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USE OF RECOMBINANT DNA TECHNIQUES FOR THE PRODUCTION
OF A MORE EFFECTIVE ANTHRAX VACCINE

FINAL REPORT

Donald L. Robertson

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<p>During the course of this contract, we have isolated and characterized each of the Bacillus anthracis toxin genes. Although the PA (pag) gene was cloned and sequenced by researchers in the Bacteriology Division of USAMRIID, the cloning and characterization of the Et (cya) and LF (lef) genes were performed in my laboratory (7,8). In addition, DNA sequence determinations for the cya (9) and lef (unpublished data of author) genes have also been completed in my laboratory.</p> <p>We have prepared an improved method for the isolation of large quantities of pX01 and pX02 from B. anthracis strains. Restriction enzyme cleavage maps for these plasmids have been constructed. We have initiated mutagenesis procedures for the modification of each of the toxin genes and these mutants are being tested for biochemical activity. In addition, wild-type and mutant toxin genes are being inserted into B. subtilis to produce larger quantities</p>			
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of these proteins for biochemical purposes and for vaccine testing. However, we have not yet placed mutant toxin genes back into B. anthracis, although the wild-type PA and EF genes have been transferred. (←) ←

In conclusion, it appears that most of the experiments outlined in the original research proposal are completed. (i) The anthrax toxin genes have each been cloned. (ii) Each of the toxin genes have been sequenced. (iii) We have generated some toxin gene mutants to be used in the construction of a safe vaccine. We are also using mutants to help elucidate the biochemical activities of these proteins. (iv) We have expressed the anthrax toxin genes in E. coli and B. subtilis and have constructed expression vectors for B. subtilis and B. anthracis. These recombinant plasmids should allow for high level expression of the toxin proteins for biochemical and immunological purposes. (v) We have identified conserved amino acid homology between EF and the Bordetella pertussis calmodulin-dependent adenylate cyclase. This relationship should help to characterize EF better. (vi) Homologies which exist between LF and EF should allow us to examine the interaction between these proteins and PA.

Overall, the research performed under this contract has allowed us to characterize the anthrax toxin genes and to construct important gene mutants. This research is absolutely required for the construction of a safe recombinant DNA derived anthrax vaccine.

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SUMMARY OF RESEARCH

Research Goals. The overall goal of the present research is to construct a safe and effective human anthrax vaccine using recombinant DNA techniques. We plan to isolate and characterize the *Bacillus anthracis* toxin genes for protective antigen (PA), lethal factor (LF) and edema factor (EF). The individual toxin genes will be cloned and expressed in *E. coli* and *B. subtilis*. The toxin genes will be modified using site-specific mutagenesis or deletion mutagenesis procedures to generate gene mutants which lack biochemical activity but which are still fully immunologic for use in a recombinant vaccine. These mutant genes can then be inserted back into *B. anthracis* Sterne with the selective removal of wild-type genes. These mutant *B. anthracis* strains will be tested in animals, such as the mouse or guinea pig, for vaccine efficacy.

We will also characterize the *B. anthracis* plasmids pX01 and pX02 (1-3). Since we plan to insert the toxin genes back into *B. anthracis* to construct a recombinant vaccine host, we need to know a complete restriction map of pX01, which contains the toxin genes. In addition, in order to understand the expression of the toxin genes and of the capsule (2-4), we need to physically characterize these plasmids as completely as possible.

Research Achievements. During the course of this contract, we have isolated and characterized each of the *B. anthracis* toxin genes. The PA (*pag*) gene was cloned and initially characterized in the Bacteriology Division of USAMRIID (5). In addition, the DNA sequence for *pag* was also determined by them (6). The cloning and characterization of the EF (*cya*) and LF (*lef*) genes were performed in my laboratory (7,8). The DNA sequences for the *cya* (9) and *lef* (unpublished data of author) genes have also been completed in my laboratory.

An improved method for the isolation of large quantities of pX01 and pX02 from *B. anthracis* strains was developed at Brigham Young University (10). Initial restriction enzyme cleavage maps have also been constructed. We have also initiated mutagenesis procedures for the modification of each of the toxin genes. These mutants are currently being tested for biochemical activity. In addition, these gene mutants are being inserted into *B. subtilis* to produce larger quantities of these proteins and for vaccine testing.

FOREWORD

The investigators (Principal Investigator and Graduate Students) have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA Molecules (May, 1986). Supplemental guidelines pertaining to the subcloning of the individual *B. anthracis* toxin genes in sporulation competent *B. subtilis* was approved by the NIH committee on toxins March 13, 1986. All recombinant DNA research has also been registered with and approved by the Brigham Young University Institutional Biosafety Committee.

TABLE OF CONTENTS

SUMMARY OF RESEARCH	1
FOREWORD	3
TABLE OF CONTENTS	4
RESULTS	5
Isolation and characterization of the edema factor gene (cya)	5
Characterization of the LF gene (lef)	7
Mutagenesis of the anthrax toxin genes	8
Transcription start sites for the anthrax toxin genes	9
Expression of toxin genes in <i>B. subtilis</i> and <i>B. anthracis</i>	10
Isolation and characterization of pX01 and pX02	11
CONCLUSIONS	13
LITERATURE CITED	15
FIGURE 1. Restriction map of pX01	17
FIGURE 2. Restriction map of pX02	18
FIGURE 3. Restriction map of the PA and LF gene regions on pX01	19
FIGURE 4. Anthrax toxin signal peptides	20
APPENDIX I: Nucleotide Sequence of the EF gene	21
APPENDIX II. EF amino acid sequence	24
APPENDIX III. Homology Comparison between EF and pertussis cyclase	25
APPENDIX IV: Nucleotide Sequence of the LF gene	26
APPENDIX V. LF amino acid sequence	29
PUBLICATIONS	30
PERSONNEL	32
DISTRIBUTION LIST	33

RESULTS

Isolation and characterization of the edema factor gene (*cya*). The edema factor is a calmodulin-dependent adenylate cyclase (11,12). We have cloned and sequenced the EF gene (*cya*). The DNA sequence and deduced amino acid sequences (9) were reported in previous annual reports and are shown in Appendix I and II. A paper describing the cloning and expression of EF in *E. coli* has been published (8) and a manuscript describing the DNA sequence and its deduced amino acid sequence has been submitted and should soon be accepted by Gene (9).

Several interesting structural features for EF are part of its deduced amino acid sequence. (i) EF apparently contains a 33 amino acid signal peptide which conforms to known *Bacillus* leader sequences in that it starts with charged (mostly positive) and hydrophilic residues (amino acids 1-10), followed by a central core of hydrophobic amino acids (residues 11-23) and then several hydrophilic residues (amino acids 24-33) prior to the start of the mature protein. Proteolytic cleavage apparently occurs at an Ala-Met peptide bond, near the start of a proposed α -helix (see Figure 4A), consistent with signal processing after an Ala or Gly in bacilli (13). PA apparently contains a 29 amino acid leader sequence (6) and LF appears to contain a 33 amino acid leader (see below). Figure 4B shows a comparison between the amino acid sequences near the ends of the EF, PA and LF signal peptides and the apparent position of proteolytic cleavage. Similar amino acids at the ends of these signal peptides may be required for signal peptidase recognition or for secretion. (ii) A very strong *Bacillus* ribosome binding site immediately upstream from the start of the EF protein coding region is present (AAAGGAGGT) which is similar to the identical PA and LF ribosome binding sites (AAAGGAG). (iii) Amino acid residues 347 to 355 of the EF-precursor

protein contains the sequence Gly-x-x-x-x-Gly-Lys-Ser (where x=any amino acid) which is a perfect match to a consensus sequence present in prokaryotic and eukaryotic ATP and GTP binding proteins (14). The Lys residue is part of the ATP binding site of these proteins and appears to be part of the EF ATP binding site as well. That is, using site-specific mutagenesis procedures, we have replaced this Lys within EF with an Asn and cyclase activity was reduced 90-95% (unpublished data of author). (iv) We have also identified a domain in EF which could represent its putative calmodulin-binding site. As described in the EF sequencing paper (9), calmodulin-binding proteins often contain an α -helical region with charged or hydrophilic residues on one side and hydrophobic residues on the other. Such an amphiphilic helical region is present in EF located between amino acid residues 313-323 of the EF-precursor (see Appendix II). (v) No homology between the EF gene or its deduced EF amino acid sequence was observed with either the *E. coli* or yeast adenylate cyclases. However, there is at least three regions of homology in the amino acid sequence between EF and the *B. pertussis* calmodulin-dependent adenylate cyclase. The putative calmodulin-binding site, identified above, is conserved in the *B. pertussis* adenylate cyclase as well (15,16).

