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Characterization And Cloning Of Coxiella burnetii

Janelle McLean

Carroll College, Helena, MT

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CHARACTERIZATION AND CLONING OF Coxiella burnetii

Submitted in Partial Fulfillment of the Requirements for Graduation with Honors to the Department of Biology at Carroll College, Helena, Montana

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April 3, 1992
This thesis for honors recognition has been approved for the Department of Biology.

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April 3, 1992
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>ii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vi</td>
</tr>
<tr>
<td>INTRODUCTION AND LITERATURE REVIEW</td>
<td>1</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>7</td>
</tr>
<tr>
<td>RESULTS AND CONCLUSIONS</td>
<td>13</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>21</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>31</td>
</tr>
</tbody>
</table>
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ABSTRACT

Coxiella burnetii, an obligate intracellular parasite, is the etiological agent of the disease Q-Fever, which can occur in acute and chronic forms. The acute form is rarely serious, but chronic Q-Fever may result in endocarditis, which is usually fatal. It is therefore of interest to determine which strains of Coxiella burnetii lead to the acute versus the endocarditis-associated sickness, and what the mechanisms involved in each are. This past summer at Rocky Mountain Laboratories in Hamilton, Montana, under the direction of Dr. Ted Hackstadt, I began to research the Coxiella burnetii 9-Mile strain, which is associated with the acute form of the disease. DNA characterization of this bacterium by restriction mapping was performed as well as attempts with antibodies to determine which proteins were cloned into recombinants. These could be compared to those of endocarditis-associated strains. With lambda gt11 as a cloning and expression vector, DNA was inserted into various plasmids, proteins of the systems were expressed, and ligations and transformations of plasmids with Escherichia coli strains were carried out. These
transformants were then used in plasmid mini-preparations for isolation and identification of the DNA we were successful in cloning.
LIST OF FIGURES

Fig. 1.  Restriction map of Lambda Bacteriophage gt11 vector.............................. 4

Fig. 2.  Plasmid pBR322..................................................... 5

Fig. 3.  Plasmids pUC18 and pGEMEX-1............ 6

Fig. 4.  Characterization of DNA samples by restriction mapping.................. 15

Fig. 5.  Electrophoretic gel of pBR322 plasmid mini-prep............................. 17

Fig. 6.  Results of plasmid mini-prep (with pUC18) digested with EcoRI......... 19
LIST OF TABLES

Table 1. Lambda dilutions for plate lysates..... 8

Table 2. Concentrations of pUC18 ligated with DNA to SURE E. coli cells for transformation......................... 12

Table 3. Titration of viral stocks.............. 13
INTRODUCTION AND LITERATURE REVIEW

*Coxiella burnetii* and Q-Fever

*Coxiella burnetii* is an obligate intracellular parasite and the etiological agent of Q-Fever, an infectious disease which is more commonly found in livestock, but often diagnosed in humans as well. *Coxiella burnetii*, an extremely infectious bacterium and among the most stable in its environment, is classified within the family Rickettsiaceae (6). This bacterium was originally called a rickettsia, but it is now known that it differs in many ways (i.e. genomic composition, vector of transmission, filterability, mode of intracellular growth, size, antibiotic sensitivity, fatty acid composition, and resistance to temperature and pH), and is of its own genera (1,7,15). It has a genome size of 1.04 X 10^9 daltons and enters the host cell by a passive parasite-directed phagocytosis (1). *C. burnetii* has adapted to the acidic environment of the phagolysosome in which it lives, and in fact, depends on the acidic pH for metabolic and transport processes, surviving best at a pH of approximately 5 or less.
The bacterium multiplies within the host cell, and after the initial infection, all host cells are infected within a few days (8).

The agent is found in arthropods, fish, birds, rodents, marsupials, and livestock (1). Ticks usually maintain the disease in nature, but they rarely infect humans. Instead, cattle, sheep, and goats are usually the source of human infection through their milk, urine, meat, feces, or placental material. Inhalation of dust and aerosols are also ways to infect humans (6,15).

