Cell, Vol. 34, 693–697, September 1983, Copyright © 1983 by MIT

0092-8674/83/090693-05 $02.00/0

Cloning of the Protective Antigen Gene of Bacillus anthracis

Michael H. Vodkin and Stephen H. Leppla
Pathology Division
United States Army Medical Research Institute of Infectious Diseases
Fort Detrick
Frederick, Maryland 21701

Summary

The tripartite protein toxin of Bacillus anthracis consists of protective antigen (PA), edema factor (EF), and lethal factor (LF). As a first step in developing a more efficacious anthrax vaccine, recombinant plasmids containing the PA gene have been isolated. A library was constructed in the E. coli vector pBR322 from Bam HI-generated fragments of the anthrax plasmid, pBA1. Two clones producing PA were identified by screening lysates with ELISA (enzyme-linked immunosorbent assay). Western blots revealed a full-size PA protein in the recombinant E. coli, and a cell elongation assay demonstrated biological activity. Both positive clones had a 6 kb insert of DNA, which mapped in the Bam HI site of the vector. The two inserts are the same except that they lie in opposite orientations with respect to the vector. Thus PA is encoded by the plasmid pBA1.

Introduction

Bacillus anthracis secretes three distinct proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF), which collectively comprise the toxin of the anthrax bacillus. These proteins interact in animal test systems in combinations that produce two distinct pathological presentations. Protective antigen interacts with LF, causing death in rats (Beall et al., 1962), whereas PA in combination with EF produces localized edema in the skin of guinea pigs and rabbits (Thorne et al., 1960; Stanley et al., 1960). PA is thought to mediate the effects of EF and LF by binding to receptors of sensitive eucaryotic cells (Molnar and Altenbern, 1963). Recently, EF has been shown to be a calmodulin-dependent adenylate cyclase that dramatically elevates cyclic AMP concentrations in eucaryotic cells (Leppla, 1982).

Gladstone (1946) first demonstrated in 1948 that a soluble component from B. anthracis culture supernatants induces a protective immunity against subsequent challenge by live organisms. In later reports PA has been implicated as this immunogen, either by itself or in combination with another toxin component. For example, Fish et al. (1968) observed that enrichment for PA from a crude preparation improved the efficacy of the vaccine. Stanley and Smith (1963), on the other hand, reported that the combination of PA and EF was more effective than PA alone. The current vaccine licensed for human use consists of alum-precipitated supernatant material from fermenter cultures of B. anthracis V770-NP1-R (Brachman et al., 1962). The growth conditions utilized for these fermenter cultures result in a supernatant that consists primarily of PA and contains barely detectable levels of EF and LF (Ristroph and Ivins, 1983; and unpublished data).

A principal goal of this research is development of an anthrax vaccine with enhanced efficacy and decreased reactogenicity. Though the cause of the reactogenicity is uncertain, it is probably caused by other Bacillus antigens, which may include LF and EF. PA produced from recombinant E. coli would be free of such antigens. The current vaccine is given in a series of six injections, followed by yearly boosters, which appear necessary to maintain protective titers of antibody. Availability of pure PA may allow larger doses to be given in each injection, so as to induce protective titers of antibody more quickly and safely.

B. anthracis contains a large plasmid (pBA1) that either regulates or encodes the production of the toxin (Mikesell et al., 1983). Plasmid-cured strains lose toxic activity (PA and/or LF) and regain it after transformation by the native plasmid DNA (Mikesell et al., 1983). By ELISA (enzyme-linked immunosorbent assay), we estimate that one cured strain produces no more than 1/2500 the amount of PA made by its wild-type parental strain (unpublished data). Such a large decrease in PA production favors the hypothesis that pBA1 codes directly for PA. However, in strains of B. thuringiensis, a species closely related to B. anthracis, the δ-endotoxin gene is apparently encoded by chromosomal DNA, plasmid DNA, or both (Schnepf and Whiteley, 1981; Klier et al., 1982; Held et al., 1982; Gonzalez, et al., 1982).

We report here the construction of a library of the plasmid DNA from a strain of B. anthracis. At least two of the recombinant E. coli clones in the library code for PA and express the protein. These E. coli clones offer many opportunities for clinical, diagnostic, and research applications. PA can now be isolated without contamination by other Bacillus proteins and indeed, even without growing B. anthracis. PA from the recombinant E. coli may eventually form the basis of an improved anthrax vaccine. Furthermore, recombinant technology will allow the examination of various domains of PA (e.g., immunogenicity, EF and LF binding, and binding to eucaryotic receptor sites).

