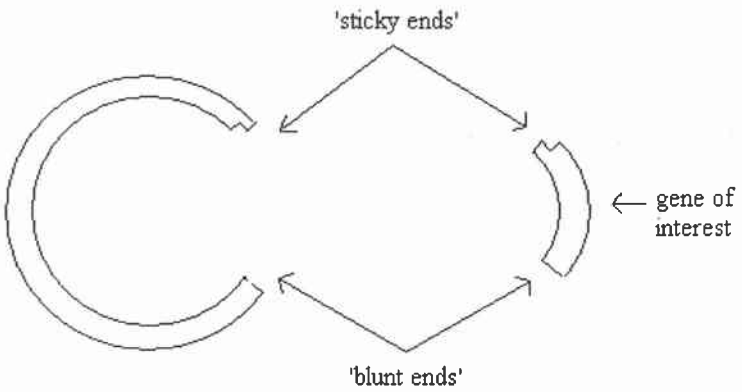

FIGURE 14. Restriction of plasmid and gene of interest exhibiting 'sticky ends' and 'blunt ends'.

Using Phase 1 primers, the pMal vector is cut by *Xmn* I and either *Bam* H1 or *Hin* dIII, depending on the insert of choice, in two separate digest reactions. The first digestion reaction using restriction enzyme *Xmn* I, composed of 28.5 µl water, 5 µl (1:10) BSA, 5 µl NE Buffer 2, 10 µl vector pMal C2, and 1.5 µl Xmn1 enzyme, to total a 50 µl reaction. In addition, a 20 µl digestion reaction was produced to assure that the vector was present and the enzyme was working, which consisted of 3 µl vector, 2 µl NE Buffer 2, 2 µl 10X BSA, 12.5 µl water, and 0.5 µl Xmn 1 enzyme. This experiment was performed on ice. Then, the samples were placed in a 37° C water bath for 2.5 hours, which was followed by a 65° C water bath to heat inactivate the enzymes for 20 minutes.

Next, the digestion reaction was cleaned using Quiagen PCR purification kit and eluted into 30 µl water. This step is necessary to remove any excess debris from the reaction and will leave only the

cut vector to be present.

QIAquick PCR Purification Kit Protocol

1.) Add 5 volumes of Buffer PB to 1 volume of the digest reaction and mix.

2.) Place a QIAquick spin column in a provided 2-ml collection tube.

3.) To bind DNA, apply the sample to the QIAquick column and centrifuge 30-60 seconds.

4.) Discard flow-through. Place QIAquick column back into the same tube.

5.) To wash, add 0.75 ml Buffer PE to column and centrifuge 30 - 60 seconds.

6.) Discard flow-through. Place QIA quick column back into the same tube. Centrifuge column for an additional 1 minute at maximum speed.

7.) Place QIA quick column in a clean 1.5 μl microfuge tube.

8.) To elute DNA, add 30 μl deionized water to the center of the QIA quick column, let stand for 1 minute, and centrifuge for 1 minute.

Both the second digest reaction of the pMal vector and the insert are cut using *Bam* HI (for pbp3 and ftsW) or *Hin* dIII (for p60). The same protocol is used for cutting the vector and insert with the appropriate enzyme; the recipe is as follows: 28 μl (vector or insert), 5 μl buffer, 5 μl 10X BSA, 1.0 μl appropriate restriction digest enzyme, and 11 μl water. In addition, a control is included containing 2 μl vector, 2 μl buffer, 2 μl 10X BSA, 13.5 μl water, and 0.5 μl enzyme. After placing the cut vector and insert into a 37° C water bath for 2.5 hours, heat inactivating the enzymes at 65° C for 20 minutes, and cleaning the samples using QIAquick PCR Purification Kit Protocol, gel electrophorize the samples on an agarose gel to be certain they are still present and to obtain intensities of bands for ligation reaction.

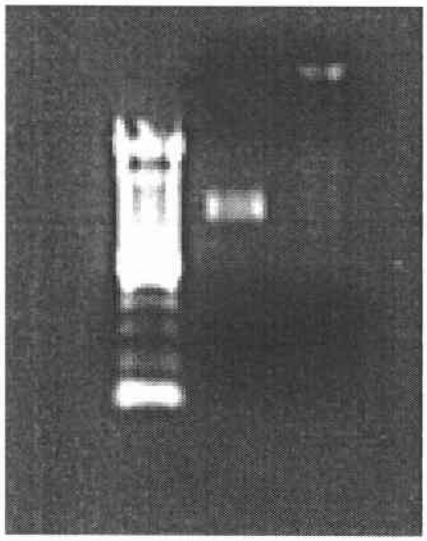

FIGURE 15. Phase 1 pbp3 insert and vector. Lane 1 is 100 bp ladder, lane 2 is the cut pbp3 insert, lane 3 is the cut pMal vector

Using Phase 2 primers, the compatibility of the two restriction digest enzymes, *Eco* RI and *Bam* HI, separately allows the vector and insert to be cut twice in one reaction. The amounts used for digestion of the inserts, pbp3 and p60, are: 30 μl insert, 5 μl NE Buffer U (EcoR1), 5 μl 10X BSA, 1.5 μl EcoR1 enzyme, 1.5 μl BamH1 enzyme, 7 μl water for a 50 μl total reaction. The digestion reaction for the pMal vector includes: 25 μl vector, 5 μl NE Buffer U (EcoR1), 5 μl 10X BSA, 1.5 μl EcoR1 enzyme, 1.5 μl BamH1 enzyme, 12 μl water for a 50 μl total reaction. The reaction tubes are then placed in 37° C water bath for 2.5 hours, followed by, a 65° C water bath for 20 minutes. 8 μl of the digestion reaction plus 3 μl tracking dye are combined and then gel electrophorized on a DNA agarose gel to be certain the digestions were successful. The samples were cleaned using the Gel Purification Protocol (pg.12), which is different than the cleaning used in Phase 1. The weight of the agarose slices containing the cut inserts and vector are: ftsW= 0.251, pbp3= 0.263, pMal= 0.418. After cleaning, the samples are gel electrophorized on a DNA agarose gel and the bands are used to obtain intensities for the succeeding ligation reaction.