The first recorded outbreak of Q-Fever was in Brisbane, Australia in 1935, among the workers in a slaughterhouse. The cause was unknown at the time, but in 1938, Coxiella burnetii was discovered as its agent in ticks near Nine Mile Creek by Missoula, Montana. The acute form of Q-Fever has nonspecific symptoms, and is therefore often difficult to identify, but common symptoms include: sudden onset, headache, fever, chills, general malaise, pneumonia, anorexia, muscular pain, and liver damage (1,5,8,16). The acute form is generally not serious and can be treated with antibiotics, most commonly chloramphenicol and tetracyclines (13,16). Occasionally, chronic or subacute forms of the disease can occur which are much more serious, such as endocarditis, which involves inflammation of the heart lining and valves. The endocarditis-associated disease
more often than not results in death, as antibiotic therapy is usually not effective.

It is known that *Coxiella burnetii* undergoes a phase variation, in which there is a transition from a virulent (Phase I) to avirulent (Phase II) form as it is passed into eggs or tissue culture. If phase II is then injected into animals, the organism reverts back to phase I (1, 6,). Testing with antibodies has shown that unique lipopolysaccharides (LPS's) seem to be the phase-dependent antigen (6, 7). The two different phases share the presence of common surface proteins, but they are not always accessible to serve as antigens. It is now believed that phase I LPSs sterically block access of antibodies to outer membrane proteins which give phase II serospecificity (6). Therefore, the LPS is the only unique antigen that is different between phases. It was previously believed that there was no variation between the strains of *Coxiella burnetii*, but genetic analysis and analysis of LPS structure in *Coxiella burnetii* isolates associated with endocarditis have shown that there is variation (6). The phase I LPS's are the main phenotypic difference between acute-type strains and strains related to endocarditis. While LPS's are suspected as major determinants of virulence, other components may still be involved and are being studied (5, 6). Currently, detection of strain differences is by
analysis of their plasmids by restriction endonuclease or by electrophoresis of their LPSs.

**Lambda gt11 Vector**

The lambda gt11 bacteriophage is a virus engineered by Young and Davis and commonly used as a cloning and expression vector for constructing cDNA and genomic libraries (2,11). Inserts of up to 8.3 kb can be cloned into the vector and then screened with the use of antibody probes. This phage contains the lacZ gene from *E. coli* which contains a single EcoRI site for insertions (Figure 1). The insertion of foreign DNA inactivates the lacZ gene, which keeps the phage from producing blue plaques on a lacZ' host (e.g. *E. coli* Y1090) on plates which contain X-gal (3,4).

Figure 1. Restriction map of Lambda Bacteriophage gt11 vector (3).
**Plasmids Used**

Plasmid pBR322 is a popular cloning vector which contains a gene conferring resistance to tetracycline (tet\(^R\) gene), and a gene for resistance to ampicillin (amp\(^R\) gene) (12). These genes contain common restriction sites which allow for simple DNA insertion and make screening by antibiotics convenient. This plasmid is 4.363 kilobase (kb) pairs long (Figure 2).

Plasmids pUC18 and PGEMEX are also commonly used vectors. These plasmids are 2.686 kb and 4.300 kb long respectively, and are only carbenicillin resistant (Figure 3).

![figure](image-url)

Figure 2. Plasmid pBR322 (9).
In-frame sequence of the pGEMEX-1 vector multiple cloning region.

