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

ANNUAL REPORT

Donald L. Robertson

June 30, 1987

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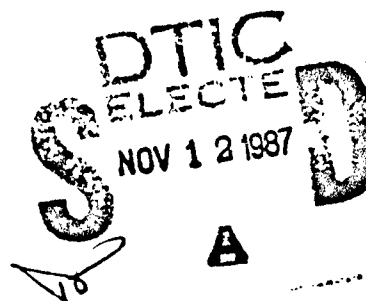
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We report an improved isolation procedure for the preparation of pX01 and pX02 plasmids of <i>B. anthracis</i>. These plasmids have been physically characterized with regard to buoyant density, GC content and size analysis using restriction enzyme digestions. Restriction maps of these DNAs have been generated. pX01 is 175 kbp and pX02 is 95 kbp. The location of the toxin genes, protective antigen (PA), lethal factor (LF) and edema factor (EF), have been positioned on pX01. (continued on reverse)			
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19. The EF gene has been cloned and sequenced. Unique features of the EF gene include a putative ATP binding site, a 33 amino acid leader sequence and a very good ribosome binding site. The direction and location for the start of each of the toxin genes have also been determined. EF has been expressed in enzymatically active form and is full-length. Expression vectors in E. coli have been used to produce large quantities of this protein. Expression vectors for LF and PA in both E. coli and B. subtilis have been used for enhanced expression of these genes as well.

In order to generate a safe anthrax vaccine using recombinant DNA techniques, we have begun experiments to specifically mutant each of these toxin genes to generate non-functional proteins that retain most, if not all, of their immunogenic properties. For example, the trypsin cleavage site of PA is being altered to remove the Arg-Lys-Lys-Arg sequence which is cleaved to activate PA. In addition, the putative ATP binding site of EF is being mutated to prevent enzyme activity. These experiments should generate mutant toxin proteins which will be safe vaccine components which will then be tested in animals.

SUMMARY OF RESEARCH

The overall goal of the present research is to construct a safe, effective human anthrax vaccine using recombinant DNA techniques. These studies are broken down into three phases:

Phase I. Isolation and characterization of the Bacillus anthracis toxin genes for protective antigen (PA), lethal factor (LF) and edema factor (EF). The individual toxin genes will be cloned in expression vectors for large scale production of toxin proteins using E. coli and B. subtilis. These experiments should provide enhanced production of the different toxin components which are made in low levels in E. coli.

Phase II. Generation of mutant toxin proteins from cloned toxin genes defined in Phase I. Mutations derived from deletion analysis or site-specific mutagenesis of the cloned toxin genes will be generated using in vitro manipulations of the recombinant plasmid DNAs. Mutations of potential use for vaccine construction will be identified as those which are non-toxic but still immunologically active and protective.

Phase III. Insertion of mutant genes back into B. anthracis with the selective removal of wild-type genes. Then, testing of these mutant strains will be performed in animals, such as the mouse or guinea pig.

The research outlined in this annual report describes the cloning and characterization of the individual B. anthracis toxin genes. These genes are being expressed in B. subtilis and E. coli and are being specifically mutated to generate mutant derivatives which lack biochemical activity but maintain immunological properties. In addition, a physical characterization of the B. anthracis plasmids with regard to size, genetic complexity, GC% and restriction enzyme mapping is also described.

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.

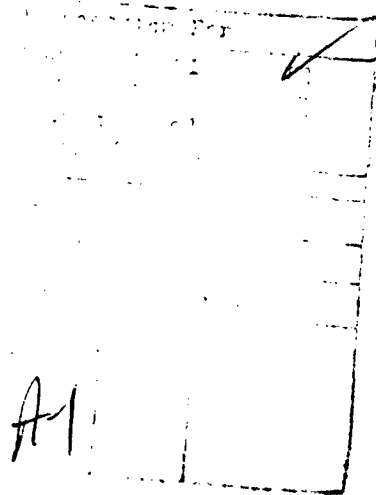


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BACKGROUND

As discussed in the summary, the goal of the experiments performed in this laboratory is to develop a more effective human anthrax vaccine for the protection of U.S. Army troops using recombinant DNA techniques. The current human anthrax vaccine consists of alum-precipitated supernatant material from fermenter cultures of B. anthracis which consists predominantly of PA (protective antigen) (9). Unfortunately, this vaccine may not be effective against all strains of B. anthracis since several virulent strains have been classified as "vaccine resistant" with regard to this human vaccine (19). Clearly, an effective vaccine must afford immunological protection against all strains of B. anthracis and against all forms of infection, including aerosol.

Virulent strains of B. anthracis contain two different plasmids. The toxin plasmid (pX01) is necessary for expression of the three toxin proteins (5,13) and the capsule plasmid (pX02) is necessary for production of the poly-D-glutamic acid capsule (2,16). In order to be able to insert mutant toxin genes back into B. anthracis for the production of a safe vaccine strain it has been necessary to characterize these plasmids. Studies designed to physically characterize these plasmids are now essentially complete. These analyses have included bouyant density centrifugation, DNA melting analysis and restriction endonuclease mapping of these DNAs. These characterizations should be helpful in generating recombinant vaccine strains of B. anthracis.

Each of the anthrax toxin genes are now cloned. The PA and EF genes have been sequenced. Experiments which are aimed at expressing these toxin genes in large quantities in E. coli and B. subtilis are in progress. In addition, we are specifically mutating the different toxin genes in

order to generate mutant toxin proteins which are still immunogenic but biochemically non-functional to be used in the vaccine development.

MATERIALS AND METHODS

The procedures described here pertain to the isolation and characterization of *B. anthracis* plasmids pX01 and pX02 and to the cloning and analysis of the PA, LF and EF genes. They are included to provide information prior to publication for the benefit of other investigators.

Isolation of pX01 and pX02. Our plasmid isolation procedure is a modification of the protocol described by Green et al. (2). A single colony of the Sterne (25) or Pasteur (ATCC 6602) strain of *B. anthracis* was incubated at 37°C in a shaking incubator for 14 to 16 hr in one liter Penassay broth (Difco) or L-broth (10 g tryptone, 5 g yeast extract, 5 g NaCl per liter [pH 7.5]). The cells are harvested by centrifugation at 8,000 rpm (Sorvall GSA rotor) and the pellet resuspended in 50 ml E-buffer (0.04 M Tris-acetate [pH 7.9], 2 mM EDTA). Cells are lysed by the addition of 100 ml of freshly-made lysis solution (50 mM Tris Base, 15% w/v sucrose, 3% sodium dodecyl sulfate [SDS], 0.5 M NaOH) and incubated with occasional stirring at 55-60°C for 30 min. After centrifugation at 10,000 rpm (GSA rotor) for 30-60 min to pellet cellular debris and unlysed cells, the plasmid-containing supernatant was extracted once or twice with an equal volume of unbuffered phenol/chloroform (1:1). The aqueous phase was then incubated on ice for 5-10 min and neutralized with 25 ml ice-cold 2 M Tris-HCl [pH 7.0]. The solution was then adjusted to 0.3 M NaOAc [pH 5.4] and ethanol precipitated using two volumes of 95% ethanol. Following a 30 min incubation at -20°C, the nucleic acid was collected by centrifugation at 10,000 rpm. The pellet was dissolved in 10 ml of RNase buffer (100 mM Tris-HCl [pH 7.4], 10 mM EDTA) containing 100 µg/ml RNase A and incubated at 37°C for 30 min. This solution was adjusted to 1% SDS and 50 µg/ml proteinase K and incubated at 65°C for 20 min. After extraction