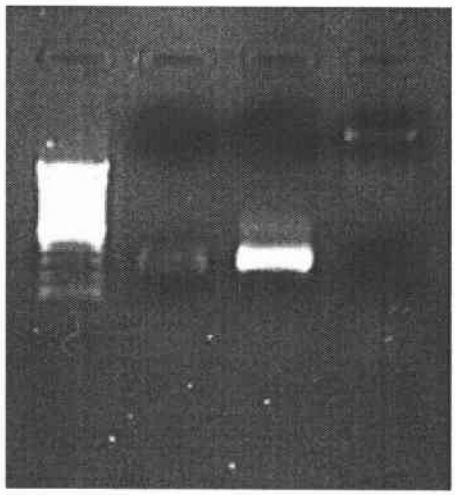

FIGURE 16. Phase 2 cut inserts and vector. Lane 1 is 100 bp ladder, lane 2 is cut ftsW, lane 3 is cut pbp3, lane 4 is cut pMal vector. Band size, location, and intensity are very important to obtain proportions for succeeding ligation reaction.

Ligation of vector and insert

In constructing a recombinant DNA molecule, the cut pMal must be ligated to the gene of interest to be cloned: this is performed using another microbial enzyme, DNA ligase. This ATP-dependent enzyme is capable of forming covalent phosphodiester bonds between annealed DNA molecules, thus creating recombinant DNA (FIGURE 17).

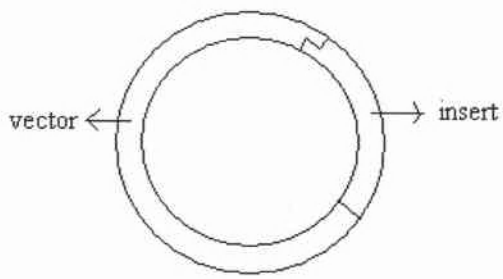

FIGURE 17. Ligated vector and insert with 'blunt end' and 'sticky end'.

The rule of thumb for determining amounts to use for ligation reaction involves a 3:1 insert-to-vector ratio based on the preceding agarose gel. This is because the size of the pMal vector is bigger than the size of the inserts and because it is more desirable to have more inserts than vectors so that the vectors don't ligate to other vector plasmids. If intensities look the same, then an approximate 3:1 ratio is

present because naturally the vector is seen bigger because of its size. Therefore, based on Phase 1 gel (FIGURE 15), the recipe used for inserting pbp3 into the pMal vector is: 0.5 μl ligase enzyme, 4 μl 5X ligase buffer, 10 μl vector, 4 μl pbp3 insert, 1.5 μl water is needed for a 20 μl ligation reaction. Based on Phase 2 gel (FIGURE 16), the ligase reaction for (ftsW or pbp3, respectively) and pMal vector contained the following: 7 μl vector (ftsW) or 10 μl vector (pbp3), 7 μl insert (ftsW) or 4 μl insert (pbp3), 0.5 μl ligase enzyme, 4 μl 5X ligase buffer, and 1.5 μl water. The reaction is performed on ice, then placed in a 16° C water bath overnight, and followed by running an DNA agarose gel to be certain the ligated vector and insert are present.

Transformation of recombinant DNA into *E. coli*

Transformation is defined as the introduction of the recombinant DNA molecule into a suitable host, such as *E.coli* (17). Each recombinant vector is incorportated into a separate host cell, which is cultured, giving a collection of transformants used for subcloning into plasmid vectors. Phase 1 transformants had 15 – 40 colonies. Phase 2 transformants had as few as 1-15 colonies.

Transformation procedure

1.) Remove competent cells from -70° C freezer; thaw on wet ice. Place required number of autoclaved 1.5 ml microcentrifuge tubes on wet ice.
2.) Gently mix cells, then aliquot 100 μl competent cells into chilled microcentifuge tubes.
3.) Add 5 μl of the DNA ligation reaction directly to a second tube containing 50 μl competent cells, moving the pipette through the cells while dispensing. Gently tap tube to mix.
4.) Incubate cells on ice for 30 minutes.
5.) Heat-shock cells 45 seconds at 37° C. Do not shake.
6.) Place on ice for 2 minutes.
7.) Add 0.95 μl of room temperature YT Broth
8.) Shake at 225 rpm for 1 hour at 37° C for expression.
9.) Spread 100 μl of the undiluted reaction onto LB plates containing 100 μg/ml ampicillin. (FIGURE 18).
10.) Transfer the rest to 1.5 ml microfuge tubes, spin down, and resuspend in 100 μl YT broth and spread onto LB plate containing 100 μg/ml ampicillin. (FIGURE 18).
11.) Incubate overnight at 37° C.