Figure 3. Plasmids pUC18 and pGEMEX (9,10).
MATERIALS AND METHODS

An overnight culture of *E. coli* Y1090 was grown (Appendix A) to be used as recombinant bacteria. Q1-1, Q1-10, Q1-4, Q3-4, Q4-5, Q1-12, and gt11 were picked from frozen lysates into 0.5-ml SM each. These were then vortexed and diluted 1:100 (10-ul lambda + 990-ul SM). These dilutions were then plated with Y1090, the virus was extracted, and viral stocks were made (Appendix B). Viral stocks were titrated to determine if a significant difference existed in the number of plaques between the plates of $10^6$ and $10^8$ dilutions. Plate lysates were set up to obtain lambda (gt11, Q1-1, Q1-10, Q1-4, Q3-4, Q4-5, and Q1-12) DNA (Appendix C). After much replating, the proper lambda dilution with 100-ul of Y1090 was determined to give moderately heavy plaque plates (Table 1). With this DNA, restriction mapping could be started.
<table>
<thead>
<tr>
<th>Lambda DNA</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>gt11</td>
<td>10-ul $10^2$ dilute + 100-ul Y1090</td>
</tr>
<tr>
<td>Q1-1</td>
<td>100-ul $10^1$ dilute + 100-ul Y1090</td>
</tr>
<tr>
<td>Q1-4</td>
<td>10-ul $10^2$ dilute + 100-ul Y1090</td>
</tr>
<tr>
<td>Q1-10</td>
<td>50-ul undilute + 100-ul Y1090</td>
</tr>
<tr>
<td>Q1-12</td>
<td>50-ul undilute + 100-ul Y1090</td>
</tr>
<tr>
<td>Q4-5</td>
<td>50-ul undilute + 100-ul Y1090</td>
</tr>
<tr>
<td>Q3-4</td>
<td>10-ul $10^2$ dilute + 100-ul Y1090</td>
</tr>
</tbody>
</table>

Table 1. Lambda dilutions for plate lysates.

Restriction endonuclease digestions were performed with EcoRI, KpnI, SstI, Bam HI, Hind III, and XbaI. These enzymes were used alone and/or were combined with each other to characterize the DNA. Upon completion of the digests, the DNA was loaded into 0.75% agarose gels and electrophoresed (Appendix D). After electrophoresis, a picture of the gel was taken and the distance from the well to each DNA band was measured in millimeters. The lambda Hind III molecular weight marker was used as a reference. The distance to each marker on the ladder was plotted on the X-axis of logarithmic paper while the size of the band (0.6 kb, 2.0 kb, 2.3 kb, 4.4 kb, 6.5 kb, 9.4 kb, and 23 kb) was plotted on the Y-axis. The picture of the gel and the measurements of the lanes of DNA were then examined to see if any lane had a band of DNA which varied from those of the other lanes. If an unusual
measurement were found, the length in millimeters of this band could be compared to the standard curve of the lambda Hind III marker on the graph. The approximate size of that band (in kb) could then be determined. The restriction map of lambda gt11 is known, and we therefore knew the possibilities of different sized fragments of the gt11 when digested with varying restriction endonucleases. We also then knew the distance of different restriction sites from the EcoRI site on the gt11. From this we could confirm which fragments of DNA were not the lambda gt11, but instead fragments of our DNA samples. We could then determine the restriction map of each sample.

Screening for direct protein expression to identify proteins of the *Coxiella burnetii* involved the use of affinity purified antibodies to recombinant protein (Appendix E). Nitrocellulose filters were used to express the protein and alpha-9-Mile antiserum was used to bind to the recombinant protein. The antibody was eluted, reacted with *Coxiella burnetii* to determine which proteins were cloned, and a Western Blot was performed to visualize the proteins (Appendix F).

Before the lambda DNA samples could be ligated into a plasmid, the DNA had to be digested with EcoRI and the fragments to be used had to be purified from the agarose gel after electrophoretic separation. DNA purification
was obtained by following the Gene Clean protocol (Appendix G). To assure that an adequate amount of DNA was recovered with the Gene Clean method, a small amount of DNA was tested by electrophoresis and by optical density on a fluorometer. Plasmid pBR322 was then cut with EcoR1 and the phosphoryl groups were cleaved off the ends with calf intestinal phosphatase (CIP) to prevent religation of the plasmid ends (Appendix H). DNA was ligated into the pBR322 and transformed into SURE E. coli cells, a competent, commercial strain of E. coli (Appendix I). These transformants were plated on LB + Carbenicilllin plates. Colonies were picked from the transformation plates twice: one day after they were plated, and then again the following day for new colonies which had not been previously picked. We picked all available colonies, and tested the ligase to make sure the lambda DNA had been ligated into the plasmid properly. It was determined that the ligase was working properly, and the transformation was repeated, adjusting to 5- ul of DNA instead of 1-ul and 7-ul of Ligase Buffer instead of 5-ul in the ligation. The selected colonies were then plated on "master plates," which served as a method to distinguish the colonies from one another and to store the desired colonies.