Results

Construction of Library from pBA1

pBA1 was estimated to be 168.4 ± 7.3 kb (X ± SE) as determined by electron microscopy. For purposes of cloning, a Bam HI digest with a pattern of 11 or 12 fragments was utilized (see Figure 4a). After ligation, transformation, and screening, a library of 110 colonies was collected. The DNAs (pSE 1–40) were extracted from 40 colonies and analyzed by gel electrophoresis before and after cleavage by Bam HI (data not shown). These analyses confirmed that all detectable restriction fragments generated by Bam HI digestion of pBA1 had been cloned in the E. coli transformants.

Screening of the Library

Forty colonies were screened immunochemically for PA production, initially using a dot blot assay. As controls, pure PA was diluted with a lysate from E. coli HB101 (pBR322). Although this method, which utilizes a single antibody, resulted in high background, colonies that produced PA (or proteins that reacted with anti-PA antibody) could be distinguished from those that did not. The sensitivity for detecting PA was 5–10 ng/ml. Subsequent verification was performed by an ELISA, which employed a capture antibody and had a greatly decreased relative background (Figure 1). Two colonies (24 and 36) harboring plasmids designated pSE 24 and 36 consistently yielded positive results in both assay systems. Colony 36 often produced 2 to 4 fold more PA (10 ng/ml) than colony 24.

Further Characterization of PA-Reactive Material in Transformed E. coli

Although the immunochemical test showed that a peptide related to PA was being produced, these methods could not confirm whether the product was full-size and biologically active. Lysates from colonies 24, 36, and HB101 (pBR322) were analyzed by Western blotting (Figure 2). Only the lysates from colonies 24 and 36 displayed a prominent PA-reactive band, which comigrated with PA purified from B. anthracis (85,000 daltons). Moreover, when a lysate from colony 36, and purified PA were mixed, only one band was seen. These data show that the initial positive response demonstrated by ELISA was due to a full-size gene product.

To determine whether a biological activity characteristic of PA was present in the recombinant E. coli lysates, cell elongation assays were performed. These assays were based on the demonstration (Leppla, 1982) that the combination of PA and EF causes a dramatic morphological alteration in CHO cells, due to the elevation of intracellular cAMP concentrations by the EF adenylate cyclase activity. Either PA or EF alone has no effect. CHO cells were treated with various combinations of purified PA, purified EF, and E. coli lysates. As anticipated, the combination of PA and EF resulted in cell elongation; cytoplasmic projections similar to dendrites could be seen at the ends of many cells. Lysates from colonies 24 and 36 could substitute for PA but not for EF (Figure 3b) in the assay. The complementation was specific, in that a lysate from E. coli HB101 (pBR322) in combination with pure EF did not produce the altered cell shape (Figure 3a). Moreover, titration of PA activity in the lysates agreed with previous estimates, based on the ELISA, that the lysates contain 5–10 ng PA/ml (based on original culture volume). These experiments show that the cloned gene product is biologically active.

Analysis of pSE 24 and 36 DNA

On agarose gels, pSE 24 and 36 have an identical size, estimated at 10.3 kb. To generate a restriction map, DNAs of colonies 24 and 36 were extracted and digested by

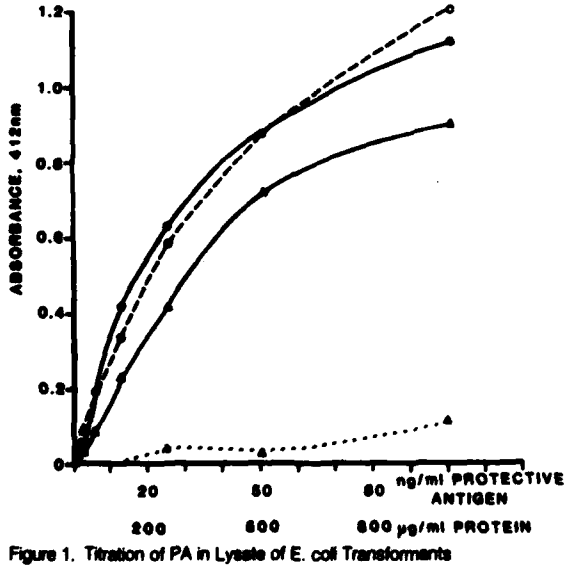

Figure 1. Titration of PA in Lysate of E. coli Transformants

The wells of microtiter plates previously coated with affinity-purified goat anti-PA antibody (5 µg/ml) were filled with 100 µl of purified PA (0–100 ng/ml) or with deoxycholate lysates of E. coli transformants (0–1000 µg/ml total protein), and with serial dilutions of these samples, made in phosphate-buffered saline, 0.05% Tween 20, 10% fetal calf serum. PA bound by the capture antibody was detected by subsequent incubations with affinity-purified rabbit anti-PA antibody, a peroxidase-staph protein A conjugate, and the chromogenic substrate ABTS. The plate was scanned at 414 nm, and the absorbance was plotted against concentration of either PA or total protein. O----O, purified PA; ●----●, lysate from colony 36; Δ----Δ lysate from colony 24; ▲----▲; lysate from HB101 (pBR322).