with equilibrated phenol/chloroform (aqueous phase contained 50 mM Tris-HCl [pH 8.6], 0.1 M NaCl, 2.5 mM EDTA, 1% 2-mercaptoethanol) and ethanol precipitated, the DNA pellet was dissolved in TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) buffer and adjusted to 0.5 M NaCl. This semi-pure plasmid was then chromatographed on a NACS-37 reverse phase ion-exchange column (Bethesda Research Labs) to remove genomic DNA. A gradient of 0.50-0.80 M NaCl (in TE buffer) was used to elute the supercoiled plasmid. After ethanol precipitation and a wash with 95% ethanol, the plasmid was ready for further analysis.

Generation of EF recombinant DNA library. Toxin plasmid pX01 was partially cleaved with MboI and then ligated into BamHI cleaved pUC8 as described previously (10). Recombinant E. coli from this DNA library were grown as colonies on grided L-agar plates and then transferred to nitrocellulose filters, lysed and screened immunologically (10). Nitrocellulose filters of identically grided plates were also prepared for hybridization and processed as described by Maniatis et al. (17). They were hybridized to a synthetic oligonucleotide which had been labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. The filters were hybridized overnight at 30°C with the radiolabeled oligonucleotide in 6X SSPE, 5X Denhardt's solution and 0.5% SDS. After hybridization the filters were washed three times for 15 min in 6X SSPE, 0.5% SDS at 30°C and autoradiographed overnight at -70°C with Kodak XAR-5 film. Following autoradiography plasmids pEF68, pEF194 and pEF215 were isolated which hybridized to the EF oligonucleotide. An additional recombinant plasmid, designated pSE42, which contained a single BamHI fragment cloned into the BamHI site of pBR322 (13) also hybridized to this oligonucleotide.

Determination of EF, PA and LF gene orientations. In order to determine

gene orientation and the start of the different toxin genes, we have generated several recombinant plasmids which contained the appropriate DNAs which hybridized to the amino terminus-specific oligonucleotides for each gene. The 2.1 kbp HindIII fragment from pPA26 (13), the 2.2 kbp PstI fragment from pLF7 (10) and the 2.2 kbp HindIII fragment from pSE42 (see Figure 5) were each cloned into pTZ18R (11). Following the preparation of single-stranded plasmid (11) for each of these recombinant plasmids, radiolabeled oligonucleotides specific for the PA, LF or EF genes were hybridized to the appropriate DNAs. Based on which strand hybridized to a specific oligonucleotide, the direction of toxin gene transcription was determined. Then, using the same radiolabeled oligonucleotides we synthesized DNA using the Klenow fragment of E. coli DNA polymerase I. After a 60 min incubation at 37°C, this polymerization mixture was ethanol precipitated, dissolved in restriction enzyme buffer and digested with different restriction endonucleases. These DNA mixtures were then electrophoresed on a 4% denaturing polyacrylamide gel (53). Following autoradiography, the size of the resultant radiolabeled DNAs (from the start of the primer to the enzyme cleavage site) was determined. Based on the known restriction maps of these cloned DNAs, we could precisely position the start of each toxin gene.

RESULTS

pX01 and pX02 plasmid isolation. The plasmid isolation procedure described here is a variation of the protocol described by Green et al. (2), but includes NACS-37 chromatography to isolate supercoiled DNA. This chromatography step efficiently separates small amounts of genomic DNA from plasmid. The avirulent B. anthracis Sterne strain (25), which produces toxin but no capsule, contains pX01 and the Pasteur strain (ATCC 6602), which is capsule positive but toxin negative, contains pX02 (2). The different steps in plasmid isolation are listed in the legend of Figure 1 and are essentially identical for pX01 and pX02. Since B. anthracis cells are almost completely refractile to lysozyme digestion, we employed a heat-alkaline treatment to lyse the cells (2,21) as described in Methods. Supercoiled plasmid is clearly present in these preparations (see Figure 1). Lane 3 shows the DNA in its final state of purification, but without NACS chromatography. As the plasmid isolation proceeded, a shift in the proportion of supercoiled DNA (upper band) to the relaxed or linearized plasmid (lower band) often occurred. This conversion was also observed after prolonged storage of supercoiled DNA, and manipulations which tended to shear the DNA, such as freeze-thaw cycles and repeated phenol/chloroform extractions, converted supercoiled DNA to the relaxed or linearized form. As a result, the originally isolated supercoiled DNA often migrated in the relaxed region of the agarose gels following storage (data not shown). The region of the gel which contained relaxed pX01 and pX02 is the same region other investigators (2,5,22) suggested that genomic DNA migrated. It should be noted that these large plasmids are easily sheared, converting them from supercoiled to relaxed or linear DNA, even banding them in CsCl gradients converts supercoiled to relaxed

DNA. Therefore, since genomic DNA bands at the same position as the relaxed plasmid (unpublished observation of the author), CsCl gradients cannot be used to effectively separate genomic DNA from the relaxed plasmid. A typical yield of pX01 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.

The plasmid DNA shown in Figure 1, lane 8, can be used for restriction endonuclease analysis (see lane 9), but in order to remove any remaining genomic DNA we used NACS-37 reverse-phase ion exchange chromatography to isolate pure plasmid. Supercoiled DNA eluted at 0.69 M NaCl, which is characteristic of supercoiled DNA using NACS resins. Figure 2A shows pX01 DNA that was purified using NACS chromatography and then digested with different restriction endonucleases. The lack of background fluorescence indicated that genomic DNA was removed. Figure 2B shows purified pX02 DNA, but without NACS chromatography. Some contaminating genomic DNA is clearly still visible. Tables I and II show the sizes of restriction enzyme cleaved pX01 and pX02, respectively. Total size of pX01 is about 175 kbp and pX02 is 95 kbp. When these DNAs are cleaved with EcoRI, which cleaves many times, several DNA bands are doublets and must be counted more than once to give the correct plasmid size.