To test the results of the transformation, four plasmid mini-preparations of each DNA type were set up
with overnight cultures of LB media and carbenicillin (Appendix J). The mini-preps were then digested with EcoRI and electrophoresed on 0.75% agarose gel. To better evaluate selected mini-prep cultures which may have had favorable inserts, another digest was performed with EcoRI as the restriction enzyme in one lane, and BamHI as the second restriction enzyme in the lane next to it. These plasmid mini-preps were performed twice each. The entire procedure was then repeated with newly transformed colonies which were picked and grown in overnight cultures for a second screening.

A second plasmid, PGEMEX 1, was also digested with EcoRI by the same procedure. Only DNA from Q1-1, Q1-10, Q4-5, and Q1-12 was ligated with the PGEMEX. These were then transformed into E. coli SURE cells and plated. Transformations were also attempted with the PGEMEX into another strain of competent E. coli cells, JM109.

The final plasmid which was used for ligation and transformation was puc18. This was also digested with EcoRI and ligated as described. Laboratory grown SURE cells were used. These E. coli are not as efficient as the commercial SURE cells, but they were adequate for our purposes. The ligations and transformations were performed several times, and the amount of lambda DNA insert to be ligated was adjusted from 5-ul to 7-ul to enhance the ligations. The concentrations of the plasmid
to the SURE cells were also varied in the transformations with the puc18 (Table 2). Again, random colonies were picked from the transformation plates and plated on master plates. Plasmid mini-preps were grown from these master plates using the same procedure as above, digested with EcoRI, and electrophoresed on 0.75% agarose gel.

<table>
<thead>
<tr>
<th>Tube</th>
<th>DNA Source</th>
<th>Amount DNA</th>
<th>Amount SURE Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Q1-1</td>
<td>1-ul</td>
<td>20-ul</td>
</tr>
<tr>
<td>2</td>
<td>Q1-1</td>
<td>9-ul</td>
<td>160-ul</td>
</tr>
<tr>
<td>3</td>
<td>Q1-10</td>
<td>1-ul</td>
<td>20-ul</td>
</tr>
<tr>
<td>4</td>
<td>Q4-5</td>
<td>1-ul</td>
<td>20-ul</td>
</tr>
<tr>
<td>5</td>
<td>Q4-5</td>
<td>9-ul</td>
<td>140-ul</td>
</tr>
<tr>
<td>6</td>
<td>Q1-12</td>
<td>1-ul</td>
<td>20-ul</td>
</tr>
<tr>
<td>7</td>
<td>pUC18*</td>
<td>0.1-ul</td>
<td>20-ul</td>
</tr>
<tr>
<td>8</td>
<td>Q1-10</td>
<td>9-ul</td>
<td>140-ul</td>
</tr>
<tr>
<td>9</td>
<td>Q1-12</td>
<td>9-ul</td>
<td>140-ul</td>
</tr>
</tbody>
</table>

* pUC18 was used as a control

Table 2. Concentrations of pUC18 ligated with DNA to SURE E. coli cells for transformation.
RESULTS AND CONCLUSIONS

The first titration of viral stocks did not prove to be adequate, as there were too many plaques and not a significant difference between the $10^6$ and $10^8$ dilutions. The second titration however, proved to be sufficient for us to begin preparing for the restriction mapping (Table 3).