As mentioned above, we have also compared the EF amino acid sequence with the calmodulin-dependent adenylate cyclase of *Bordetella pertussis*, the causative agent of whooping cough. The pertussis cyclase appears to function independently of the pertussis toxin, but is a required virulence factor since strains which lack cyclase activity are avirulent (17). Glaser et al. (16) recently showed that the cyclase catalytic domain is about 450 amino acids in length and is part of a larger precursor polypeptide of 1706 amino acids. We performed a homology search between the entire EF (800 amino acids) and pertussis cyclase (1706 amino acids). Three major

regions of homology (labeled #1, #2 and #3 in Appendix III) were observed. These homologous domains are part of the catalytic domain of the pertussis cyclase and are located within the carboxyl terminal 500 amino acids of EF. Domain #1 contains the consensus ATP binding site which is surrounded by highly conserved amino acids. This high degree of amino acid conservation indicates a close evolutionary relatedness for these two proteins. The putative calmodulin-binding site is conserved for these proteins and is shown in Appendix II and III.

Characterization of the LF gene (*lef*). We have also cloned the *B. anthracis* LF gene (*lef*) and have determined its entire DNA sequence. We easily identified the start of the LF gene since the first 15 amino acids of the mature LF was previously determined by Dr. J. Schmidt (USAMRIID). The LF DNA sequence and the deduced amino acid sequence are shown in Appendix IV. The LF gene contains a good ribosome binding site (AAAGGAG) which is identical to the proposed PA gene ribosome binding site. The LF-precursor apparently contains a 33 amino acid signal sequence (see Figure 4A) which is removed during secretion. This signal sequence conforms to consensus *Bacillus* leader peptides (and to the EF and PA signal peptides) in that it starts with a polar or charged region followed by 23 non-polar, hydrophobic amino acid residues. After this 33 amino acid leader peptide, the next 16 amino acids correspond exactly to the LF amino acid sequence determined by Dr. Jim Schmidt (USAMRIID), except for one amino acid. Amino acid position +10 of the mature protein (+43 of LF-precursor) is a His (based on the DNA sequence) whereas it was previously reported to be a Lys (based on LF protein sequencing). Interestingly, there is a single Cys in the LF leader, although no Cys residues are in the mature protein. The entire protein sequence of LF is also shown in Appendix V.

There appears to be extensive amino acid homology between LF and EF in the first 300 amino acids of each protein. We have detected 10 closely related domains and three of these highly conserved domains are underlined (labelled #1, #2 and #3) in Appendix II and Appendix V. These homologous regions could represent domains which are required for association with PA prior to cellular uptake. Since these conserved domains in LF and EF are charged, interactions with PA may occur through a series of electrostatic interactions.

Mutagenesis of the anthrax toxin genes. Using site-specific mutagenesis procedures, we have altered the EF gene in order to modify its enzyme activity and to construct EF expression vectors. First, the previously identified ATP binding domain in EF, which conforms to the consensus ATP binding site of other prokaryotic and eukaryotic ATP and GTP binding proteins (14), has a Lys residue which is involved in ATP binding. This amino acid was changed to an Asn in EF. When this mutant EF was isolated from *E. coli*, adenylate cyclase activity was reduced about 90-95% indicating that this Lys is probably involved in ATP binding. However, since total activity was not abolished, other residues are probably also involved. Of particular interest, is the presence of a His two residues prior to this Lys. This His is also conserved in the *B. pertussis* adenylate cyclase (see the ATP binding domain in Appendix III).

We have also removed a *Bgl*III cleavage site within the EF gene and inserted a new *Bgl*III recognition site immediately prior to the start of the protein coding sequence. In another experiment, we inserted a *Bgl*III cleavage site immediately downstream from the PA promoter so that we could fuse the PA promoter to the EF gene. This hybrid toxin gene, when inserted into pBS42 (18) and transformed into *B. subtilis*, expressed EF at a level

at least as great as *B. anthracis* Sterne. We are in the process of determining the precise amount produced using an ELISA or Western blot. EF was secreted from *B. subtilis* and was enzymatically active in an adenylate cyclase assay. Since PA expression is regulated by bicarbonate (19) in *B. anthracis* (Dr. J. Bartkus, USAMRIID, personal communication), we are attempting to transfer this PA promoter-EF gene plasmid into *B. anthracis* by electroporation. Hopefully, this plasmid, when introduced into *B. anthracis*, will produce regulated high levels of EF for purification and analysis. EF gene mutants can also be generated and transferred to *B. anthracis* using this plasmid construction.

Several mutagenesis experiments have also been initiated with the PA gene. Since expression of PA in *B. anthracis* appears to be significantly greater than either LF or EF, we are fusing the PA promoter to both the EF and LF genes for higher levels of expression. In addition, we have started experiments to specifically alter PA. Specifically, we are mutating the Arg-Lys-Lys-Arg sequence (Dr. S. Leppla, USAMRIID, personal communication) in PA which is cleaved with a trypsin-like enzyme when bound to its cellular receptor. After cleavage, the amino terminal 20,000 daltons of PA is removed and PA can now bind either LF or EF. Therefore, by preventing cleavage, LF or EF will not bind and cannot enter the cell. We will alter the amino acids at this location in PA to examine specificity of cleavage and to substitute amino acids which could prevent cleavage. These alterations should also prevent the binding of LF or EF and make these toxin components essentially inactive.

Transcription start sites for the anthrax toxin genes. We have used radiolabeled oligonucleotides, specific for each of the different toxin genes, to determine the start site for toxin gene transcription. Using

mRNA (isolated from *B. anthracis* Sterne) as template, each oligonucleotide was used to prime DNA synthesis (using reverse transcriptase) towards the 5'-end of the respective toxin mRNA. This newly synthesized radioactive DNA was denatured and electrophoresed on a denaturing polyacrylamide gel. Using this approach, we have successfully identified the start sites for PA and LF gene transcription. The PA promoter is apparently located immediately upstream from the start of its coding region with transcription starting about 25 bases before the first start codon for PA translation (6). Likewise, the apparent start for LF gene transcription occurs 25 bases prior to the ATG start codon for LF translation (about nucleotide 456 in Appendix IV). We have not yet been able to localize EF gene transcription. This failure is probably due to the low level of EF mRNA produced in *B. anthracis* which is at least 10-fold lower than either the PA or LF mRNA concentrations (unpublished data of author).

Expression of toxin genes in *B. subtilis* and *B. anthracis*. In an effort to express the anthrax toxin genes in *B. subtilis*, we have cloned each of the toxin genes into *B. subtilis* expression plasmids. Initially, we fused these genes to a regulated promoter and a good ribosome binding site which is present in pSI-1 (20). Using site-specific mutagenesis procedures, we have introduced new *Xba*I recognition sites immediately before the start codons for the PA, EF and LF genes. Following cleavage with *Xba*I, each of the toxin genes was ligated into plasmid pSI-1. When transformed into *B. subtilis*, transcription of the inserted toxin genes is regulated by the *lac* repressor and IPTG (18,20). For example, the amount of PA produced by this fusion was close the expression of PA from PA1 (21).