Plasmid DNA melting curves. For T_m analysis, the NACS-purified plasmid DNAs were sonicated in TE buffer to a size of approximately one kbp. The absorbance at 258 nm was monitored while the temperature was varied from 60-100°C in 0.5°C increments (Figures 4). The sharp hyperchromic shift for both plasmid DNAs indicated that denaturation was taking place. The T_m 's (the average of at least 6 experiments) for pX01 and pX02 was 82.5°C

$\pm 0.3^{\circ}\text{C}$ and $82.2^{\circ}\text{C} \pm 0.3^{\circ}\text{C}$, respectively. These DNA melting points correspond to GC contents of 32.2% for pX01 and 31.5% for pX02.

Bouyant density determinations. For CsCl banding, approximately 0.5 μg of plasmid DNA was radiolabeled using $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ by nick translation. Following purification, the radiolabeled DNAs were added to 3.5 ml of a 53.0% CsCl solution in TE buffer. After an isopycnic gradient was generated the DNA samples were fractionated into 0.15 ml aliquots and the amount of radioactivity in each sample was determined using liquid scintillation. The density of each fraction was determined from the refractive index at 20°C . Analysis of several different banding profiles showed that pX01 and pX02 had average densities of 1.6905 and 1.6908, respectively. These values corresponded to GC contents of 31.1% for pX01 and 31.4% for pX02. These values are close to the GC% of *B. anthracis* genomic DNA which is 32.2%.

Restriction maps of pX01 and pX02. The restriction maps for pX01 and pX02 are essentially completed. Figure 3 shows the current map for pX02. Construction of this restriction map used the Southern Cross mapping procedure recently developed by Potter and Dressler (20). As shown in Figure 3, we have a restriction map for the enzymes PstI, SstI, BamHI and ClaI as well as for BglII and PvuII (not shown) but not yet for EcoRI and HindIII, which cleave pX02 at least 20 times each. Using recombinant plasmids of an EcoRI digest, we should be able to generate a restriction map for these two enzymes as well.

The restriction map for pX01 (Figure 4) is essentially finished for the enzymes PstI, SstI, BamHI, PvuII, ClaI and SalI. However, due to the size of pX01, in comparison to pX02, we generated a restriction map using recombinant λ phage DNAs and BamHI/SstI cleaved pX01 DNA isolated from agarose gels. There are still two areas of some ambiguity, but these are

in the process of being refined. The map shown in Figure 4 is essentially complete with little or no variation for the enzymes shown (PstI, SstI, BamHI). The positions of the LF, PA and EF genes are also shown. We do not have a λ recombinant which contains both LF and PA genes. These restriction maps should be beneficial in examining variations between different *B. anthracis* strains, and for genetic modifications, such as for transposon mutagenesis, for construction of mutant toxin derivatives and for DNA sequencing studies.

Cloning of edema factor gene. The edema factor is a calmodulin-dependent adenylate cyclase that has been well characterized (3,4). Although previous attempts to clone the EF gene were unsuccessful, we have now cloned and expressed this gene in *E. coli*. The EF gene was cloned as a 6.5 kbp BamHI fragment in pBR322. This plasmid, designated pSE42, along with other recombinant plasmids isolated from a pX01 library, was positively identified as containing at least part of the EF gene by hybridizing with a radioactively labeled EF-specific oligonucleotide. The sequence of this oligonucleotide (5'-ATGAAPGAXCAPTAP-3'; where X=G,A and P= T,C) was deduced from the amino acid sequence for the first 5 amino acids of EF (Met-Asn-Glu-His-Tyr; determined by Dr. J. Schmidt [USAMRIID]). A restriction map of pSE42 is shown in Figure 5.

The 2.2 kbp HindIII fragment from pSE42 (designated as EF2.2 in Figure 5), which hybridized to the labeled oligonucleotide (data not shown) was cloned into plasmid pTZ18R (36) in both orientations. pTZ18R contains the IG region from M13 and can be used to produce single-stranded DNA for strand-specific hybridization. One of these recombinant plasmids, designated pEF2.2-11, hybridized with the radiolabeled oligonucleotide. Based on the orientation of the 2.2 kbp HindIII fragment in this plasmid, the

deduced direction of transcription of the EF gene was in a counter clockwise direction as depicted in Figure 5.

In order to precisely position the start of the EF gene, we used the labeled EF oligonucleotide for primer elongation. Following polymerization with the Klenow fragment of DNA polymerase I, the reaction mixtures were cleaved with HindIII. The size of the resultant DNA from the start of the labeled primer to the HindIII cleavage site was about 800 bp, indicating that the amino terminus of EF is about 800 bp to the right of the HindIII recognition site with transcription proceeding in the leftward direction. Cleavage of the same elongation product with XbaI generated a fragment of about 450 bp, consistent with the map positions of XbaI and HindIII. Clearly, pSE42 contains enough DNA to encode the entire EF gene.

pSE42 was sent to Dr. Francine McCutchen of Meloy Laboratories for DNA sequencing. The entire EF gene sequence has now been determined and is shown in Appendix I. Several interesting features of the EF sequence are present. (i) EF apparently contains a 33 amino acid leader sequence (beginning at nucleotide 554 to 643 on the DNA sequence) which is probably necessary for secretion. A similar 29 amino acid leader sequence was also found for PA (LTC. J. Lowe and Dr. S. Welkos, personal communications). (ii) A very strong ribosome binding site immediately upstream from the start of the EF protein is present (between nucleotides 528 and 537). (iii) Amino acid residues 314 to 321 (corresponding to nucleotides 1582 to 1605 of the DNA sequence) of the mature protein contain the sequence Gly-X-X-X-X-Gly-Lys-Ser which is a perfect match to a consensus sequence which was recently reported to be present in prokaryotic and eukaryotic ATP binding proteins (52) and which is part of their ATP binding sites. It is probable that this sequence is part of the EF ATP binding site as

well. (iv) No homology between the EF gene sequence or the deduced EF protein sequence was observed with the reported nucleotide and protein sequences from GenBank, including yeast and *E. coli* adenylate cyclases. (v) There appears to be no good consensus promoter immediately upstream from the start of the EF sequence, although a DNA sequence located at positions 317-348 seems to resemble a *B. subtilis* promoter, albeit a poor one. It should be noted that the PA promoter, which immediately precedes the PA gene, is a perfect match to a consensus *B. subtilis* promoter (data not shown).

Position for the start of the LF and PA genes. Using radiolabeled oligonucleotides specific for the first five amino acids of PA and LF, we have determined the position for the start of these genes as well. When the PA oligonucleotide, which corresponds to the first 5 amino acids of mature PA (Glu-Val-Lys-Gln-Glu), was hybridized and elongated using Klenow DNA polymerase and then digested with HindIII, a DNA about 300 bases was generated. After these experiments were performed, the PA sequence was determined and the actual distance between the position of the PA oligonucleotide and the HindIII site is 295 bases. Likewise, when the LF oligonucleotide, which corresponds to the beginning of LF (10) was used in these experiments, a DNA about 800 bases was generated when cleaved with EcoRI. Consequently, the start of the LF protein occurs about 800 bases to the right of the EcoRI cleavage site located to the left of the ClaI site in pLF7 (see Figure 2 in reference 10).