<table>
<thead>
<tr>
<th>PLATE</th>
<th>DILUTION</th>
<th>TOTAL # PLAQUES/PLATE</th>
</tr>
</thead>
<tbody>
<tr>
<td>gt11</td>
<td>$10^8$</td>
<td>744</td>
</tr>
<tr>
<td>Q1-1</td>
<td>$10^8$</td>
<td>668</td>
</tr>
<tr>
<td>Q1-10</td>
<td>$10^8$</td>
<td>780</td>
</tr>
<tr>
<td>Q1-4</td>
<td>$10^8$</td>
<td>796</td>
</tr>
<tr>
<td>Q1-12</td>
<td>$10^3$</td>
<td>624</td>
</tr>
<tr>
<td>Q4-5</td>
<td>$10^8$</td>
<td>596</td>
</tr>
<tr>
<td>gt11</td>
<td>$10^6$</td>
<td>1132</td>
</tr>
</tbody>
</table>

Table 3. Titration of viral stocks. A significant difference in the number of plaques per plate between $10^8$ dilutions and $10^6$ was desired before restriction mapping was attempted.

Characterization of the DNA samples by restriction mapping led to clarification of Q1-1, Q1-4, Q1-10, Q1-12, Q4-5, and Q3-4 DNA fragments in the gt11 cloning vector
(Figure 4). Because of human error in measurement, the indistinct line of the DNA band or well in the lane being measured, and the fact that most of these restriction digests were run on different gels, measurements of the DNA during the mapping are approximate values. It was suspected that Q4-5 and Q3-4 are the same, but of different orientation. The map seems to confirm this. It was also suspected that Q1-1 and Q1-10 were the same, but repeated digests showed a BAM HI site in the Q1-10 that was not present in Q1-1. Q1-4 and Q1-12 proved to be unique. The length of the inserts varied from approximately 1.1 kb in Q1-4 to 5.8 kb in Q1-12.

Screening for protein expression by antibody selection proved to be unsuccessful. This procedure was tried twice, but in neither attempt could we see the unique proteins desired after the Western Blot was completed. We viewed the SDS-acrylamide gel before the second Western Blot and thought the gel looked promising with respect to recombinant proteins to be identified. It may have been the long and detailed protocol, along with inexperience, that accounted for this method not working. However, it will be attempted again in the future.
Figure 4. Characterization of DNA samples by restriction mapping.

E...EcoRI
K...KpnI
S...SstI
B...Bam HI
H...Hind III
Electrophoretic gels showed we had been successful in purifying lambda DNA with the Gene Clean Method. Our first attempts at transformation were with the pBR322 plasmid and SURE *E. coli* cells. Approximately 45 colonies grew on these transformation plates. When we did a second pick of these plates the following day, no colonies were obtained. The second transformation only grew 17 colonies, with none on the Q1-12 plate. When the plasmid mini-preparation was performed with the transformants, the screening digest showed no inserts and very few plasmids. The mini-prep was repeated because it was realized that in the first step of the mini-prep, 5-ml of the overnight culture were centrifuged instead of 1.5-ml, and this may have made a significant difference in the results. In the second attempt, the plasmid could be seen at 4.4 kb in the digest gels, but again, no inserts were found. A frequently occurring band did show up in most lanes of the digest gel, but this was not the proper size to be an insert. We were looking for inserts of 1 kb, and this band was at approximately 2 kb. It is most likely that this band represents undigested plasmid, and is the pBR322 in a supercoiled form resistant to digestion (Figure 5).
Figure 5. Electrophoretic gel of pBR322 plasmid mini-prep. The band at approximately 4 kb is the plasmid, while the band at approximately 2 kb is most likely the supercoiled undigested form. No inserts were found.

The second screening of the plasmid mini-preps with pBR322 did not produce any cloned inserts when digested with EcoRI. When these mini-preps were repeated however, we found possible inserts in five lanes of Q1-1 DNA and in three lanes of Q1-10 DNA. When these eight mini-preps were double checked with EcoRI and Bam HI endonuclease, we found one lane of Q1-1 origin to contain an insert of the proper length.

By this time, transformations had been performed with the PGEMEX plasmid. The first two transformations
were not successful, and it was realized that these transformations had mistakenly been plated on LB + Carbenicillin + Tetracycline plates. PGEMEX is not resistant to tetracycline, and therefore would not grow on plates containing it. When the transformations were attempted again with LB + Carbenicillin plates, colonies still did not appear, and we decided to attempt using pUC18 as the plasmid.