We have also constructed a plasmid using the T7 promoter cloned upstream from the toxin gene. We cloned the T7 RNA polymerase gene (22) into pSI-1

so that transcription would be controlled by the *lac* promoter, which is inducible with IPTG. Part of this recombinant plasmid which contained the T7 polymerase gene and the erythromycin resistance gene from pE194, was integrated into *B. subtilis* genomic DNA (23,24). *B. subtilis* with this DNA should express T7 RNA polymerase after the addition of IPTG. These cells can then be transformed with a replication competent plasmid containing one of the *B. anthracis* toxin genes (e.g., *cya*, *pag*, or *lef*) cloned downstream from the T7 promoter for gene expression. Although we have not yet tested these recombinants in *B. subtilis*, plasmids containing the toxin genes express toxin in *E. coli* using the T7 polymerase (21). *B. subtilis* containing these plasmids should produce high level, regulated expression of the toxin genes in a safe bacterial host. Toxin is secreted from *B. subtilis* and can be used for purification of individual toxin components.

Isolation and characterization of pXO1 and pXO2. We have developed an efficient plasmid isolation procedure to isolate pure supercoiled pXO1 and pXO2 DNA. This procedure involves chromatography using NACS-37 resins and effectively separates small amounts of genomic DNA from plasmid (10). Our purification protocol does not use CsCl bouyant density gradients since these large plasmids are easily sheared, converting them from supercoiled to relaxed or linear DNA. A typical yield of pXO1 from a one liter culture of *B. anthracis* was about 200 μ g, which is close to the maximum amount of DNA expected per liter of culture if these plasmids were present as single copies within *B. anthracis* cells.

Using pure pXO1 and pXO2, we characterized these DNAs using thermal denaturation and bouyant density procedures. Using a T_m analysis, the melting temperatures for pXO1 and pXO2 were $82.5^\circ\text{C} \pm 0.3^\circ\text{C}$ and $82.2^\circ\text{C} \pm 0.3^\circ\text{C}$, respectively. These values correspond to GC contents of 32.2% for

pXO1 and 31.5% for pXO2. Similar experiments using CsCl banding gave GC-contents of 31.1% for pXO1 and 31.4% for pXO2. These values are close to the GC% of *B. anthracis* genomic DNA which is 32.2%.

The restriction maps for pXO1 and pXO2 have been determined for several enzymes which cleave a few times, such as *Pst*I, *Bam*HI, *Cla*I, *Sst*I, *Bgl*III and *Pvu*II (Figures 1 and 2). Experiments to map the more frequent cutting enzymes, such as *Eco*RI and *Hind*III, are presently being completed. We have generated recombinant DNA libraries for pXO1 and pXO2 in bacteriophage λ as well as in plasmids in order to generate a complete map for the most common restriction enzymes. A detailed restriction enzyme map of the LF and PA gene regions on pXO1 is also shown in Figure 3.

In a final effort to generate a complete gene map of pXO1 and pXO2, we are identifying the number and location of the different RNA transcripts from these plasmids. This project involves the identification of the different promoters and the RNAs made from them. Basically, we are cleaving pXO1 and pXO2 with an enzyme which cleaves these DNAs many times, such as *Mbo*I or *Sau*3A, generating DNA fragments which can ligate to *Bam*HI cleaved plasmids. Using *B. subtilis* plasmids which have been cleaved with *Bam*HI located prior to a promoterless chloramphenicol resistance gene (25), we will insert the pXO1 or pXO2 DNA fragments into these promoter identification plasmids. After transformation of these recombinant plasmids into *B. subtilis*, we will identify bacteria which are now resistant to chloramphenicol. These plasmids will contain a functional promoter (derived from pXO1 or pXO2) driving the transcription of the chloramphenicol resistance gene. The recombinant DNA inserts prepared from these promoter expression plasmids will then be mapped on pXO1 or pXO2. The size and direction of RNA transcription will also be determined. This procedure is very powerful and should allow us to

identify and position most, if not all, of the functional promoters from the *B. anthracis* plasmids, assuming that all these promoters will also function in *B. subtilis*. However, with the recent discovery that we can transform *B. anthracis* using electroporation, we will also be able to transfer these promoter plasmids to *B. anthracis* for promoter identification directly in the parent organism.

CONCLUSIONS

It appears from the data described in this report, that most of the experiments outlined in the original research proposal are essentially completed. (i) The anthrax toxin genes are each cloned. (ii) Each of the toxin genes have also been sequenced. We will be able to study gene expression and to characterize the toxin proteins better. (iii) We can generate toxin gene mutants for the construction of a safe vaccine and to elucidate the biochemical activities of these proteins. (iv) We have expressed the anthrax toxin genes in *E. coli* and *B. subtilis* and have constructed expression vectors, especially for *B. subtilis* and *B. anthracis*, which should allow for high level expression of the toxin proteins for biochemical and immunological purposes. (v) We have determined homology between EF and the pertussis calmodulin-dependent adenylate cyclase which should allow us to better characterize EF based on conserved domains. In addition homology between LF and EF should allow us to examine the interaction between these proteins and PA. (vi) We have not yet placed mutant toxin genes back into *B. anthracis*, although the wild-type PA and EF genes have been transferred. Overall, our research has allowed us to characterize the anthrax toxin genes and to construct important gene mutants. This research is absolutely

required for the construction of a safe recombinant DNA derived anthrax vaccine.

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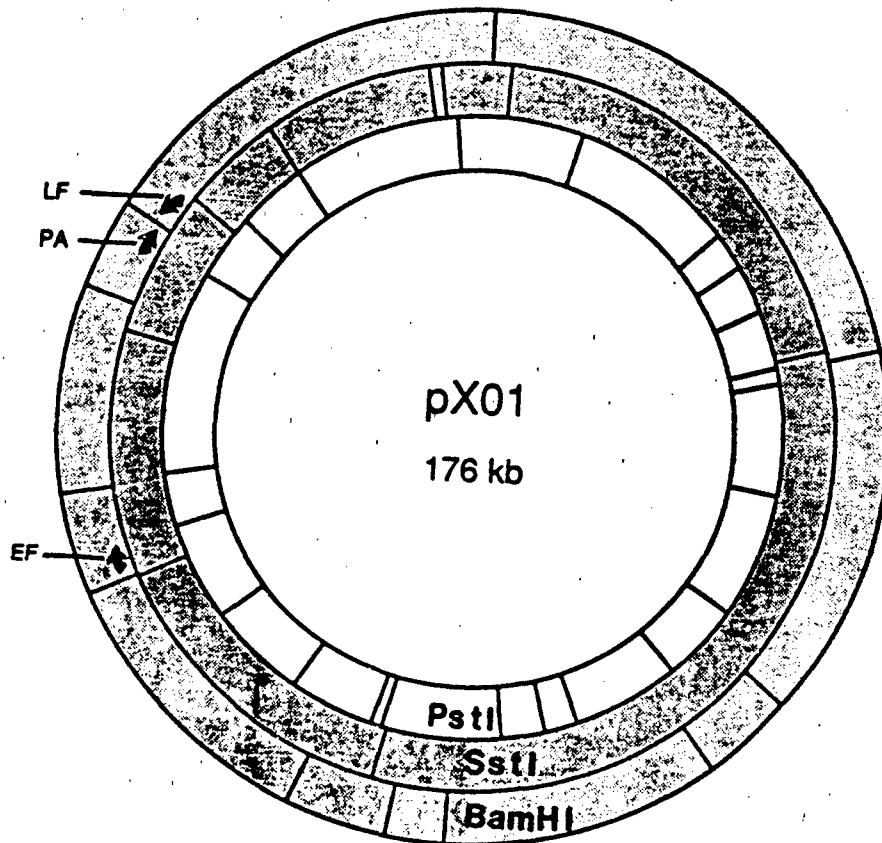


FIGURE 1. Restriction map of pX01. The positions of the LF, PA and EF genes are depicted. The sizes of DNA fragments for each enzyme are not included due to the lack of space.