Expression of EF in *E. coli*. We have not been able to detect expression of EF in *E. coli* carrying pSE42, even though it contains the entire EF gene. Therefore, we attempted to express the EF gene by fusing it to the lac promoter. The 4 kbp EcoRI to BamHI fragment from pSE42 (see Figure

5) was subcloned into pTZ18R. This construction, designated pEF42, positioned the EF gene about 400 bases downstream from the lacZ promoter, but in the same transcriptional orientation. When this plasmid was grown in E. coli, EF was produced. IPTG, an inducer of the lac promoter, increased EF production at least 2-fold. pTZ18R also contains the T7 RNA polymerase promoter downstream from the lac promoter. Using an E. coli strain provided by Dr. W. Studier (Brookhaven National Laboratories) which contains a copy of T7 RNA polymerase under the control of the lac promoter (14), we monitored expression of EF after induction of T7 RNA polymerase by IPTG. In the presence of IPTG, we detected enzymatically active EF and full-length EF (89,000 Da) was observed in Western blots (S. Leppla, personal communication). We have also fused the EF gene, using site-specific mutagenesis, directly to the lac promoter and detected large quantities of full-length protein and enzyme activity.

Expression of elevated levels of B. anthracis toxin protein in E. coli and B. subtilis. Each of the B. anthracis toxin genes, PA (13), LF (10), and EF (manuscript in preparation), have been cloned and expressed in E. coli. We are now attempting to enhance expression in E. coli and in B. subtilis. For example, using the T7 RNA polymerase expression system developed by Dr. W. Studier (14), we have successfully expressed high levels of enzymatically active EF (3,4). Drs. B. Ivins and S. Welkos successfully transferred the PA gene into B. subtilis and monitored the high level of expression of PA (18). We are also generating genetic constructions which will fuse the EF gene to the PA promoter. Since the PA promoter is very active in B. subtilis (18), we should be able to produce large quantities of EF, perhaps in similar quantities to the level of PA.

In order to be able to shuttle mutant toxin genes from E. coli to B. subtilis, it will be necessary to use plasmids which replicate in both organisms (6-8,12). This conventional approach so far has been only marginally successful. However, we have transferred the PA, LF and EF genes into B. subtilis using a slightly different approach. Normal and mutant toxin plasmids are grown initially in E. coli, then, as the last step of mutagenesis, a B. subtilis plasmid is ligated to the toxin plasmid. For example, using E. coli plasmids (11) which contain the toxin genes, we have combined them with separate B. subtilis plasmids (e.g., pC194, pE194, pUB110, pBS42 [1] or pGX284 [23,24]). The resultant chimeric plasmids which contain toxin DNA, E. coli plasmid DNA and B. subtilis plasmid DNA are then transformed into B. subtilis. The advantage of using this experimental approach is that the toxin genes are easily manipulated and modified in E. coli and then transferred into B. subtilis using efficient protoplast or competent cell transformation. These chimeric plasmids are stable in E. coli and transform B. subtilis well.

Construction of mutant PA and EF genes. We have initiated experiments that should result in the construction of mutant toxin genes. If biochemically non-functional toxin mutants are generated which are still immunogenic, these proteins can be used safely as vaccine components. Dr. S. Leppla and Col. A. Friedlander have identified a site in PA which is cleaved by a trypsin-like enzyme when bound to a receptor on the cell surface (personal communications). This proteolytic cleavage of PA is required for the binding of LF or EF before endocytosis. The amino acid sequence at this location in PA is Arg-Lys-Lys-Arg. We have constructed, using site-specific mutagenesis, a mutant derivative of the PA gene in which we have inserted SstI and SalI sites which flank this cleavage

site. The intent of this alteration is to remove and to alter the basic amino acids (Arg-Lys-Lys-Arg) at this location and then to substitute other amino acids. These substitutions should prevent proteolytic cleavage and render PA non-functional but still fully immunogenic.

As described above, an amino acid sequence in EF (from amino acid residues 314 to 321 of the mature protein) which is Gly-X-X-X-Gly-Lys-Ser is a perfect match to a consensus sequence present in prokaryotic and eukaryotic ATP binding proteins (15). Since the Lys residue is part of the ATP binding site it is likely that this Lys residue is also part of the EF ATP binding site. Since the overall goal of this research contract is to generate a safe anthrax vaccine, this conserved amino acid sequence is a good candidate for site-directed mutagenesis. If this Lys residue were replaced with a non-charged amino acid, it is possible that EF will no longer bind ATP and that EF will be enzymatically inactive. These mutagenesis experiments are in progress.

CONCLUSIONS

It appears from the data reported here that Phase I and Phase II of the original proposal are nearing completion. Each of the anthrax toxin genes have been cloned and expressed in *E. coli* and to some extent in *B. subtilis*. Current research and experiments to be carried out during the first part of this next year should result in the elevated expression of mutant and wild-type toxin protein. With the research at the current level, we should be able to start experiments outlined in Phase III during this year. Therefore, during the next year, we will continue to express the toxin proteins at high levels and will test these proteins for biochemical activity.

We have also succeeded in physically characterizing the toxin plasmid

(pX01) and the capsule plasmid (pX02) of B. anthracis. These analyses should be extremely beneficial in the construction of mutant vaccine strains and for DNA sequencing analysis of these plasmids.

PUBLICATIONS

The following articles were published during this reporting period:

Robertson, D. L., and S. H. Leppla. 1986. Molecular cloning and expression on E. coli of the lethal factor gene of B. anthracis. Gene 44:71-78.

The following manuscripts were submitted for publication:

Kaspar, R. L. and D. L. Robertson. 1987. Purification and analysis of Bacillus anthracis plasmids pX01 and pX02. submitted to J. Bacteriol.

Tippetts, M. T. and D. L. Robertson. 1987. Molecular cloning of the edema factor gene of Bacillus anthracis. submitted to Gene.

The following dissertations were published:

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.

The following abstracts were published during this reporting period.

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

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TABLE I. Computer-assisted sizing analysis of pX01 DNA cleaved with different restriction enzymes^a.