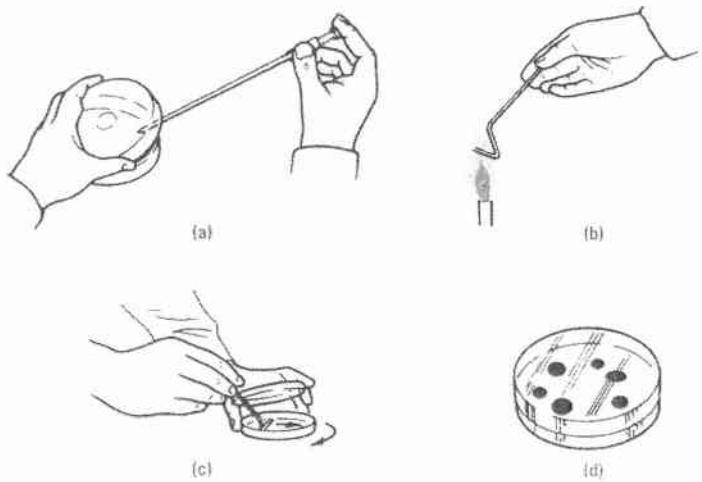

FIGURE 18. Preparation of a spread plate. (a) Transfer a small volume of cell suspension 150 μl to the surface of a solidified medium in a Petri plate. (b) Flame sterilize a glass spreader and allow to cool (8-10 sec). (c) Distribute the liquid over the surface of the plate using the sterile spreader. Make sure of an even coverage by rotating the plate as you spread: allow the liquid to be absorbed into the agar medium. Incubate under suitable conditions. (d) After incubation, the microbial colonies should be distributed over the surface of the plate. (17)

PCR Screening

Because a single transformed host cell can be grown to produce clones of genetically identical cells, each carrying the gene of interest, the technique of gene cloning is used for selection and growth of the transformed cell (7). PCR Screening involves screening for the positive recombinant DNA products using PCR, to amplify the gene of interest.

Protocol for PCR Screening

1.) Pick a colony with a toothpick and swirl in a GeneAmp 0.5 μl microcentrifuge tube containing 25 μl water. Then touch it to a fresh antibiotic containing plate and place in incubator.

2.) Heat the tubes to 100° C for 1 minute in a heating block. (Use PCR machine program 12, which denatures dsDNA by heating to 99° C separating the individual strands of the target DNA).

3.) Centrifuge the tubes at 15,000 rpm for 2 minutes.

4.) Pull off 10 µl, which should contain most of the pellet. The remaining 15 µl is what will get amplified by PCR.

5.) Prepare the PCR master mix as follows:

11 colony screens	4 colony screens
75 µl de-ionized water	25 µl de-ionized water
3 µl upstream primer	1 µl upstream primer
3 µl downstream primer	1 µl downstream primer
30 µl 10X Taq PCR buffer	10 µl 10X Taq buffer
3 µl *Taq* polymerase	1 µl *Taq* polymerase
6 µl PCR nucleotides	2 µl PCR nucleotides
120 µl total	40 µl total

6.) Add the 10 µl of the master mix containing appropriate primers to each of the mini prep tubes.

7.) Add 1 drop of mineral oil to each tube.

8.) Perform PCR. Remember a positive and negative control.

9.) Analyze amplification products on an agarose gel.

Grow up positive clones

pMal was designed so that insertion of a foreign DNA fragment disrupts the gene lacZ (*β*-galactosidase), which has an easily detectable phenotype. *β*-galactosidase converts colorless X-gal to a dark blue derivative. When this enzyme is included in selective agar plates, X-gal allows distinction from lac(+) transformants (blue colonies) that receive a recircularized vector plasmid (with no foreign DNA inserted) from lac(-) transformant that contain the recombinant molecule which will grow to produce a white colony (17).

The transformants were replica-plated onto a new plate containing IPTG and X-gal to isolate colonies using the technique of preparing a streak plate (FIGURE 19). The initial isolation and subsequent transfer of microbes between containers can be achieved using a sterile inoculating loop. A wire loop is repeatingly sterilized by heating the wire in the hottest part of a Bunsen burner flame until the whole wire becomes red hot. After cooling for roughly 8 seconds (without touching any other object), it is ready for use. The positive samples were plated with the pMal vector without an insert as a control. Observations for Phase 1 results of replica-plating were poor with no signs of intergrading the X-gal color component. Phase 2 results were very distinguishable blue or white colonies.

Next, inoculate 10 ml of LB broth with positive colonies from plates used in the previous step. To accomplish this, add 100-µl (2M) glucose plus 20 µl (50 µg/ml) Ampicillin to 10-ml LB broth. Then, add 1 sterile loop full of cell culture from plates. Put on 37° C shaker at 2500 rpm for 8 hours. After expression, half of the 10 ml culture is used to make glycerol stocks, for back up positive clone cultures, and the other half is prepared for DNA sequencing using QIAGEN prep Mini prep kit Protocol or CTAB.

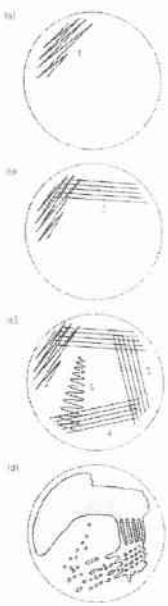

FIGURE 19. Preparation of a streak plate for single colonies. (a) Using a sterile metal loop, take a small sample of the material to be streaked. Distribute the sample over a small sector of the plate (area 1), then flame the loop and allow to cool. (b) Make several small streaks from the initial sector into the adjacent sector (area 2), taking care not to allow the streaks to overlap. Flame the loop and allow to cool. (c) Repeat the procedure for areas 3 and 4, re-sterilizing the loop between each step. Finally, make a single long streak as shown for area 5. (d) The expected result after incubation at the appropriate temperature (e.g. 37°C for 24 hours): each step should have diluted the inoculum, giving individual colonies within one or more sectors on the plate. Further sub-culture of an individual colony should give a pure (clonal) culture. (17)