The first transformation with puc18 and the laboratory-made SURE cells did not work, but a second attempt at the transformation produced many colonies. When colonies from these plates were plated on master plates of carbenicillin, IPTG, and X-Gal, 13 colonies from Q1-1, 13 colonies from Q1-10, and two colonies from Q4-5 appeared white, indicating that the lacZ gene had been interrupted and cloned into. Plasmid mini-preps from these overnight cultures proved successful, with five of 22 preparations showing an insert of approximately 1 kb. Of these five, three of the inserts were of Q1-1 origin, and the other two were of Q1-10 origin (Figure 6).
Figure 6. Results of plasmid mini-prep (with pUC18) digested with EcoRI. Five preparations (designated with stars) proved to be successful in cloning a 1 kb segment of *Coxiella burnetii*.

In summary, cloning 1 kb fragments of the 9-Mile strain of *Coxiella burnetii* DNA was successful, and this can now be used for further study. In the near future, much of this will be repeated to obtain a larger amount of cloned DNA and to try procedures again which may have failed due to lack of experience with the techniques.
The immediate goal is to try expressing the lambda DNA in larger amounts in the plasmids. Also, to attempt using antibody selection against cloned proteins and carrying out the same procedures with the endocarditis-associated Priscilla strain are objectives. These results, along with some additional testing, can then be used to compare the two strains. The use of monoclonal antibodies specific for endocarditis-associated strains could detect and characterize these strains. Specific antibodies can then be developed if surface antigens that vary between strains are determined.
APPENDIX A

Growing Y1090 (ampicillin resistant)

1. Add the following:
   10-ml LB
   100 ul maltose
   100 ul carbenicillin
2. Inoculate with E. coli
3. Shake O/N at 37 degrees C.

APPENDIX B

Making Viral Stocks

1. Spin approximately 10-ml Y1090 5 min. @ 5000 RPM
2. Resuspend in 4-ml 10 mM MgSO₄
3. Melt top agar 10 min. in autoclave; 45 degrees C. to cool
4. Pick lambda DNA from frozen lysates (gt11, Q1-1, Q1-10, Q1-4, Q4-5, Q1-12, Q3-4)
5. Vortex and dilute 1:100 (10-ul of lambda + 990-ul SM)
6. Add 100-ul Y1090 to 100-ul undilute lambda and 1:100 lambda in separate tubes
7. 20 min. @ RT
8. Plate on LB with 3-ml lambda top agar
9. Incubate @ 42 degrees C. 2-3 hr.
10. Incubate @ 37 degrees C. O/N
APPENDIX C

Plate lysates/Amplification and LambdaSorb

1. Mix 100-ul Y1090 + 50-ul supe from picked plaque - 20 min @ RT
2. Plate in 3-ml lambda top agar over LB
3. Incubate 3 hr. @ 42 degree C.
4. Shift to 37 degree C. O/N
5. Add 5-ml SM - 2 hr. w/ rotation
6. Collect SM
7. Add 5-ml SM - 2 hr. w/ rotation
8. Pool supes
9. Centrifuge @ 3.5 K RPM for 5 min.
10. Transfer supe to clean tube
   *
11. Add 10-ul LambdaSorb/ml (about 85 ul)
12. 2 hr. @ 4 degree C. w/ occasional mixing
13. Centrifuge @ 3.5 K for 5 min.
14. Discard supe
15. Add 5-ml SM and mix
16. Centrifuge 3.5 K for 5 min.
17. Resuspend in 1-ml lambda dil. - transfer to microfuge tube
18. Microfuge 1 min. @ 12 K RPM
19. Decant supe
20. Add 200-ul T10E10, pH 8
21. Heat to 70 degree C. for 10 min.
22. Chill to 4 degree C.
23. Microfuge 1 min. @ 12 K
24. Save supe

* If preparing plate lysates: Stop here and add a few drops of chloroform to each bottle of supe to kill bacteria
APPENDIX D

Enzyme Digests (Endonuclease or restriction enzymes)