BamHI + SalI		PstI		ClaI	
Measured Mobility	Measured Size	Measured Mobility	Measured Size	Measured Mobility	Measured Size
(cm)	(kb)	(cm)	(kb)	(cm)	(kb)
1.53	25.4	1.79	20.7	1.81	20.4
1.62	23.6	1.91	19.0	2.06	17.1
1.65	23.1	2.02	17.6	2.24	15.2
1.86	19.7	2.54	12.6	2.61	12.1
2.26	15.0	2.73	11.3	2.69	11.6
2.30	14.6	2.73	11.3	2.77	11.1
3.55	7.6	2.83	10.7	2.80	10.9
3.71	7.1	3.01	9.8	3.24	8.7
3.75	7.0	3.20	8.9	3.79	6.9
3.94	6.5	3.96	6.4	3.83	6.8
4.02	6.3	3.98	6.4	4.00	6.3
4.23	5.8	4.12	6.1	4.33	5.6
4.33	5.6	4.26	5.8	4.42	5.5
5.73	<u>3.7</u>	4.45	5.4	4.61	5.2
		4.63	5.1	4.68	5.0
Total	171.1	4.76	4.9	4.82	4.8
		5.01	4.6	4.97	4.6
		5.53	4.0	4.98	4.6
		6.09	<u>3.4</u>	5.08	4.5
		Total	174.1	5.17	4.4
				5.51	<u>4.0</u>
				Total	175.3

^apX01 DNA was cleaved with the indicated enzymes, electrophoresed and the ethidium bromide-stained bands photographed. The extrapolated sizes are shown along with the distances migrated in the agarose gel. DNA bands that appeared more intense than expected we counted as doublets. (The mobility distances shown in this Table are those of the Polaroid negative of the ethidium bromide stained gel and are about 40% of the actual migration distances.) λ DNA, cleaved with HindIII, was used as molecular weight standards to establish the sizing curve.

TABLE II. Computer-assisted sizing analysis of pX02 cleaved with ClaI^a.

Measured Mobility <u>(cm)</u>	Calculated Size <u>(kb)</u>
1.330	24.8
1.540	19.7
1.800	15.5
2.130	11.3
3.040	6.2
3.420	5.2
3.840	4.4
3.870	4.4
5.340	2.6
5.640	<u>1.2</u>
Total	95.3

³pX02 was cleaved with ClaI and then processed exactly as described in TABLE I.

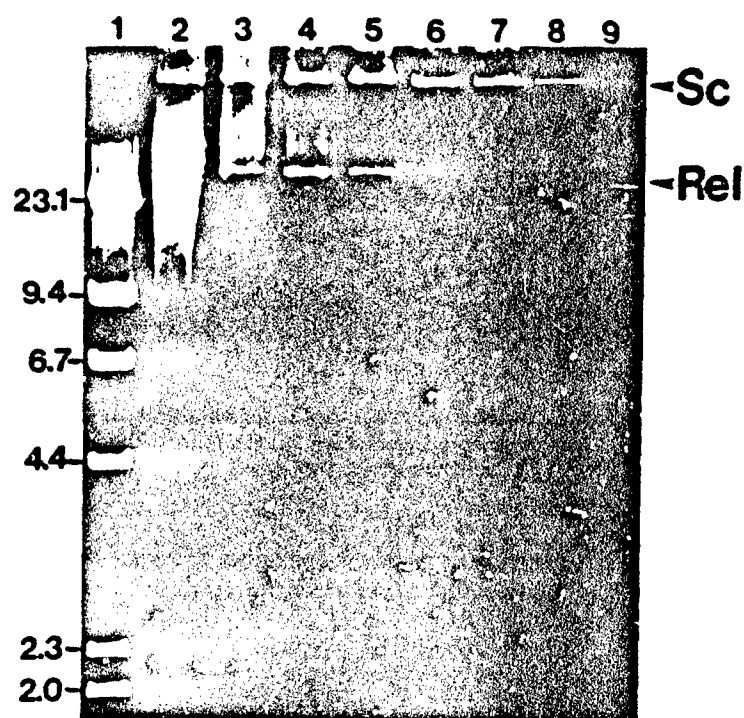


Figure 1. pX01 DNA following each of the different steps in the plasmid isolation. Lane 1, *Hind*III digested λ DNA. Lane 2, pX01 after the heat-alkali treatment and centrifugation. Lane 3, plasmid DNA after neutralization with 2 M Tris-HCl [pH 7.0]. Lane 4, DNA after the first unbuffered phenol/chloroform extraction. Lane 5, pX01 DNA following second unbuffered phenol/chloroform extraction. Lane 6, plasmid DNA dissolved in RNase buffer. Lane 7, plasmid DNA following RNase A and proteinase K treatment and extraction with buffered phenol/chloroform. Lane 8, pX01 DNA dissolved in TE buffer. Lane 9, pX01 DNA (same DNA shown in lane 8) digested with 10 units of *Bam*HI. (Sc-Supercoiled plasmid DNA. Rel-Relaxed or linearized plasmid DNA.)

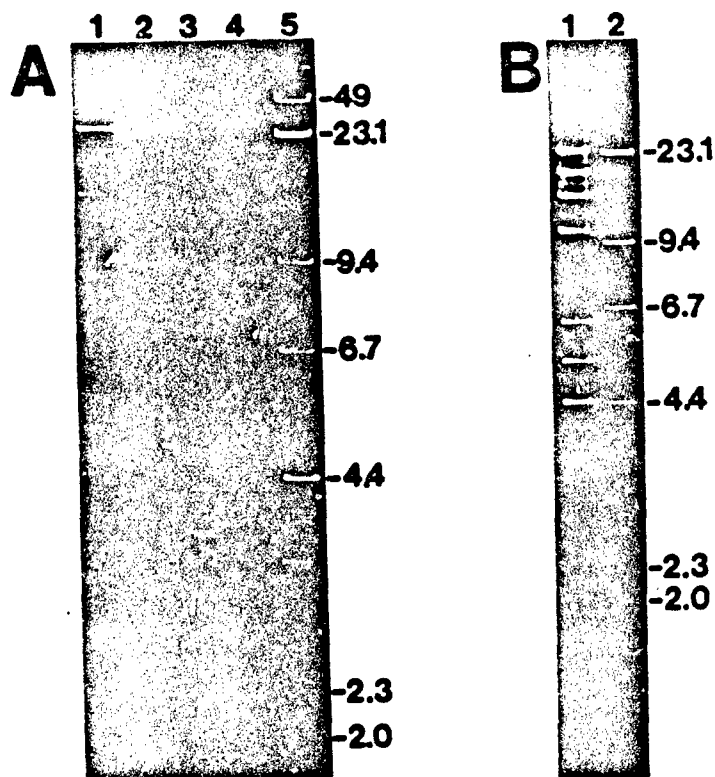


Figure 2. Analysis of plasmid DNAs with various restriction endonucleases. (A) pX01 DNA (purified over NACS-37 ion-exchange column) digested with *Bam*HI and *Sal*I (lane 1), *Bam*HI and *Sst*I (lane 2), *Pst*I (lane 3), and *Cla*I (lane 4). Lane 5 is *Hind*III cleaved λ DNA with fragment sizes shown in kilobases. (B) pX02 DNA (not purified with NACS chromatography) digested with *Cla*I (lane 1). Lane 2 is *Hind*III cleaved λ DNA. A more complete restriction enzyme analysis of pX01 and pX02 will be published elsewhere (DLR, S. Simpson and T. Bragg, personal communication).