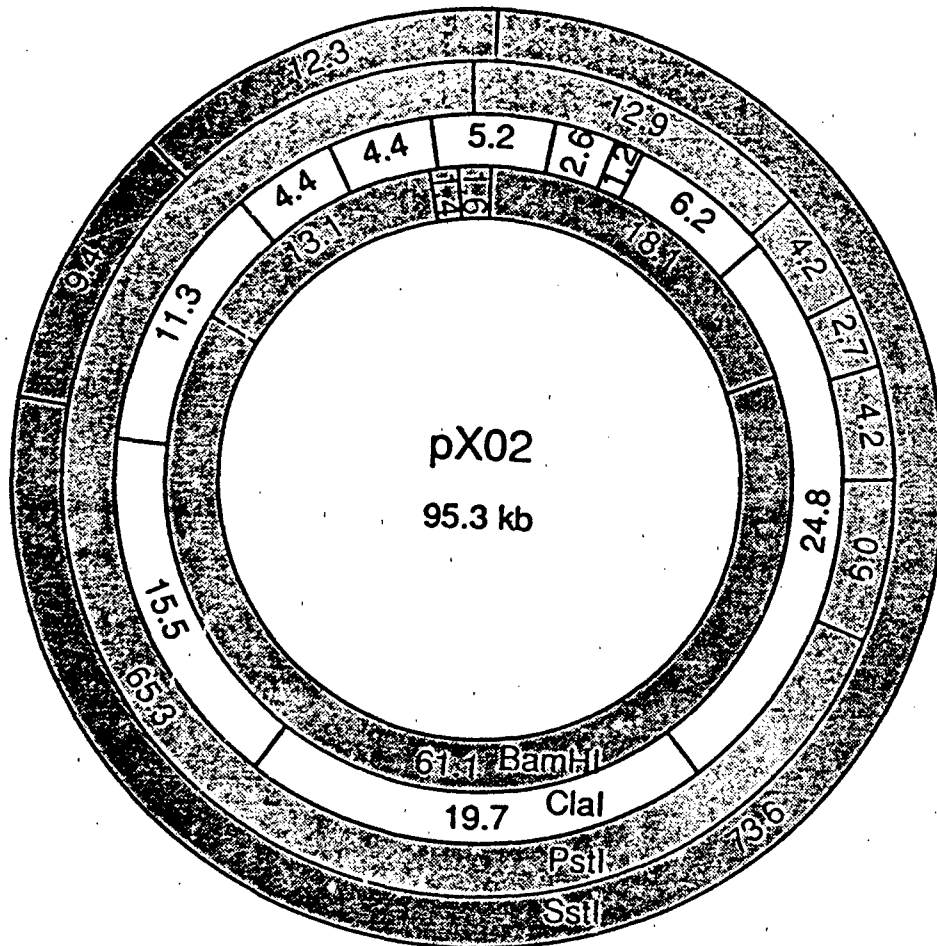
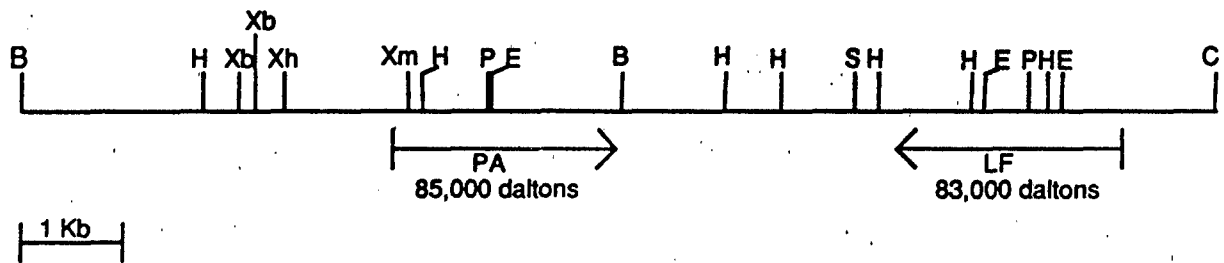


FIGURE 2. Restriction map of pX02.

PA and LF gene regions of pXO1



B - *Bam* HI
 H - *Hin* dIII
 Xb - *Xba* I
 Xh - *Xho* I
 Xm - *Xmn* I
 P - *Pst* I
 E - *Eco* RI
 S - *Sst* I
 C - *Cla* I

FIGURE 3. Restriction map of the PA and LF gene regions on pXO1.

APPENDIX I: Nucleotide Sequence of the EF gene.

10 20 30 40 50 60 70 80 90
 TTA C T T T T T T A T A T A C T G A A T T A A A A A G T C C A A G C A C T T A T A T C G T A A T A G A T G C T T T C T A T T G A C C T T A T A G T C C T T G A A G T T A C G A C T

100 110 120 130 140 150 160 170 180
 G A U C A A T T A T G A G A C G T T T G C G C T A A C C T G C T G A A T T C A A A A T C C G A C T T A G A A A T A C A C A T A T A G A A A T A A A C A A C C T A A T C C A T G T C A

190 200 210 220 230 240 250 260 270
 C T G T A C C G T T T T T T A C T A A A T A A A C G A A A T C A G T G T A A A A A T G A A C A G C T G A A C T T T A T C A A C T T A G A A T C T C T T T T T T A C T T T A A A T

280 290 300 310 320 330 340 350 360
 G C C T A G C T G T T T T T T C T A A T G T T T G T A T T T C T A A A T A T A T T T A A A T A T G A A T T G T A G C T G T G C C A A G A G T T A T A A T T A A T T T A A A T A A
 -35 (putative promoter site) -10

370 380 390 400 410 420 430 440 450
 G A T T A T A T T T G T A A A T A A A A T T G L A A T T T A A C A T G T A G A A T A A A G A G A T T T T T A G T T T T A T T A A C A G A G A T G A A A A T C C A T A A A A C C G T A A

460 470 480 490 500 510 520 530 540
 A T G T G A T T T C T A A A T T A G T T T A A A A T A A A A A C A A G G A T T T G C T C A G A C T T G A G A T G A A T A T C T A A A T A T C A A G A A C C A A A G G A G G T T T A
 ribosome binding site

+1 550 560 570 580 590 600 610 620 630
 A G A T G A C T A G A A A T A A A T T T A T A C C T A A T A A G T T T A G T A T T A T A T C C T T T C A G T A T T A C T A T T T G C T A T A T C C T C C T C A C A G C T A T A
 Met Thr Arg Asn Lys Phe Ile Pro Asn Lys Phe Ser Ile Ile Ser Phe Ser Val Leu Leu Phe Ala Ile Ser Ser Ser Gln Ala Ile
 33 amino acid leader sequence

640 650 660 670 680 690 700 710 720
 G A A G T A A A T G C T A T G A A T G A A C A T T A C A C T G A G A G T G A T A T T A A A A G A A A C C A T A A A A C T G A A A A A A T A A A A C T G A A A A G A A A A A T T T
 Glu Val Asn Ala Met Asn Glu His Tyr Thr Glu Ser Asn Ile Lys Arg Asn His Lys Thr Glu Lys Asn Lys Thr Glu Lys Glu Lys Phe
 1st amino acid of EF