Purify the plasmid

DNA can be extracted by 1) complexation with the detergent cetrymethylammonium bromide (CTAB), or 2) anion-exchange column chromatography (Quiagen prep Mini prep) (17). Cell wall digestion requires a lysozyme solution and additional treatment of STET. STET is added as a catalyst to reach the cell wall layer because gram-negative bacteria are relatively insensitive to lysozyme. STET will also inactivate any bacterial DNases in the solution, preventing enzymatic degradation of plasmid DNA during extraction. Additional chemical purification and removal of macromolecules by NaCl further increases the purity of the sample. Rinsing with 70% ethanol removes any salt from the sample; followed by centrifugation will recover the DNA pellet.

Glycerol Stocks

1. Spin down at 50000 rpm for 10 minutes
2. Resuspend in 0.5 ml glycerol
3. Store in –70° C freezer

QIAGEN prep Mini prep kit protocol

1. Resuspend pelleted bacterial cells in 250 µl of Buffer P1 and transfer to a microfuge tube.
2. Add 250 µl of Buffer P2 and gently invert the tube 4 – 6 times to mix.
3. Add 350 µl of Buffer N3 and invert the tube immediately but gently 4 – 6 times.
4. Centrifuge for 10 minutes. During centrifugation, place a QIAprep spin column in a 2-ml collection tube.
5. Apply the supernatants from step 4 to the QIAprep column by decanting or pipetting.
6. Centrifuge 30 – 60 seconds. Discard the flow-through.
7. Wash QIAprep spin column by adding 0.5 ml of Buffer PB and centrifuging 30 – 60 seconds. Discard the flow-through.
8. Wash QIAprep spin column by adding 0.75 ml of Buffer PE and centrifuging 30 – 60 seconds.
9. Discard the flow-through, and centrifuge for an additional 1 minute to remove residual wash buffer.
10. Place QIAprep column in a clean 1.5-ml microfuge tube. To elute DNA, add 50 µl of Buffer EB (10mM Tris-CL, pH 8.5) or water to the center of each QIAprep column, let stand for 1 minute, and centrifuge for 1 minute.

CTAB Plasmid Mini Prep

1. Grow 5 ml culture overnight
2. Spin cells 10 minutes. Resuspend in 600 µl STET (do not vortex or centrifuge). Transfer to microfuge tube.
3. Add lysozyme, 24 µl of 25 mg/ml stock. Room temperature for 5 minutes.
4. Boil tubes 1 minute, Spin 10 minutes, remove glob with toothpick.
5. Add 5 µl RNase of 10 mg/ml, incubate 10 minutes at 68° C or 30 minutes at 37°C
6. Add 1/10 volume 5% CTAB, mix and let sit for 5 minutes
7. Spin 10 minutes, resuspend pellet in 300 µl 1.2 M NaCl, add 750 µl Ethanol
8. Spin 15 minutes, wash pellet in 70% ethanol, dry pellet, resuspend in 25 µl water.

Sequence the DNA sample(s)

The Central Services laboratory in ALS requires 1.2 pmoles primer and template at a 600ng concentration (for rounded-up 6 kb sequence) in a final volume of 12 µl in water. A Perkin Elmer MBA 2000 calorimeter, provided by Dennis Hruby's laboratory, was used to calibrate the original DNA concentrations. The concentration for Phase 1 pbp sample 4 was 0.020 µg/µl. The concentrations for Phase 2 fts sample 4, sample 5, sample 6, were 0.035 µg/µl, 0.04 µg/µl, 0.03 µg/µl, respectively. The concentrations for Phase 2 pbp sample 3 and sample 5 were 0.025 µg/µl and 0.045 µg/µl, respectively. Each sample was diluted using the formula, initial (VC)= final (VC), where V=volume and C=concentration.

For example,

The equation used for Phase 2 fts sample 5 was:

(40 ng/ 1µl) X (volume to add) = (600 ng/ 12 ng) X (12 µl)

volume to add = 15 µl

The equation used for diluting the MalE primer was:

(44.4 pmoles/µl) X (volume to add) = (1.2 pmoles/ 12 µl) X 12 µl

volume to add = 0.027 µl (Therefore, I diluted the primer 1:100, then added 3 µl for sequencing.)

Next, align returned sequence from Central Services with original section of gene from *C. trachomatis* genome using MacVector and website (http://chlamydia-www.berkeley.edu:4231/). See attached sequence for confirmed Phase 1 pbp3-sample 4 clone. Phase 2 clones aligned perfectly with HP sequence, a clone that neighboring lab technician easily obtained.

Induction

The lacIq gene of the pMal vector encodes the *lac* repressor, which turns off transcription from the P-tac promoter until IPTG is added (16). The samples are then electrophorized on a SDS protein gel, which was assembled using the SDS-PAGE Gel recipe. The gel is stained overnight with Coomassie blue, and then destained for a couple of hours the next day.