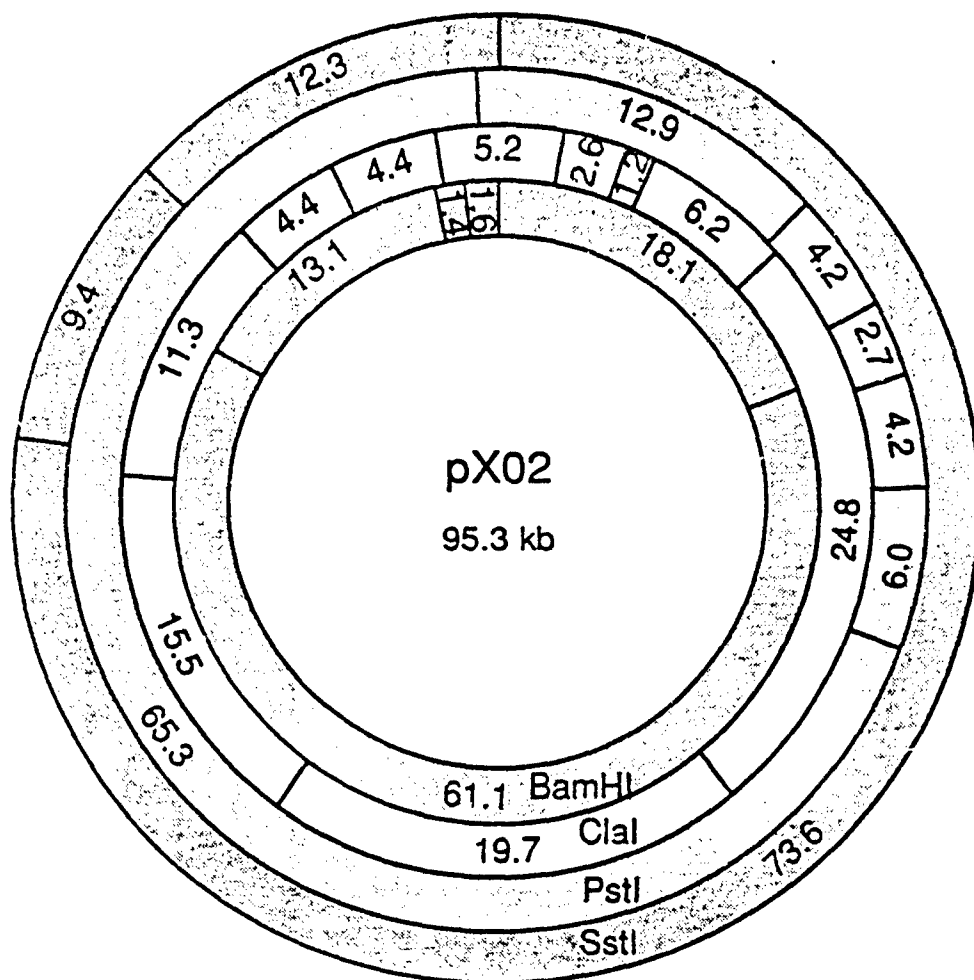


Figure 3. Restriction map of pX02.

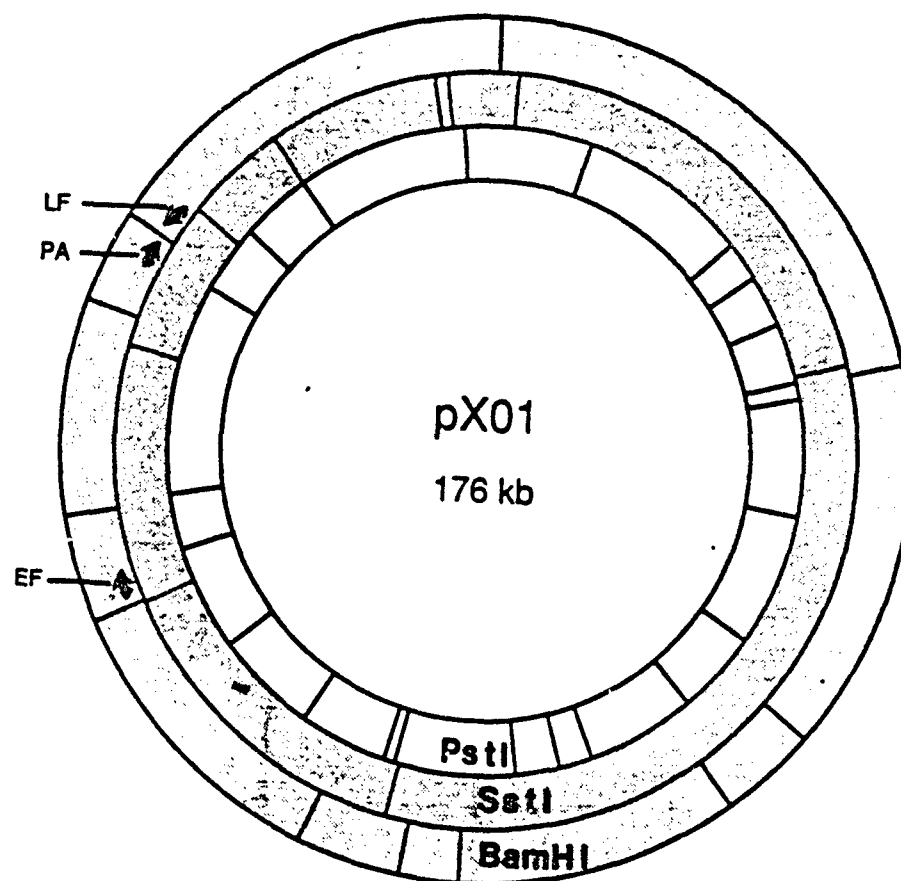


Figure 4. Restriction map of pX01. The positions of the LF, PA and EF genes are depicted. The sizes of DNA fragments for each enzyme are not included due to the lack of space.