730 740 750 760 770 780 790 800 810
 A A A G A C A G T A T T A A T A A C T T A G T T A A A C A G A A T T T A C C A A T G A A A C T T T A G A T A A A T A C A G C A G A C A C A A G A C T T A T T A A A A A G A T A
 Lys Asp Ser Ile Asn Asn Leu Val Lys Thr Glu Phe Thr Asn Glu Thr Leu Asp Lys Ile Gln Gln Thr Gln Asp Leu Leu Lys Lys Ile

820 830 840 850 860 870 880 890 900
 C C T A A G C A T G T A C T T G A A A T T T A T A G T G A A T T A G G A G G A G A A A T C T A T T T T A C A G A T A T A G A T T T A G T A G A A C A T A A C G A G T T A C A A G A T
 Pro Lys Asp Val Leu Glu Ile Tyr Ser Glu Leu Gly Gly Glu Ile Tyr Phe Thr Asp Ile Asp Leu Val Glu His Lys Glu Leu Gln Asp

910 920 930 940 950 960 970 980 990
 T T A A G T G A A G A G A G A A A A T A G T A T G A A T A G T A G A G G T G A A A A G T T C C G T T T G C A T C C G T T T T G T A T T T G A A A A G A A A A G G G A A A C A
 Leu Ser Glu Glu Glu Lys Asn Ser Met Asn Ser Arg Gly Glu Lys Val Pro Phe Ala Ser Arg Phe Val Phe Glu Lys Lys Arg Glu Thr

1000 1010 1020 1030 1040 1050 1060 1070 1080
 C C T A A A T T A A T T A A A T A T G A A A G A T T A T G C A A T T A A T A G T G A A G A A A G T A A A G A G T A T A T T A T G A A A T T G G A A A G C C G A T T T C T C T T
 Pro Lys Leu Ile Ile Asn Ile Lys Asp Tyr Ala Ile Asn Ser Glu Gln Ser Lys Glu Val Tyr Tyr Glu Ile Gly Lys Gly Ile Ser Leu

1090 1100 1110 1120 1130 1140 1150 1160 1170
 G A T A T T A A G T A A G G A T A A A T C T C T A G A T C C A G A G T T T T T A A A T T A A T T A A G A G A T T T A A G C G A T G A T A G T G A T A G T A G C G A C C T T T T A
 Asp Ile Ile Ser Lys Asp Lys Ser Leu Asp Pro Glu Phe Leu Asn Leu Ile Lys Ser Leu Ser Asp Asp Ser Asp Ser Ser Asp Leu Leu

1180 1190 1200 1210 1220 1230 1240 1250 1260
 TTTAGTCAAAAATTTAAAGAGAAGCTAGAATTGAATAATAAAAGTATAGATATAAATTTTATAAAAAGAAAATTTAACTGAATTTTCAGCAT
 PheSerGlnLysPheLysGluLysLeuGluLeuAsnAsnLysSerIleAspIleAsnPheIleLysGluAsnLeuThrGluPheGlnHis

1270 1280 1290 1300 1310 1320 1330 1340 1350
 GCGTTTTCTTTAGCGTTTTCTTATTATTTTGCACCTGACCATAGAAOGGTATTAGAGTTATATGCCCCCGACATGTTTGAGTATATGAAT
 AlaPheSerLeuAlaPheSerTyrTyrPheAlaProAspHisArgThrValLeuGluLeuTyrAlaProAspMetPheGluTyrMetAsn

1360 1370 1380 1390 1400 1410 1420 1430 1440
 AAGTTAGAAAAAGGGGATTGAGAAAATAAGTGAAGTTTGAAGAAAGAAGGTGTGGAAAAAGATAGGATTGATGTGCTGAAAGGAGAA
 LysLeuGluLysGlyGlyPheGluLysIleSerGluSerLeuLysLysGluGlyValGluLysAspArgIleAspValLeuLysGlyGlu

1450 1460 1470 1480 1490 1500 1510 1520 1530
 AAAGCACTTAAAGCTTCAGGTTTAGTACCAGAACATGCAGATGCTTTTAAAAAATGCTAGAGAATTAATACATATATCTTTTTAGG
 LysAlaLeuLysAlaSerGlyLeuValProGluHisAlaAspAlaPheLysLysIleAlaArgGluLeuAsnThrTyrIleLeuPheArg

1540 1550 1560 1570 1580 1590 1600 1610 1620
 CCTGTTAATAAGTTAGCTACAAACCTTATTAAGTGGTGTGGCTACAAAGGATTGAATGAACATGGAAAGAGTTGGGATGGGGCCCT
 ProValAsnLysLeuAlaIhrAsnLeuIleLysSerGlyValAlaThrLysGlyLeuAsnGluHisGlyLysSerSerAspTrpGlyPro

1630 1640 1650 1660 1670 1680 1690 1700 1710
 GTAGCTGGATACATACCATTTCATCAAGATTTATCTAAGAAGCATGGTCAACAATTAGCTGTCTAGAAAAGAAAATTTAGAAAATAAAAA
 ValAlaGlyTyrIleProPheAspGlnAspLeuSerLysLysHisGlyGlnGlnLeuAlaValGluLysGlyAsnLeuGluAsnLysLys

1720 1730 1740 1750 1760 1770 1780 1790 1800
 TCAATTACAGAGCATGAAGGTGAAATAGGTAATAACCATTAAAGTTAGACCATTAAAGAATAGAAGAGTTAAAGGAAAATGGGATAATT
 SerIleThrGluHisGluGlyGluIleGlyLysIleProLeuLysLeuAspHisLeuArgIleGluGluLeuLysGluAsnGlyIleIle

1810 1820 1830 1840 1850 1860 1870 1880 1890
 TTGAAGGGTAAAAAGAAAATGATAATGGTAAAAATATTATTGTTAGAAATOGAAATAATCAGGTATATGAATTTAGAATTAGCGATGAA
 LeuLysGlyLysLysGluIleAspAsnGlyLysLysTyrTyrLeuLeuGluSerAsnAsnGlnValTyrGluPheArgIleSerAspGlu

1900 1910 1920 1930 1940 1950 1960 1970 1980
 AACAAAGAAAGTACAATACAAGACAAAAGAAGGTAAATTAAGTGTGTTTAGGGGAAAAATTCAATTGGAGAAATATAGAAGTGATGGCTAAA
 AsnAsnGluValGlnTyrLysThrLysGluGlyLysIleThrValLeuGlyGluLysPheAsnTrpArgAsnIleGluValMetAlaLys

1990 2000 2010 2020 2030 2040 2050 2060 2070
 AATGTAGAAGGGTCTTGAAGCCGTTAACAGCTGACTATGATTTATTTGCACTTGCCCAAGTTTAAACAGAAAATAAAAAACAAATACCC
 AsnValGluGlyValLeuLysProLeuThrAlaAspTyrAspLeuPheAlaLeuAlaProSerLeuThrGluIleLysLysGlnIlePro

2080 2090 2100 2110 2120 2130 2140 2150 2160
 ACAAAAGAATGGATAAAGTAGTTAACACCCCAAATTCATTAGAAAAGCAAAAAGGTGTTACTAATTTATTGATTAAATATGGAATTGAG
 ThrLysArgMetAspLysValValAsnThrProAsnSerLeuGluLysGlnLysGlyValThrAsnLeuLeuIleLysTyrGlyIleGlu

2170 2180 2190 2200 2210 2220 2230 2240 2250
 AGGAAACCGGATTCAACTAAGGGAAGTTTATCAAATGGCAAAAACAAATGCTTGATCGTTTGAATGAAGCAGTCAAATATACAGGATAT
 ArgLysProAspSerThrLysGlyThrLeuSerAsnTrpGlnLysGlnMetLeuAspArgLeuAsnGluAlaValLysTyrThrGlyTyr