Protocol for inducing the P-tac promoter

1. Inoculate 1 Liter LB/glucose/ampicillin medium with 6 ml out of 10 ml overnight culture. Grow at 37° C, shaking, at 2 x 10^8 cells/ml (OD600 = 0.4 to 0.6).
2. Remove a 1-ml sample, microcentrifuge 2 minutes, and discard supernatant. Resuspend cells in 50 µl of 1X SDS buffer and set aside on ice for analysis on Protein Gel Electrophoresis (uninduced cells).
3. Add 3 ml of 0.1 M IPTG to remainder of culture. Incubate 2 hours at 37° C with good aeration.
4. Remove another 1-ml sample, microcentrifuge 2 minutes, and discard supernatant. Resuspend cells in 100 µl of 1X SDS buffer and set aside on ice for analysis on Protein Gel Electrophoresis (uninduced cells-2 hours).
5. Centrifuge remaining cells 20 minutes at 9000 x g, for 15 minutes at 4° C, and discard supernatant. Resuspend cells in 50 ml column buffer. Freeze samples at –70° C.

SDS-PAGE GEL recipe:

12.5% Mini Gels (resolving)	2 Gels
33.5/0.3% acrylamide	7.4 ml
1 M Tris pH 9.1	7.6 ml
Water	4.15 ml

10% SDS	200 µl
3% APS	500 µl
Temed	10 µl

Mini Gels (Stacking)	2 Gels
30:0:44 acrylamide	1.3 ml
1 M Tris pH 6.8	1.25 ml
Water	7.35 ml
10% SDS	100 µl
3% APS	100 µl
Temed	10 µl

Extracting the protein through column chromatography

Column chromatography is used as a final means to collect the purified protein. It is at this point that the MBP binds to the amylose resin in the column, which is then eluted with maltose, to obtain the fusion protein. This is followed by affinity purification for MBP, which is facilitated by binding to amylose resin to isolate the fusion protein.

The protocol for column chromatography is as follows:

1.) Prepare buffer, LB broth/amp/glucose, inoculate overnight.

2.) Inoculate 1 liter rich broth plus glucose & ampicillin with 10 ml of an overnight culture of cells containing the fusion plasmid. (Glucose is necessary in the growth medium to repress the maltose genes on the chromosome of the *E. coli* host.

3.) Grow ≈ six hours. Add IPTG to a final concentration of 0.3 mM. Incubate the cells at 37° C for two hours. The period of time and the temperature to use during expression depends on several factors (stability of the protein, host strain, etc.) and variations can be tried to find optimum conditions for expression.

4.) Harvest the cells by centrifugation at 4000 x g for 20 minutes and discard the supernatant. Resuspend the cells in 50 ml Column buffer

5.) Freeze sample overnight at –70° C freezer. Thaw in cold water.

6.) Place sample in an ice-water bath and sonicate in short pulses of 15 seconds or less for a total time of two minutes.
7.) Centrifuge at 9000 x g for 30 minutes. Save the supernatant (crude extract). Dilute the crude extract 1:5 with Column buffer.
8.) Pour the amylose resin in a 2.5 x 10 cm column. Wash the column with 8 volumes of Column Buffer to total 120 ml.
9.) Load the diluted crude extract at a flow rate of 1ml/minute for a 2.5 cm column.
10.) Wash with 12 column volumes of Column Buffer to total 180 ml.
11.) Elute the fusion protein with 200 ml Column Buffer plus 0.72 g (10 mM) maltose. Collect 20 fractions of 1 ml each.
12.) Pool the protein-containing fractions.

Samples 2-11 for each pbp and fts were run on a SDS-Page Protein gel to evaluate which samples contain the most amount of protein (FIGURE 25 and 26).

Western Blot

Western blot is used to detect, in a mixture of proteins or fragments of proteins, those that react with the same antibody. In this technique, the antibody reactive proteins in a mixture are analyzed by first resolving the proteins in that mixture by denaturing gel electrophoresis (7). After electrophoresis, the gel is placed in contact with a sheet of nitrocellulose by an electric current. The proteins are bound to the nitrocellulose sheet, and then the antigen-antibody reactions are visualized using chemiluminescence.

The primary antibody used was 17 F12, which has anti-IncA properties associated with the HP protein. If our supposed clone is actually the HP clone, then we will observe the same band for the pbp3, ftsw, and Hp clone.

Western Blot

1.) Perform electrophoresis on an SDS/Page gel @110 Volts until the dye was close to bottom
2.) Proteins were transferred onto Nitrocellulose membrane.

Order of apparatus:

a. black side down

b. brillo pad

c. waltman paper

d. gel

e. nitrocellulose

f. whatman paper

g. brillo pad

h. white side up

3.) A stir bar was placed at bottom of Blot Box, ice pack was added, blot buffer was used

4.) Blot ran at 0.9 A for 1 hour

5.) Put into bags with 15 ml blot block and put into freezer overnight

6.) Incubate the blot on shaker for 1 hour in 10 ml primary antibody (17F12, anti-IncA monoclonal antibody) diluted to 1:5000 in blot block (3 ul/15ml).

7.) Rinse 3 X (5-10) minutes with blot wash in tuberware container.

8.) Incubate the blot on shaker for 1 hour with 1:5000 dilution of secondary antibody (chicken anti-mouse)

9.) Rinse 3 X (5-10) minutes with blot wash in tuberware container.

Chemiluminescence

Chemiluminescent detection systems have emerged as the best all-around method for detection of Western blots (18). It is possible to record and store the blot results permanently because they are generated on film. Either alkaline phosphatase (AP) or horseradish peroxidase (HRP) conjugated secondary antibodies are utilized in combination with specific chemiluminescent substrates to generate the light signal (FIGURE 20).