1. Add to microfuge tube:
   10-ul DNA
   1.5-ul salt buffer (10X Low Salt w/ KpnI and SstI, 10X High Salt with EcoRI, Hind III, Bam HI, and XbaI)
   1-ul 0.1M MgCl₂
   1-ul enzyme (use only 0.5-ul for EcoRI)
2. Incubate @ 37 degree C. for 1.5 hr.
3. Add 3.5-ul Sample Buffer to each tube

Agarose gel (.75%):
   0.75 g Agarose
   98 ml H₂O
   2 ml 50X TAE
   4-5 ul EtBr

Running buffer:
   1.47 l Distilled H₂O
   30 ml 50X TAE
   72 ul EtBr
APPENDIX E

Affinity Purified Antibodies to Recombinant Protein

1. Plate lambda with Y1090 in 3-ml lambda top agar to give near confluent lysis
2. Incubate 3 hr. @ 42 degree C.
3. Overlay with NC filters saturated with 10 mM IPTG
4. Incubate 2 hr.
5. Invert filters and incubate at least 2 hr. more
6. Block with BLOTTO
7. Rinse 2X with PBST
8. Incubate with antisera in BLOTTO - 2 hr. minimum
9. Rinse 3X with PBST
10. Elute with 5 ml 0.25 glycine, pH 2.8, 150 mM NaCl - 5 min.
11. Neutralize with 1 ml 1M Tris-HCl, pH 7.5
APPENDIX F

Western Blot Procedure

1. Remove gel and place in transfer buffer. Layer in order: a. Scotch brite pad, b. prewetted Whatman filter, c. gel, d. prewetted NC filter, e. prewetted Whatman filter, f. Scotch brite pad - clamp between gel holder and insert into transblot
2. Immerse in transfer buffer
3. Increase power to 32 V. Amperage should be 0.9 – 1.1 A.
Transfer 2 hr.
4. Remove nitrocellulose - immerse in blocking soln: PBST, PBSA, or BLOTTO. Rocking platform for 5 min.
5. Rinse 2X with PBST
6. Antibody (1:50 - 1:300) in blocking soln. 2 hr. to O/N @ RT on rocking platform
7. Rinse 3X with PBST
8. 125I protein A (1:300) in blocking solution. 2 hr - O/N @ RT with rocking.
9. Rinse once with PBST, 6-8 X with H2O. Dry at 37 degree C. between sponge pads in non-humidified incubator. Tape on blank paper.

Stock Solutions

Transfer Buffer:

<table>
<thead>
<tr>
<th>Component</th>
<th>MW</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄</td>
<td>142</td>
<td>230.0 g</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>120</td>
<td>45.6 g</td>
</tr>
<tr>
<td>Na₃Azide</td>
<td></td>
<td>0.8 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>4.0 l</td>
</tr>
</tbody>
</table>

\[ = 0.5 \text{ M NaPO}_4, \]  
\[ \text{pH 7.4} \]  
\[ + 0.2\% \text{ Azide} \]

Dilute 1:20 (150 ml + 2850 ml H₂O) for transfer. (=25 mM NaPO₄) Prepare fresh and degas during electrophoresis.
1.5 M NaCl:  
NaCl \quad \text{mw} \quad 58.4 \quad 175.2 \text{ g}  
H_2O \quad \quad \quad \quad \quad \quad \quad \quad 2.0 \text{ l}  

Wash Buffer (PBST):  
.4 M NaPO_4 \quad 100 \text{ ml}  
1.5 M NaCl \quad 100 \text{ ml}  
Tween 20 \quad 0.5 \text{ ml}  
H_2O \quad 800 \text{ ml}  
= 50 \text{ mM NaPO}_4 -  
150 \text{ mM NaCl}  
+ 0.05\% \text{ Tween 20}  

Blocking Solution:  
.5 M NaPO_4 \quad 100 \text{ ml}  
1.5 M NaCl \quad 100 \text{ ml}  
H_2O \quad 800 \text{ ml}  
= PBS  
+ 0.5 \text{ Tween 20} = \text{ PBST}  
+ 3\% \text{ BSA} = \text{ PBSA}  
+ 5\% \text{ Non-fat dry milk}  
= \text{ BLOTTO}
Gene Clean Protocol