2260 2270 2280 2290 2300 2310 2320 2330 2340
 ACAGGGGGGATGTGGTTAACCATGCACAGCAAGATAATCAAGAGTTTCCTGAAAAAGATAACGAAATTTTATAATTAATCCAGAA
 ThrGlyGlyAspValValAsnHisGlyThrGluGlnAspAsnGluGluPheProGluLysAspAsnGluIlePheIleIleAsnProGlu

2350 2360 2370 2380 2390 2400 2410 2420 2430
 GGTGAATTTATATTAATACTAAAAATTGGGAGATGACAGGTAGATTTATAGAAAAAACATTACGGGAAAAGATTATTTATATTATTTTAAC
 GlyGluPheIleLeuThrLysAsnTrpGluMetThrGlyArgPheIleGluLysAsnIleThrGlyLysAspTyrLeuTyrTyrPheAsn

2440 2450 2460 2470 2480 2490 2500 2510 2520
 CGTTCITATAATAAAATAGCTCCTCGTAATAAAGCTTATATTGAGTGGACTGATCCGATTACAAAAGCCAAAATAAATACCATCCCTACG
 ArgSerTyrAsnLysIleAlaProGlyAsnLysAlaTyrIleGluTrpThrAspProIleThrLysAlaLysIleAsnThrIleProThr

2530 2540 2550 2560 2570 2580 2590 2600 2610
 TCAGCAGAGTTTATAAAAACTTATCCAGTATCAGAAGATCTTCAAATGTAGGAGTTTATAAAGATAGTGGCGACAAAGACGAATTTGCA
 SerAlaGluPheIleLysAsnLeuSerSerIleArgArgSerSerAsnValGlyValTyrLysAspSerGlyAspLysAspGluPheAla

2620 2630 2640 2650 2660 2670 2680 2690 2700
 AAAAAAGAAACCGTGAAAAAATGTCAGGATATTTGTCAGACTATTACAATTCACAAAATCATATTTTTCTCAGGAAAAAAGCGTAAA
 LysLysGluSerValLysLysIleAlaGlyTyrLeuSerAspTyrTyrAsnSerAlaAsnHisIlePheSerGlnGluLysLysArgLys

2710 2720 2730 2740 2750 2760 2770 2780 2790
 ATATCAATATTTTCGTGCAATCCAAGCCTATAATGAAATTGAAAATGTTCTAAAATCTAAACAAATAGCACCAGAATACAAAAATTATTTT
 IleSerIlePheArgGlyIleGlnAlaTyrAsnGluIleGluAsnValLeuLysSerLysGlnIleAlaProGluTyrLysAsnTyrPhe

2800 2810 2820 2830 2840 2850 2860 2870 2880
 CAATATTTAAAGGAAAGGATTACCAATCAAGTTCAATTGCTTCTAACACATCAAAAATCTAATATTGAATTTAAATATTGTATAAACAA
 GlnTyrLeuLysGluArgIleThrAsnGlnValGlnLeuLeuLeuThrHisGlnLysSerAsnIleGluPheLysLeuLeuTyrLysGln

2890 2900 2910 2920 2930 2940 2950 2960 2970
 TTAAACTTTACAGAAAATGAAACGGATAATTTTGAGGTCTTCCAAAAAATTATTGATGAAAAATAAATATATATAATTTGTTTTCTGAAA
 LeuAsnPheThrGluAsnGluThrAspAsnPheGluValPheGlnLysIleIleAspGluLys

2980 2990 3000 3010 3020 3030 3040 3050 3060
 ATTCATCATTTTAAAGAAGACACTAGGAATTAATAGATGTATTGAATAGTTATAGTAATGGTCTTGTATGCACATACCGCTTATACTTT

APPENDIX III. Homology Comparison between EF and pertussis cyclase.

	Calmodulin Site	ATP binding Site
	←-----→	* **
289	EKDRIDVLKGEKALKASGLVPEHADAFKCIARELNITYILFRPVNKLATNLIKSGVATKGLNEHCKSSDWGPFVAGYIPFDQDLSKHKQQQL	
1	MQQSHQAGYANAADRESGLPAAVLDGKAVAKEKNATLMFRLVNPSTSLIAEGVATKGLGVHAKSSDWGLQAGYIPVNPNSKLFGRAP	
		←-----Domain #1-----→
379	AVEKGNLENKKSITTEHEGEIGKIPL K LDIHRIEELKENGILKCKKEIDNGKCYLLESNNQVYFRISDENNEVQYCKEKKITVL	
91	EVIARADNDVNSSLAHGHTAVDLTILSKERLDYLROAGL VTG MADG WASNHAGYEQFE FRVKE TSDGRYAVQYRRK G	
	←Domain #2→	
466	GEKFNWRNIEVMAKNVEGVLKPLTADYDLFALAP SLTEIKKQIPIKRMKV VNT PMSLEKQGVINLLI KYGIER KPDST	
168	GDDF EAVKV IGNAAG IPLTADIDMFAIMPHLSNFRDSARSSVTSGDSVIDYLARTIRRAASEATGGLDRERIDLLKCIARAGARSA	
		←-----Domain #3-----→
546	KGILSNQ KQM LDRINE AVKYTYGTCG DVNNGTEQDNEEFPEKNEIFINPEGE FILTKNEMITGRFIERNIT	
253	VGTEARRQFRYDGMNIGVITDFELEVNRNALNRRHAVGAQDVVQHGTEQNN PFPEADEKIFVWSATGESQMLTRQQ IKEYICQQ R	
621	GKDYLYYFNRSYNKLAFGNKAYIEWDP IYKAKINTIPTSAEFIKRLSSIRRSSNUGVYKDSGKLEFAKESVKKIAGYLSDYNSA	
339	GEYVYFENRAYGVACKSLFDDGLGAAPGVFSQRSKFSFDVLETVPASPLRRPSLGAVERQDSG YDSLGDVGSRSFSLGEVSD MAA	
709	NHLFSQEKKKKLSIFRGIQAYNEIENVLKSKQIAPEMKNYFYLKERTINQVQLLTHQKSNIEFKLLKQLNFTENEIDNFEVFKLIDEK	
426	VEAAELEMIRQVLHACARQDDAE FGV SGASAHWQRALQ GAQAVAAQRLVHALALMTQFGRAGSINTIPEAASLSAAVFGLEASS	

1. Domains #1, #2 and #3 represent three highly conserved amino acid domains in EF (top line of each pair) and the pertussis cyclase (bottom line in each pair).
2. The numbers to the left of each line indicates the amino acid position for EF-precursor or the pertussis cyclase.
3. The asterisks (*) indicate the consensus sequences for the ATP binding site for EF and the pertussis cyclase.