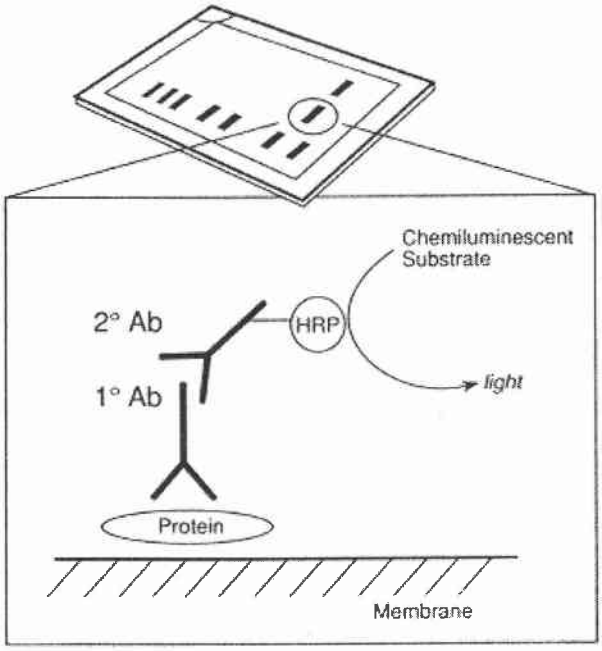

FIGURE 20. Chemiluminscence binding. After the primary antibody is bound to the target protein, a complex with HRP-linked secondary antibody is formed. LumiGLO is added and it emits light during an enzyme catalyzed reaction.(18)

Chemiluminscence was performed by:

1.) Mix equal volumes of the chemiluminesce reagents (3 ml of each) with the blot block. Rock on shaker for 2 minutes.
2.) Remove excess reagent. Cover the blot with clear plastic wrap.
3.) Expose the blot to X-ray film for 10 seconds.
4.) Place film into Chemiluminescence machine.

RESULTS

During the Phase 1 experiments, not only was one positive clone, pbp3 sample4, produced and sequenced correctly, but Phase 1 experiments also provided great depth and understanding of the experimental procedures. As illustrated in Figure 21, PCR screening samples of pbp reveal a positive clone in lane four.

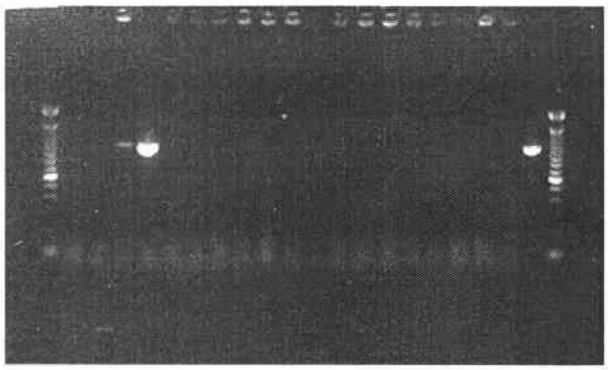

FIGURE 21. An agarose gel of Phase 1 pbp samples after PCR screening.

The reason why the pbp3-sample 4 clone wasn't transcribed into a protein or used to complete this project, was because the gene product would not induce. Brown and I tried to induce the sample at different time intervals (1 hr, 2 hrs, 4 hrs, 8 hrs, overnight) and in different amounts (10 ml, 100 ml, 500 ml, and 1 Liter). The protein exhibited inconsistent results with either no band present or, (on the same sample, different day) a very small, faint band present. Brown and I decided to start over by designing new primers, which should in the end, produce stronger proteins.

Phase 2 pbp and ftsW gene products were obtained. Figure 22 and 23 illustrates agarose gels after PCR screening with Phase 2 pbp and ftsW putative clones, respectively. The samples were induced with IPTG and the result was recorded using a SDS-PAGE protein gel, Figure 24. Figure 25 and 26 represent SDS-PAGE protein gels completed after column chromatography. These gels reveal samples 3 and 4 showing the greatest amount of protein. Western blot analysis distinguished the protein of interest from the HP clone by using 17 F12 antibody specific to the HP clone. Figure 27 and 28 are proofs of the ftsW and pbp3 Western blots, respectively, which reveals that both the HP clone and the putative ftsW and pbp3 clones bonded to 17 F12.

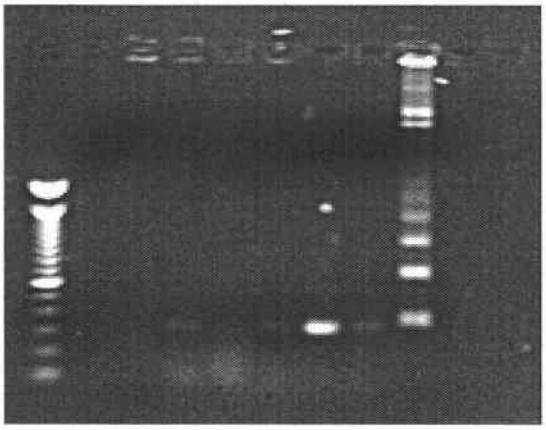

FIGURE 22. Phase 2 pbp3 samples 3 and 5 are predicted to be positive clones. The positive control is the brightest lane, but the negative control is also seen. The last lane is pure pbp3 control, which does appear to be a different molecular weight than the pbp3 samples.

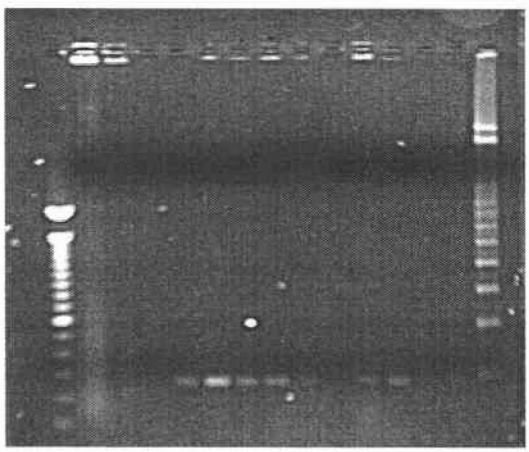

FIGURE 23. Phase 2 ftsW sample 4, 5, 6, 7, 8, 10, 11 are predicted to be positive clones. The positive and negative controls are the last two lanes, respectively, of which neither is seen.