1. Mix in microfuge tube:
   60-ul DNA (9mI/I)
   12-ul 10X High Salt
   12-ul 0.1 M MgCl₂
   6-ul EcoRI
2. Digest 1.5 hr. @ 37 Degree C.
3. Prep gel .7% Agarose
4. 75 V for 1 hr.
5. Excise 1-8 kb
6. Weigh excised band - cut to 2 mm cubes and transfer to 15 ml tube
7. Add 3 vol NaI
8. Incubate at 50 degree C. for 5 min with occasional mixing until dissolved
9. Add 10-ul Glassmilk - mix well - incubate 15 min on ice with mixing every few min
10. Pellet @ 2.5 K RPM 1 min
11. Decant supe (save)
12. Resuspend pellet in NEW buffer - Transfer to microfuge tube
13. Pellet @ 5 K 1 min.
14. Wash 3X with NEW - Blot pellet dry
15. Add 10-ul Tₜ₀E₁
16. Heat to 50 degree C. for 3 min.
17. Microfuge for 1 min. @ 5 K
18. Carefully remove supe
19. Repeat elution
20. Check on gel

Ligate into gt11 arms
APPENDIX H

CIP Procedure (Dephosphorylation of plasmid)

1. Mix in microfuge tube:
   - 2-ml plasmid
   - 1.5-ml 10X High Salt
   - 0.1 M MgCl₂
   - 8-ml H₂O
2. Digest @ 37 degree C for 2 hr.
3. Add 2-ml CIP
4. 1 hr @ 37 degree C.
5. Add 2-ml 200 mM EGTA
6. 70 degree C. for 30 min
7. Add .2-ml IpOH, 30 min @ -20 degree C.
8. Pellet @ 15 K for 5 min
9. Wash 2X with cold 70% EtOH
10. Dry
11. Resuspend in 10-ml T₁₀E₁
APPENDIX I

Ligation
1. Mix in microfuge tube:
   5-ul DNA
   1-ul plasmid
   7-ul ligase buffer (2X)
   1-ul T4 DNA ligase
2. Incubate at 16 degree C. for 4 hr

2X DNA Ligase Buffer:
1 M Tris-HCl, pH 7.5  100-ul
1 M MgCl₂         20-ul
1 M DTT           20-ul
0.05 M ATP       20-ul
dH₂O             840-ul

Transformation
1. Thaw E. coli cells on ice
2. Aliquot to chilled microfuge tubes
3. Add 1-ul DNA - mix gently
4. 30 min on ice
5. 40 sec @ 42 degree C.
6. Add 1 ml SOC
7. 1 hr @ 37 degree C. with shaking
8. Pellet @ 5 K for 5 min
9. Plate on LB + Antibiotic-resistant plates
APPENDIX J

Plasmid Mini-Preps

1. O/N cultures in LB w/shaking
2. Pellet 1.5-ml @ 5K RPM, 1 min; discard supe
3. Resuspend in 100-ul T\textsubscript{10E1}, 5 min @ RT
4. Add 200 ul NaOH/SDS Soln.; mix gently, 5 min on ice
5. Add 150 ul KAcetate Soln, Vortex 2 sec., 5 min on ice
6. Pellet @ 12 K RPM, 2 min
7. Transfer supe to clean tube, add 0.5-ml TrOH, 30 min @ -20 degree C.
8. Pellet @ 12 K RPM for 5 min
9. Discard supe, Wash 1X with 70% EtOH
10. Dry under vacuum 20 min
11. Resuspend in 50-ul TE

NaOH/SDS Solution:
- 2 ml 1 N NaOH
- 1 ml 10% SDS
- 7 ml dH\textsubscript{2}O

KAcetate Solution:
- 29.4 g KAcetate/80 ml dH\textsubscript{2}O - Autoclave
- 5 ml 90% Formic Acid
- q.s. to 100 ml w/dH\textsubscript{2}O
LITERATURE CITED


10. Pharmacia LKB Biotechnology, Certificate of Analysis