1180 1190 1200 1210 1220 1230 1240 1250 1260
 CCCACAGACTTTTCTGTAGAATTCTTGGAAACAAAATAGCAATGAGGTACAAGAAGTATTTGCGAAAGCTTTTGCATATTATATCGAGCCA
 ProThrAspPheSerValGluPheLeuGluGlnAsnSerAsnGluValGlnGluValPheAlaLysAlaPheAlaTyrTyrIleGluPro

1270 1280 1290 1300 1310 1320 1330 1340 1350
 CAGCATCGTGATGTTTTACAGCTTTATGCACCGGAAGCTTTTTAATTACATGGATAAATTTAACGAACAAGAAATAAATCTATCCTTGGAA
 GlnHisArgAspValLeuGlnLeuTyrAlaProGluAlaPheAsnTyrMetAspLysPheAsnGluGlnGluIleAsnLeuSerLeuGlu

1360 1370 1380 1390 1400 1410 1420 1430 1440
 GAACTTAAAGATCAACGGATGCTGTCAAGATATGAAAAATGGAAAAGATAAAACAGCACTATCAACACTGGAGCGGATTTTATCTGAA
 GluLeuLysAspGlnArgMetLeuSerArgTyrGluLysTrpGluLysIleLysGlnHisTyrGlnHisTrpSerAspSerLeuSerGlu

1450 1460 1470 1480 1490 1500 1510 1520 1530
 GAAGGAAGAGCACTTTTAAAAAGCTGCAGATTCCCTATTGAGCCAAAGAAAGATGACATAATTCATTCTTTATCTCAAGAAGAAAAAGAG
 GluGlyArgGlyLeuLeuLysLysLeuGlnIleProIleGluProLysLysAspAspIleIleHisSerLeuSerGlnGluGluLysGlu

1540 1550 1560 1570 1580 1590 1600 1610 1620
 CTTCTAAAAAGAATACAAATTGATAGTAGTGATTTTTTATCTACTGAGGAAAAAGAGTTTTTAAAAAGCTACAAATTGATATTCGTGAT
 LeuLeuLysArgIleGlnIleAspSerSerAspPheLeuSerThrGluGluLysGluPheLeuLysLysLeuGlnIleAspIleArgAsp

1630 1640 1650 1660 1670 1680 1690 1700 1710
 TCTTATCTGAAGAAGAAAAAGAGCTTTTAAATAGAATACAGGTGGATAGTAGTAATCCTTTATCTGAAAAAGAAAAAGAGTTTTTAAAA
 SerLeuSerGluGluGluLysGluLeuLeuAsnArgIleGlnValAspSerSerAsnProLeuSerGluLysGluLysGluPheLeuLys

1720 1730 1740 1750 1760 1770 1780 1790 1800
 AAGCTGAAACTTGATATTCAACCATATGATATTAATCAAAGTTGCAAGATACAGGAGGTTAATTGATAGTCCGTCAATTAATCTTGAT
 LysLeuLysLeuAspIleGlnProTyrAspIleAsnGlnArgLeuGlnAspThrGlyGlyLeuIleAspSerProSerIleAsnLeuAsp

1810 1820 1830 1840 1850 1860 1870 1880 1890
 GTAAGAAACAGTATAAAAAGGATATTCAAAATATTGATGCTTTATTACATCAATCCATTGGAAGTACCTTGTACAATAAAAATTATTTC
 ValArgLysGlnTyrLysArgAspIleGlnAsnIleAspAlaLeuLeuHisGlnSerIleGlySerThrLeuTyrAsnLysIleTyrLeu

1900 1910 1920 1930 1940 1950 1960 1970 1980
 TATGAAAATATGAATATCAATAACCTTACAGCAACCTTAGCTGCGGATTTAGTTGATTCCTACTGATAATACTAAAAATTAATAGAGGTATT
 TyrGluAsnMetAsnIleAsnAsnLeuThrAlaThrLeuGlyAlaAspLeuValAspSerThrAspAsnThrLysIleAsnArgGlyIle

1990 2000 2010 2020 2030 2040 2050 2060 2070
 TTCAATGAATTCAAAAAAATTTCAAAATATAGTATTTCTAGTAACTATATGATTGTTGATATAAATGAAAGGCCTGCATTAGATAATGAG
 PheAsnGluPheLysLysAsnPheLysTyrSerIleSerSerAsnTyrMetIleValAspIleAsnGluArgProAlaLeuAspAsnGlu

2080 2090 2100 2110 2120 2130 2140 2150 2160
 OGTTTGAATGGAGAATCCAATTATCACCAGATACTCGAGCAGGATATTTAGAAAAATGAAAGCTTATATTACAAAGAAACATCGGCTCG
 ArgLeuLysTrpArgIleGlnLeuSerProAspThrArgAlaGlyTyrLeuGluAsnGlyLysLeuIleLeuGlnArgAsnIleGlyLeu

2170 2180 2190 2200 2210 2220 2230 2240 2250
 GAAATAAAGGATGTACAAATAATTAAGCAATCGAAAAAGAATATATAAGGATTGATGCGAAAGTAGTGCCAAAGAGTAAAAATAGATACA
 GluIleLysAspValGlnIleIleLysGlnSerGluLysGluTyrIleArgIleAspAlaLysValValProLysSerLysIleAspThr

2260 2270 2280 2290 2300 2310 2320 2330 2340
 AAAATTCAAGAAGCACAGTTAAATATAAATCAGGAATGGAATAAAGCATTAGGGTTACCAAAATATACAAAGCTTATTACATTCAACGTG
 LysIleGlnGluAlaGlnLeuAsnIleAsnGlnGluTrpAsnLysAlaLeuGlyLeuProLysTyrThrLysLeuIleThrPheAsnVal

2350 2360 2370 2380 2390 2400 2410 2420 2430
 CATAATAGATATGCATCCAATATTGTAGAAAGTGCTTATTTAATATTGAATGAATGGAAAAATAATATTCAAAGTGATCTTATAAAAAAG
 HisAsnArgTyrAlaSerAsnIleValGluSerAlaTyrLeuIleLeuAsnGluTrpLysAsnAsnIleGlnSerAspLeuIleLysLys

 2440 2450 2460 2470 2480 2490 2500 2510 2520
 GTAACAAATTACTTAGTTGATGGTAATGGAAGATTTGTTTTTACOGATATTACTCTCCCTAATATAGCTGAACAATATACACATCAAGAT
 ValThrAsnTyrLeuValAspGlyAsnGlyArgPheValPheThrAspIleThrLeuProAsnIleAlaGluGlnTyrThrHisGlnAsp

 2530 2540 2550 2560 2570 2580 2590 2600 2610
 GAGATATATGAGCAAGTTCATTCAAAGGGTTATATGTGCCAGAATCCCGTTCTATATTACTCCATGCACCTTCAAAGGTGAGTAATTA
 GluIleTyrGluGlnValHisSerLysGlyLeuTyrValProGluSerArgSerIleLeuLeuHisGlyProSerLysGlyValGluLeu

 2620 2630 2640 2650 2660 2670 2680 2690 2700
 ACGAATGATAGTCAGGGTTTTATACACGAATTTGGACATGCTGTGGATGATTATGCTGGATATCTATTAGATAAGAACCAATCTGATTTA
 ArgAsnAspSerGluGlyPheIleHisGluPheGlyHisAlaValAspAspTyrAlaGlyTyrLeuLeuAspLysAsnGlnSerAspLeu

 2710 2720 2730 2740 2750 2760 2770 2780 2790
 GTTACAAATTCTAAAAAATTCATTGATATTTTAAAGGAAGAAGGAGTAATTTAACTTCTGATGGGAGAACAATGAAGCGGAATTTTTT
 ValThrAsnSerLysLysPheIleAspIlePheLysGluGluGlySerAsnLeuThrSerTyrGlyArgThrAsnGluAlaGluPhePhe

 2800 2810 2820 2830 2840 2850 2860 2870 2880
 GCAGAAGCCTTTAGGTTAATGCATTCTACGGACCATGCTGAACGTTTAAAAGTTCAAAAAATGCTCCGAAAACCTTCCAATTTATTAAC
 AlaGluAlaPheArgLeuMetHisSerThrAspHisAlaGluArgLeuLysValGlnLysAsnAlaProLysThrPheGlnPheIleAsn

 2890 2900 2910 2920 2930 2940 2950 2960 2970
 GATCAGATTAAGTTCATTATTAACTCATAAGTAATGTATTAATAATTTTCAAATGGATTTAATAATAATAATAATAATAATAACGGG
 AspGlnIleLysPheIleIleAsnSer