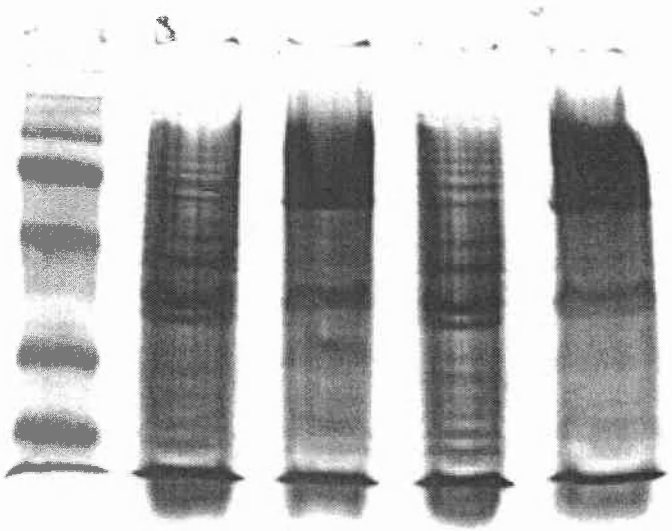

FIGURE 24. SDS-PAGE protein gel of high MW Ladder, pbp3-uninduced, pbp3-induced, ftsW-uninduced, ftsW-induced. This staining pattern was a challenge to observe due to similar molecular weights of MBP (39 kb) and protein of interest (≈41 kb)

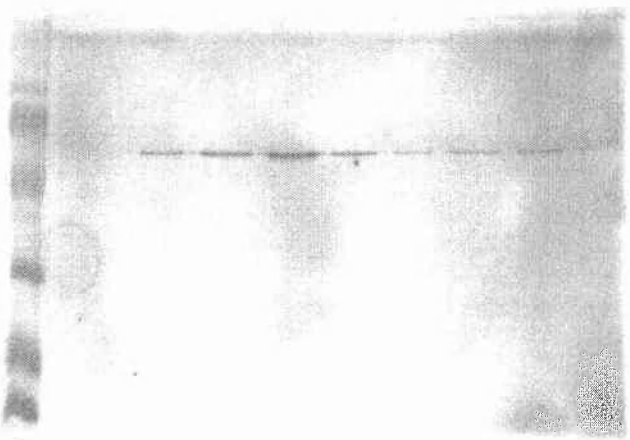

FIGURE 25. Phase 2 ftsW protein gel. The darkest bands, 2,3,4, correspond to fts samples 4,5,6.

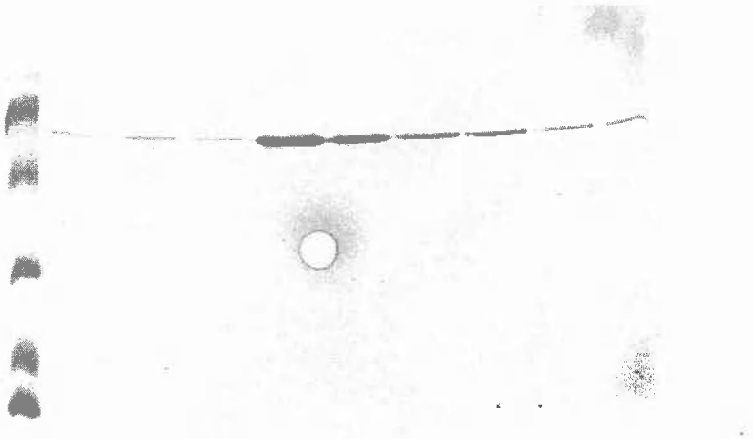

FIGURE 26. Phase 2 pbp3 protein gel. The darkest bands, 3,4,5, correspond to pbp samples 5,6,7.

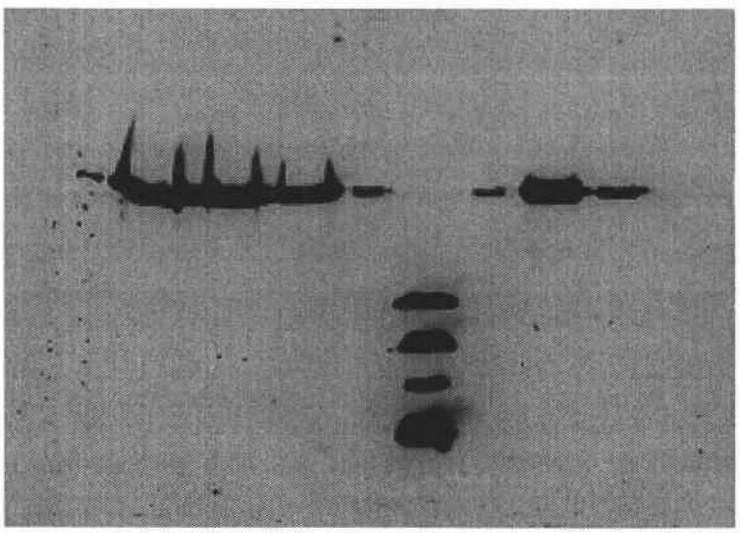

FIGURE 27. Phase 2 ftsW Western blot. Lanes 1,2,3 contain fts samples 4,5,6. Lane 7 contains HP.