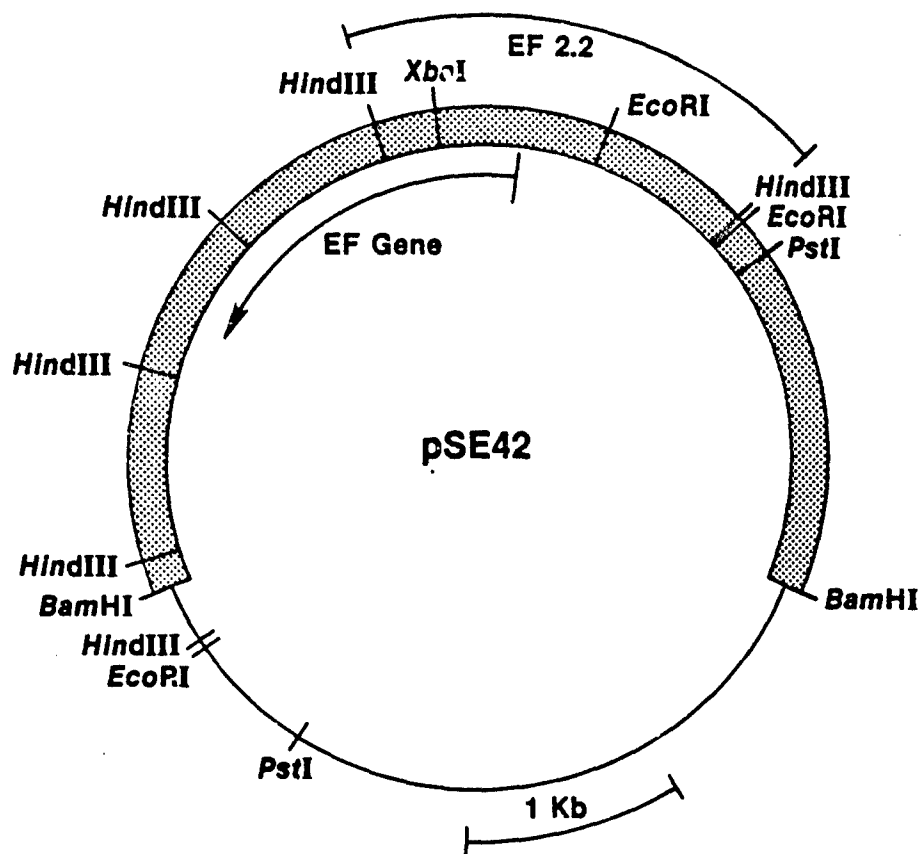


Figure 5. Restriction map of pSE42. The thin line represents pBR322 sequences and the wide line is from pX01 which contains the entire EF gene. The position for the start of EF is shown and the 2.2 *HindIII* fragment (designated EF2.2) which hybridizes with the EF oligonucleotide and contains the EF promoter.

APPENDIX I. DNA and protein sequence of EF

DNA and protein sequence of EF

```

      10      20      30      40      50      60
TTACTTTTTTATATACTGAATTAAAAAGTCCAAGCACTTATATCGTAATAGATGCTTTCT

      70      80      90     100     110     120
ATTGACCTTATAGTCCTTGAAGTTACGACTGACCAATTATGAGACGTTTGCGCTAACCTG

      130     140     150     160     170     180
CTGAATTCAAAATCGGACTTAGAAATACACATATAGAAATAAACCAACCTAATCCATGTCA

      190     200     210     220     230     240
CTGTACCGT.TTTTTTACTAAATAAACGAAATCAGTGTAATAATGAACAGCTGAACCTTAT

      250     260     270     280     290     300
CAACTTAGAATCTCTTTTTTACTTTAAATGCCTAGCTGTTTTTCTAATGTTTGTATTT

      310     320     330     340     350     360
CTAAATATATTTAAATATGAATTGTAGCTGTGTGCCAAGAGTTATAATTAATTTAAATAA
      -35 (putative promoter site) -10

      370     380     390     400     410     420
GATTATATTTGTAAATAAAATTGTAATTTAACATGTAGAATAAAGAGATTTTACTTTTA

      430     440     450     460     470     480
TTAACAGGATGAAAATCCATAAAACCGTAAATGTGATTCTAAATTAGTTTAAAAATAAAA

      490     500     510     520     530     540
AACAAAGGATTGCTCAGACTTGAGATGAATATCTAAATATCAAGAACCAAAGGAGGTTTA
      ribosome binding site

+1 550     560     570     580     590     600
AGAATGACTAGAAATAAATTTATACCTAATAAGTTTAGTATTATATCCTTTTCAGTATTA
MetThrArgAsnLysPheIleProAsnLysPheSerIleIleSerPheSerValLeu
      33 amino acid leader sequence

      610     620     630     640     650     660
CTATTTGCTATATCCTCCTCACAGGCTATAGAAGTAAATGCTATGAATGAACATTACACT
LeuPheAlaIleSerSerSerGlnAlaIleGluValAsnAlaMETAsnGluHisTyrThr
      1st amino acid of EF

      670     680     690     700     710     720
GAGAGTGATATTAAGAAACCATAAACTGAAAAAATAAACTGAAAAAGAAAAATTT
GluSerAspIleLysArgAsnHisLysThrGluLysAsnLysThrGluLysGluLysPhe

```

730	740	750	760	770	780
AAAGACAGTATTAATAACTTAGTTAAAAACAGAATTTACCAATGAACTTTAGATAAAATA					
LysAspSerIleAsnAsnLeuValLysThrGluPheThrAsnGluThrLeuAspLysIle					
790	800	810	820	830	840
CAGCAGACACAAGACTTATTAATAAGATACCTAAGGATGTACTTGAAATTTATAGTGAA					
GlnGlnThrGlnAspLeuLeuLysLysIleProLysAspValLeuGluIleTyrSerGlu					
850	860	870	880	890	900
TTAGGAGGAGAAATCTATTTTACAGATATAGATTTAGTAGAACATAAGGAGTTACAAGAT					
LeuGlyGlyGluIleTyrPheThrAspIleAspLeuValGluHisLysGluLeuGlnAsp					
910	920	930	940	950	960
TTAAGTGAAGAAGAGAAAAATAGTATGAATAGTAGAGGTGAAAAAGTTCCGTTTGCATCC					
LeuSerGluGluGluLysAsnSerMetAsnSerArgGlyGluLysValProPheAlaSer					
970	980	990	1000	1010	1020
CGTTTTGTATTTGAAAAAGGAAACACCTAAATTAATTATAAATATCAAAGATTAT					
ArgPheValPheGluLysLysArgGluThrProLysLeuIleIleAsnIleLysAspTyr					
1030	1040	1050	1060	1070	1080
GCAATTAATAGTGAACAAAGTAAAGAAGTATATTATGAAATTGGAAAGGGCATTCTCTT					
AlaIleAsnSerGluGlnSerLysGluValTyrTyrGluIleGlyLysGlyIleSerLeu					
1090	1100	1110	1120	1130	1140
GATATTAIAAGTAAGGATAAATCTCTAGATCCAGAGTTTTTAAATTTAATTAAGAGTTTA					
AspIleIleSerLysAspLysSerLeuAspProGluPheLeuAsnLeuIleLysSerLeu					
1150	1160	1170	1180	1190	1200
AGCGATGATAGTGATAGTAGCGACCTTTTATTTAGTCAAAAATTTAAAGAGAACCTAGAA					
SerAspAspSerAspSerSerAspLeuLeuPheSerGlnLysPheLysGluLysLeuGlu					
1210	1220	1230	1240	1250	1260
TTGAATAATAAAAGTATAGATATAAATTTTATAAAAGAAAATTTAACTGAATTTTCAGCAT					
LeuAsnAsnLysSerIleAspIleAsnPheIleLysGluAsnLeuThrGluPheGlnHis					
1270	1280	1290	1300	1310	1320
GCGTTTTCTTTAGCGTTTTCTTATTATTTTGCACCTGACCATAGAACGGTATTAGAGTTA					
AlaPheSerLeuAlaPheSerTyrTyrPheAlaProAspHisArgThrValLeuGluLeu					
1330	1340	1350	1360	1370	1380
TATGCCCCGACATGTTTGAGTATATGAATAAGTTAGAAAAAGGGGGATTGAGAAAAATA					
TyrAlaProAspMetPheGluTyrMetAsnLysLeuGluLysGlyGlyPheGluLysIle					
1390	1400	1410	1420	1430	1440
AGTGAAAGTTTGAAGAAAGAAGGTGTGAAAAAGATAGGATTGATGTGCTGAAAGGAGAA					
SerGluSerLeuLysLysGluGlyValGluLysAspArgIleAspValLeuLysGlyGlu					
1450	1460	1470	1480	1490	1500
AAAGCACTTAAAGCTTCAGGTTTAGTACCAGAACATGCAGATGCTTTTAAAAAAATTGCT					
LysAlaLeuLysAlaSerGlyLeuValProGluHisAlaAspAlaPheLysLysIleAla					