 2980 2990 3000 3010 3020 3030 3040 3050 3060
 ACCAGCCATTATGAAGCAACTAATTCTAGACTTGATAGTAATTCCTGGGAAGCACCCAGATAGTGTAAAAGGTGCCATTGCCAGAATGATA

 3070 3080 3090 3100 3110 3120 3130 3140 3150
 TTTTATGTGTTTCGTAGATATGAAGGCAAAAACAATGATCCTGACCTAGAACTTAATGATAATGTTATTATAATTTAATGCCTTTTATA

 3160 3170 3180 3190 3200 3210 3220 3230 3240
 GCAATATTAGTAAAAGTCCGAAAAGATCCTGTTGCAAAGCTTTTAAAGAACATATTATTCTATCAAGTGGCTGTATATTTTGTGTAATT

 3250 3260 3270 3280 3290
 TTCAATAAATTTTGTAAATTAAGCATAAGTCAAAAAACCGAAATCTCAGCTC

SstI

APPENDIX V. LF amino acid sequence

(29 aa signal peptide) ↓-Start of mature LF (780 aa)

1 MNIKKEFIKVISMSCLVTAITLSGPFVFIPLVQGAGGHGDVGMHVKEKEKNKDKENKRRKDEERNKTQEEHLK

71 EIMKHIVKIEVKGEEAVKKEAAEKLEKVPDVL EMYKAIGGKIYIVDGDITKHISLEALSEDKKKIKDI

141 YGKDALLHEHYVYAKEGYEPVLVIQSS EDYVENTEKALNVVYEIGKILSRDILSKINQPYQKFLDVLNTI
#1

211 KNASDSDGODLLEFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPOHRDVIOLYAPEAFNYMDKF
#2 #3

281 NEQEINLSLEELKDQRM LSRYEKWEKIKQHYQHWSDSLSEEGRGLLKKLQIPIEPKDDIIHSLSQEKE

351 LLKRIQIDSSDFLSTEEKEFLK LQIDIRDSLSEEEKELNRIQVDSSNPLSEKEKEFLK LKLDIQPYD

421 INQRLQDTGGLIDSPSINLDVRKQYKRDIQNIDALLHQSIGSTLYNKIYLYENMNINLTATLGADLVDS

491 TDNTKINRGIFNEFKNFKYSISSNYMIVDINERPALDNERLKWRIQLSPDTRAGYLENGKLILQRNIGL

561 EIKDVQIIKQSEKEYIRIDAKVVPKSKIDTKIQEAQLNINQEWNKALGLPKYTKLITFNVHNRYASNIVE

631 SAYLILNEWKNNIQSDLIKVTNYLVDGNGRFVFTDITLPNIAEQYTHQDEIYEQVHSGLYVPESRSIL

701 LHGPSKGVELRNDSEGFIEFGHAVDDYAGYLLDKNQSDLV TNSKKFIDIFKEEGSNLTSYGR TNEAEFF

771 AEAFLMHSTDHAERLKVQKNAPKTFQFINDQIKFIINS

The sequence contains 809 amino acids (M_r 93,798):

Ala (A)	34	Leu (L)	80
Arg (R)	27	Lys (K)	86
Asn (N)	54	Met (M)	10
Asp (D)	55	Phe (F)	29
Cys (C)	1	Pro (P)	21
Gln (Q)	41	Ser (S)	54
Glu (E)	79	Thr (T)	28
Gly (G)	35	Trp (W)	5
His (H)	21	Tyr (Y)	35
Ile (I)	74	Val (V)	40
Acidic (Asp + Glu)			134
Basic (Arg + Lys)			113
Aromatic (Phe + Trp + Tyr)			69
Hydrophobic (Aromatic + Ile + Leu + Met + Val)			273

PUBLICATIONS

The following articles were published:

Leppla, S.H., D.L. Robertson, S.L. Welkos, L.A. Smith, and M.H. Vodkin. 1986. Cloning and analysis of genes for anthrax toxin components, pp. 275-278. In *Bacterial protein toxins*, Suppl. 15. Zentralblatt für bakteriologie und hygiene. 1. Abteilung. Gustav Fischer, Stuttgart.

Robertson, D. L., and S. H. Leppla. 1986. Molecular cloning and expression in *Escherichia coli* of the lethal factor gene of *Bacillus anthracis*. *Gene* 44:71-78.

Tippetts, M.T., and D.L. Robertson. 1988. Molecular cloning and expression of the *Bacillus anthracis* edema factor toxin gene: a calmodulin-dependent adenylate cyclase. *J. Bacteriol.* 170:2263-2266.

Kaspar, R.L. and Robertson, D.L. 1987. Purification and physical analysis of *Bacillus anthracis* plasmids pX01 and pX02. *Biochem. Biophys. Res. Commun.* 149:362-368.

The following manuscripts are submitted and presently being reviewed for publication:

Robertson, D.L., M.T. Tippetts and S.H. Leppla. 1988. Nucleotide sequence of the *Bacillus anthracis* edema factor gene (*cya*): A calmodulin-dependent adenylate cyclase. submitted to *Gene*.

Robertson, D.L. 1988. Relationships between the calmodulin-dependent adenylate cyclases produced by *Bacillus anthracis* and *Bordetella pertussis*. submitted to *Infection and Immunity*.

The following abstracts were published:

Kaspar, R. L. and D. L. Robertson. Purification and analysis of *Bacillus anthracis* plasmids pX01 and pX02. *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1987.

Tippetts, M. T., D. L. Robertson and R. Leavitt. Molecular cloning and characterization of the *Bacillus anthracis* edema factor gene. *Abstr. Annu. Meet. Am. Soc. Microbiol.* 1987.

Robertson, D.L., T. Tippetts, Y. Luh, T. Bragg and R. Larson. 1988. Biochemical Analysis of the *Bacillus anthracis* Edema Factor Gene: A Calmodulin-Dependent Adenylate Cyclase. 72nd Annual Meeting of the Federation of American Societies for Experimental Biology.

The following invited seminars were given:

Donald L. Robertson. A Biochemical Analysis of the *Bacillus anthracis* Toxin Genes. 8th Annual Rocky Mountain Regional Biochemistry Conference, Pingree Park, Colorado. September, 1987.

Donald L. Robertson. A Biochemical Characterization of the *Bacillus anthracis* Toxin Genes. Brigham Young University Chemistry Department, January, 1988.

PERSONNEL

During the course of this contract, the principal investigator has been Dr. Donald L. Robertson, except for 10 months when Dr. Robertson was on sabbatical leave in the Bacteriology Division at USAMRIID. During this period, Dr. Ronald W. Leavitt served as principal investigator and directed the research of the graduate students.

Graduate students who have done research for this contract have included M. Todd Tippetts (Ph.D. awarded), Kent Hill (Ph.D. awarded), Scott Simpson (M.S. pending), Roger Kaspar (M.S. awarded), Tom Bragg (Ph.D. being completed). Dr. Robert Larson served as a post-doctoral fellow for the last year and performed research on the *B. subtilis* expression plasmids.

The following theses have been accepted:

Tippetts, M. T. 1986. Molecular cloning of the chloroplast genome of *Carthamus tinctorius* L. and of the edema factor gene from *Bacillus anthracis*. Department of Chemistry, Brigham Young University.

Kaspar, R. L. 1986. Purification and characterization of pX01 and pX02 plasmids from *Bacillus anthracis*. Department of Chemistry, Brigham Young University.

Luh, Y. 1988. Genetic Modification of the *Bacillus anthracis* Edema Factor Toxin Gene and Construction of Plasmid pBS42-EF which expresses EF in *Escherichia coli* and *Bacillus subtilis*. Department of Chemistry, Brigham Young University.

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