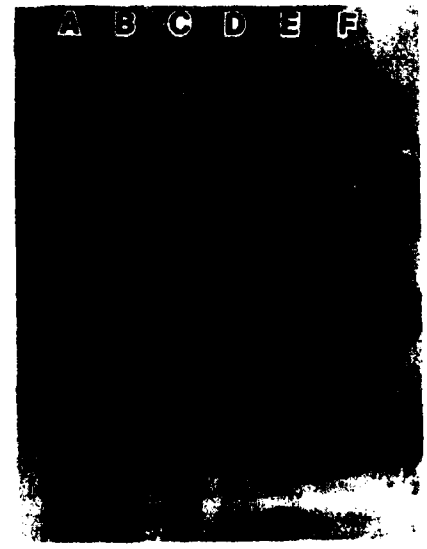

Figure 2. Western Blot of PA in Lysates from E. coli

Lysates from various strains of E. coli or PA purified from B. anthracis were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically to a nitrocellulose sheet and were reacted with rabbit anti-PA antibody, which was detected with an ELISA. Lanes A and F, lysate from E. coli HB1010 (pBR322); lane B, lysate from colony 36; lane C, lysate from colony 24; lane D, mixture of 5 ng purified PA and lysate from E. coli HB101 (pBR322); lane E, mixture of 5 ng purified PA and lysate from colony 36.

Figure 3. Elongation Response of Chinese Hamster Ovary (CHO) Cells to EF and E. coli-Produced PA

CHO cells were grown 24 hr in 24-well plates. Purified toxin components and dialyzed deoxycholate lysates of E. coli transformants were added to the indicated final concentrations. After 5 hr, the cells were photographed by phase contrast microscopy. (A) EF (200 ng/ml), and lysate from E. coli HB101 (pBR322) (100 μg/ml final concentration of total protein). (B) EF (200 ng/ml) and lysate from E. coli colony 36 (100 μg/ml final concentration of total protein).

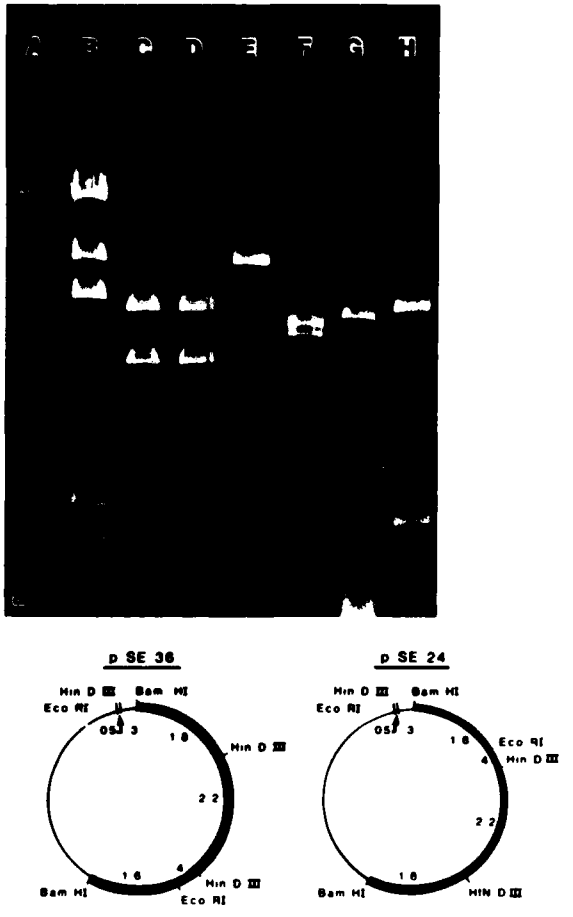

Figure 4. Restriction Digests of pBA1, pSE36, and pSE24 and Restriction Maps of pSE36 and pSE24