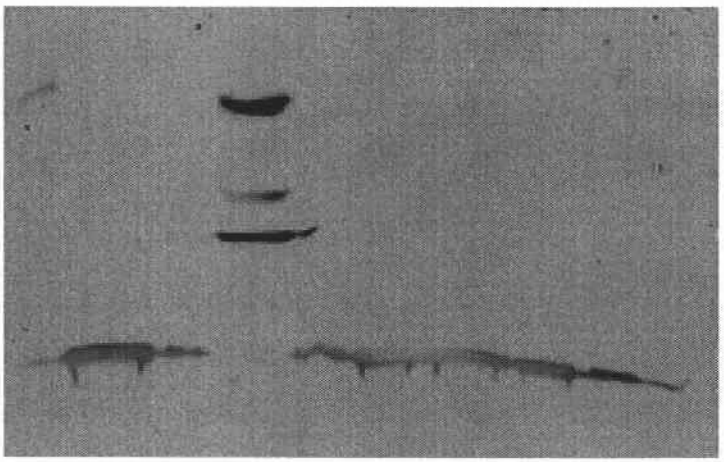

FIGURE 28. Phase 2 pbp3 Western blot. Lanes 2,3,4 correspond to pbp samples 5,6,7. Lane 8 corresponds with the HP clone.

DISCUSSION

The major problem during Phase 1 was the process of obtaining a cut vector and insert before ligation. This process was very time consuming and involved many series of trial and error before a clone was obtained. The most encountered problems were losing the vector after the second digestion, in addition to, losing the vector and/or insert after cleaning it with PCR purification kit. Most problems were solved by reviewing the necessary steps and ingredients or by asking Wendy Brown, my graduate student mentor, to supervise me while I was conducting the experiment. Two major mistakes I found I was doing after weeks of struggle, were that I was using 100X BSA buffer instead of 10X BSA buffer and that I wasn't aware that there was a specific Bam HI buffer for the Bam HI restriction enzyme. I feel I learned the most during this stage of the project and what makes up biotechnology and working in a microbiology laboratory. I learned from all of my mistakes and in doing so, I was able to independently decipher how to face the problem and what changes I should make for the next time.

The Phase 2 experiments went much faster than Phase 1 because I had learned the sequence of steps required to complete each task and what each step lead to. Another attribute of Phase 2 included

designing new primers, which was a worthwhile hands-on learning experience, in contrast to reading about it in a book. We designed primers pbp3, ftsW, and p60, but only experimented with pbp3 and ftsW because addition of a third gene in our procedures would have created disorganization. Even though our supposed clones turned out to be the HP clone, the protein is needed in the laboratory and I was able to perform the entire primary experimentation of this project with success.

Phase 2 was moving very smoothly with positive results from each step. The one warning sign we should have looked more closely at was the screening gels. (FIGURE 20 and 21). These gels show poor marking in both the positive and negative controls. Other than these gels, the signs all pointed toward obtaining clones for genes pbp3 and ftsW. It was quite a surprise to find that the pbp3 and ftsW clones did not match their original sequence, but instead both perfectly matched Theresa's HP clone sequence.

It is ambiguous how exactly the mix up of DNA samples occurred. Theresa's HP clones were very easy to clone, thus any contamination would have allowed HP to dominate over the weak pbp3 and ftsW clones. It seems likely that it must have happened toward the beginning of the procedures because of the strong correspondence and by something as simple as not sterilizing the loop properly, but it is difficult to say. An additional agarose gel pictures is included as FIGURE 29, which shows that although the negative controls are highlighted, the two samples are of different molecular weights, therefore they both couldn't be HP. Brown and I kept hopeful until the very end with proof of the Western Blot, which showed in effect, that our supposed clones were the HP clone (FIGURES 24 and 25).

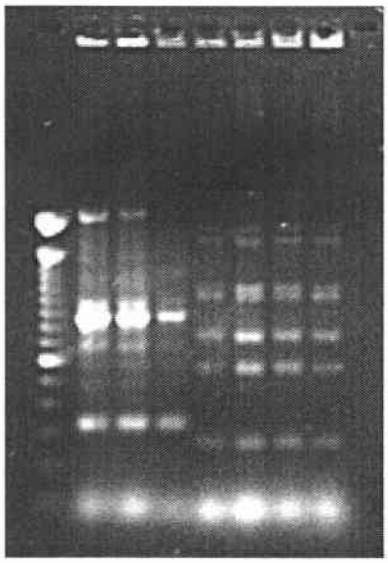

FIGURE 29. Additional gel of Phase 2: lane 1=100 bp ladder, lane 2, 3 = pbp sample 3,5, lane 4 = pbp3 negative control, lane 5,6,7= fts samples 4,5,6, lane 8= ftsW negative contol.

There are several reasons why this project had so many trials and tribulations. Some genes just cannot be clones, some are extremely difficult to clone, and some fit together perfectly. The PG-associated genes in *Chlamydia*, pbp3, ftsW, and p60, were extremely difficult to clone for reasons unknown. One reason lies within the fact that both *E. coli* and *Chlamydia* contain PG. Therefore, it could be that they both complete for it until one outlives the other or *E. coli* wouldn't accept the competing plasmid at all. Types of disfunction could have been caused when the protein disturbed the protein enzymatic activity in *E. coli* and degraded the protein. Poor usage of proper technique handling could have been the cause for poor results. Another reasons is codon bias, which is when the ratio of codons used in the heterologous gene product is different than the available tRNAs in E. coli.

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