1510 1520 1530 1540 1550 1560
 AGAGAATTAAATACATATATTCTTTTAGGCCTGTTAATAAGTTAGCTACAAACCTTATT
 ArgGluLeuAsnThrTyrIleLeuPheArgProValAsnLysLeuAlaThrAsnLeuIle

1570 1580 1590 1600 1610 1620
 AAAAGTGGTGTGGCTACAAAGGGATTGAATGAACATGGAAAGAGTTCGGATTGGGGCCCT
 LysSerGlyValAlaThrLysGlyLeuAsnGluHisGlyLysSerSerAspTrpGlyPro

1630 1640 1650 1660 1670 1680
 GTAGCTGGATACATACCATTTGATCAAGATTTATCTAAGAAGCATGGTCAACAATTAGCT
 ValAlaGlyTyrIleProPheAspGlnAspLeuSerLysLysHisGlyGlnGlnLeuAla

1690 1700 1710 1720 1730 1740
 GTCGAGAAAGGAAATTTAGAAAATAAAAAATCAATTACAGAGCATGAAGGTGAAATAGGT
 ValGluLysGlyAsnLeuGluAsnLysLysSerIleThrGluHisGluGlyGluIleGly

1750 1760 1770 1780 1790 1800
 AAAATACCATTAAAGTTAGACCATTAAAGAATAGAAGAGTTAAAGGAAAATGGGATAATT
 LysIleProLeuLysLeuAspHisLeuAlaGluGluLeuLysGluAsnGlyIleIle

1810 1820 1830 1840 1850 1860
 TTGAAGGGTAAAAAGAAATTGATAATGGTAAAAATATTATTTGTTAGAATCGAATAAT
 LeuLysGlyLysLysGluIleAspAsnGlyLysLysTyrTyrLeuLeuGluSerAsnAsn

1870 1880 1890 1900 1910 1920
 CAGGTATATGAATTTAGAATTAGCGATGAAAACAACGAAGTACAATACAAGACAAAAGAA
 GlnValTyrGluPheArgIleSerAspGluAsnAsnGluValGlnTyrLysThrLysGlu

1930 1940 1950 1960 1970 1980
 GGTAAAATTACTGTTTTAGGGGAAAAATTCAATTGGAGAAATATAGAAGTGATGGCTAAA
 GlyLysIleThrValLeuGlyGluLysPheAsnTrpArgAsnIleGluValMetAlaLys

1990 2000 2010 2020 2030 2040
 AATGTAGAAGGGTCTTGAAGCCGTTAACAGCTGACTATGATTTATTTGCACTTGCCCCA
 AsnValGluGlyValLeuLysProLeuThrAlaAspTyrAspLeuPheAlaLeuAlaPro

2050 2060 2070 2080 2090 2100
 AGTTTAACAGAAATAAAAAACAAATACCCACAAAAAGAATGGATAAAGTAGTTAACACC
 SerLeuThrGluIleLysLysGlnIleProThrLysArgMetAspLysValValAsnThr

2110 2120 2130 2140 2150 2160
 CCAAATTCATTAGAAAAGCAAAAAGGTGTTACTAATTTATTGATTAAATATGGAATTGAG
 ProAsnSerLeuGluLysGlnLysGlyValThrAsnLeuLeuIleLysTyrGlyIleGlu

2170 2180 2190 2200 2210 2220
 AGGAAACCGGATTCAACTAAGGGAACTTTATCAAATTGGCAAAAACAAATGCTTGATCGT
 ArgLysProAspSerThrLysGlyThrLeuSerAsnTrpGlnLysGlnMetLeuAspArg

2230 2240 2250 2260 2270 2280
 TTGAATGAAGCAGTCAAATATACAGGATATACAGGAGGGGATGTGGTTAACCATGGCACA
 LeuAsnGluAlaValLysTyrThrGlyTyrThrGlyGlyAspValValAsnHisGlyThr

2290	2300	2310	2320	2330	2340
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GGTGAATTTATATTAACTAAAAATTGGGAGATGACAGGTAGATTTATAGAAAAAACATT					
GlyGluPheIleLeuThrLysAsnTrpGluMetThrGlyArgPheIleGluLysAsnIle					
2410	2420	2430	2440	2450	2460
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2470	2480	2490	2500	2510	2520
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2530	2540	2550	2560	2570	2580
TCAGCAGAGTTTATAAAAACTTATCCAGTATCAGAAGATCTTCAAATGTAGGAGTTTAT					
SerAlaGluPheIleLysAsnLeuSerSerIleArgArgSerSerAsnValGlyValTyr					
2590	2600	2610	2620	2630	2640
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LysAspSerGlyAspLysAspGluPheAlaLysLysGluSerValLysLysIleAlaGly					
2650	2660	2670	2680	2690	2700
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TyrLeuSerAspTyrTyrAsnSerAlaAsnHisIlePheSerGlnGluLysLysArgLys					
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2770	2780	2790	2800	2810	2820
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GlnIleAlaProGluTyrLysAsnTyrPheGlnTyrLeuLysGluArgIleThrAsnGln					
2830	2840	2850	2860	2870	2880
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2890	2900	2910	2920	2930	2940
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2950	2960	2970	2980	2990	3000
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Lys					
3010	3020	3030	3040	3050	3060
TAAATAGATGTATTGAATAGTTATAGTAATGGTCTTGTATGGACATACCGCTTATACTTT					

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