(a) Plasmids were extracted and digested by Bam HI, Eco RI, and Hind III. Lane A, pBA1-Bam HI; lane B, λ-Hind III; lane C, pSE24-Bam HI; lane D, pSE36-Bam HI; lane E, pSE24-Eco RI; lane F, pSE36-Eco RI; lane G, pSE24-Hind III; lane H, pSE36-Hind III. (b) Restriction maps were constructed from the above data and the restriction sites published for pBR322 (Covarrubias et al., 1981).

several restriction enzymes (Figure 4a) known to cleave the vector at a single site. Both pSE 24 and 36 have inserts of identical size that match the 6 kb fragment of Bam HI-digested pBA1. However, the Eco RI and Hind III digests of pSE 24 and 36 differ markedly, and analysis of these differences indicates that the 6 kb inserts are in opposite orientations in the vector (Figure 4b). Since PA is produced from the 6 kb insert regardless of its orientation, this BAM HI fragment must contain both transcriptional and translational elements able to function in E. coli.

Discussion

A library was constructed by cloning the fragments of pBA1 into the Bam HI site of pBR322. Of 40 colonies screened immunologically for PA production, two have a positive response, and a protein was demonstrated in lysates from both colonies that comigrated with PA and reacted with anti-PA. One of the biological functions associated with PA was present specifically in the E. coli lysates. Thus we conclude that the PA gene from B. anthracis has been cloned and that E. coli expresses a functional product, albeit at relatively low levels (10 ng/ml).

These results provide the first evidence that the recently discovered pBA1 directly codes for PA. Because of the complicated precedents of duplicate toxin genes in chromosomal and plasmid DNA of B. thuringiensis (Schnepf and Whitely, 1981; Klier et al., 1982; Held et al., 1982; Gonzales et al., 1982), it is not possible to exclude the possible existence of a PA gene in chromosomal DNA, nor is it possible to predict the location for genes encoding the other toxin components. The library of pBA1 is currently being screened for the presence of EF by techniques like these used for detecting PA.

Expression of PA by the 6 kb insert regardless of its orientation within the vector indicates the presence of a

Bacillus promoter and a ribosome-binding site. There is no obvious reason for the elevated level of PA production by colony 36 relative to colony 24, though it is possible that regulatory elements of the tetracycline-resistance gene of pBR322 have some effect. The Bacillus promoter must be quite strong in the homologous system as B. anthracis can produce up to 10 μg PA/ml culture supernatant (Ristroph and Ivins, 1983). It would be of interest to compare the strength of the PA promoter to that of other Bacillus promoters and to determine its usefulness for obtaining expression of other Bacillus genes.

A gene of 2.4–2.5 kb is required to encode a protein having the molecular weight of PA. The cloned fragment carrying the PA gene contains an additional 3.5 kb of extraneous DNA. If this extraneous DNA codes for other polypeptides, there could be complications in evaluating or producing a vaccine. Since neither EF nor LF could be detected by ELISA from the lysates of colonies 24 and 36, it is doubtful that the 6 kb insert contains an intact gene for either protein.

A number of specific genes from various species of Bacilli have been cloned into E. coli. They include genes from amylase (Yang et al., 1983; Willemot and Cornelis, 1983), sucrase (Fouet et al., 1982), penicillinase (Gray and Chang, 1981; Imanka et al., 1981), glucose dehydrogenase (Vasantha et al., 1983), δ-endotoxin (Schnepf and Whitely, 1981; Klier et al., 1982; Held et al., 1982), and for sporulation (Ollington et al., 1981; Dubnau et al., 1981; Rosenblath et al., 1981; Hirochika et al., 1981). In several cases, it has been possible to compare expression in E. coli with that in the donor species. Generally, expression was much less in E. coli for amylase, sucrase, penicillinase, and δ-endotoxin, even though the copy number was higher in E. coli. The one exceptional case involved glucose dehydrogenase, for which the recombinant E. coli had a greater specific activity. There appears to be a systematic problem for expression of Bacilli genes in E. coli, and this problem indeed affects the plans for testing the recombinant PA clones. The present level of PA in colony 36 is insufficient to conduct meaningful vaccine trials. Therefore it will be necessary to insert strong E. coli promoters in the recombinant DNA to boost PA production.

Experimental Procedures

Bacterial Growth and Plasmid Purification and Analysis

The strains used in this study were B. anthracis var. Sterne and a rifampin-resistant derivative selected from it by growth on solid medium containing 5 μg/ml of the drug. No detectable loss of toxin production nor curing of plasmid elements was noted in the (spontaneous) Rif^r mutant. Cultures of B. anthracis were grown at 37°C in a 14 l fermenter containing R medium (Ristroph and Ivins, 1983). Total cellular DNA was extracted by the method of Miura (1967), starting with at least 5 g wet weight of packed cells, and plasmid DNA was subsequently purified through two cycles of isopynic ultracentrifugation in CsCl/ethidium bromide gradients (Maniatis et al., 1982). Dye was removed from the DNA solutions with isoamyl alcohol (Maniatis et al., 1982), and the samples were dialyzed 12 hr against TE buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA). DNA was concentrated by ethanol precipitation and used either for electron microscopy (Mikesell et al., 1983) of intact plasmid or for restriction endonuclease digestion studies. The enzymes employed in this study were Bam HI, Bgl I, Eco RI, Hind III, Kpn I, Pst I, Sal I, Xba I, and T4 DNA ligase (Bethesda Research Laboratories). They were used according to the instructions of the manufacturer, except that a 10-fold excess of all restriction enzymes was used. Digests were analyzed by electrophoresis on 0.8% agarose gels (Maniatis et al., 1982) in TBE buffer.

Cloning of DNA

Vector (pBR322) and anthrax plasmid (pBA1) DNAs were digested to completion with Bam HI. This enzyme cleaves pBA1 into 11 or 12 fragments ranging in size from 4 to 25 kb. These fragments are two to ten times the size of the DNA required to code for PA (MW 85,000, unpublished data). The ligated DNA was introduced into E. coli HB101 via transformation (Bolivar and Backman, 1979), and transformants were initially selected on L agar (2% agar, 1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 20 μg/ml ampicillin. Ampicillin-resistant transformants were then screened for the inability to grow on L agar containing 10 μg/ml tetracycline.

Screening for Production of PA

Native proteins were extracted from cell pellets of putative recombinants by treatment with lysozyme and 0.1% sodium deoxycholate (Schleif and Wensink, 1981). The clarified lysates were assayed for PA by a double antibody sandwich ELISA (Voller et al., 1979). The capture and soluble antibodies were derived from goat and rabbit, respectively. A goat was immunized with 100 μg of "Michigan antigen," (Puziss et al., 1963), in Freund's complete adjuvant. The goat was then boosted twice at intervals of 8 weeks and bled 8 weeks after the last injection. A rabbit was immunized with 50 μg of purified PA in Freund's complete adjuvant. Booster injections, which contained 50 μg of PA in Freund's incomplete adjuvant, were given seven times at average intervals of 2 weeks. The rabbit was then bled 1 month after the last injection. PA-apecific antibodies were purified on columns containing PA linked to CNBr-activated Sepharose 4B (Pharmacia). The columns were prepared and operated according to the instructions of the manufacturer. A capture antibody was adsorbed to microtiter plates by incubating it in the wells at a concentration of 5 μg/ml in 0.05 M Na borate (pH 8.9). Nonspecific binding sites were saturated by overlaying with 1 mg/ml bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The microtiter plates were then washed with 0.05% Tween 20 in PBS (this washing is done after each of the three next steps). The samples and PA standards were then serially diluted in 10% fetal calf serum, 0.05% Tween 20 in PBS. The samples and standards were incubated in the plates for 2 hr at 37°C. A second (soluble) antibody to PA was added at a concentration of 1 μg/ml in 0.05% Tween 20 in PBS. Horseradish peroxidase covalently linked to Staph A protein (Surolia and Pain, 1981), at a final concentration of 500 ng/ml, was allowed to react with bound rabbit globulin and the peroxidase was detected colorimetrically with 2, 2′azino-di-(3-ethyl-benzthiazoline sulfonic acid) (ABTS) (Sigma) as the substrate (Engvall, 1980). The microtiter plates were scanned at 414 nm by a Flow Titertek Multiskan. The assay was capable of detecting PA at a concentration of 5 ng/ml.

A dot blot assay was also used for screening cultures for PA production. The E. coli from 10 ml cultures were pelleted and resuspended in 500 μl of 1 mg/ml lysozyme in TES buffer (0.05 M Tris-HCl, pH 8.5; 1 mM EDTA, 15% sucrose). After 10 min at room temperature, 100 μl of 25% SDS were added and the sample was boiled for 5 min. Aliquots (5 μl) of the lysates or of purified toxin components were spotted on gridded 85 mm nitrocellulose membranes (BA 85, Schleicher and Shuell), which were then incubated for 20 min in 0.05 M Hepes (pH 7.5), 0.1 M NaCl, 3% BSA. The membranes were then washed in the previous buffer containing 0.1% BSA. Following this, the membranes were incubated with affinity-purified rabbit anti-PA antibody for either 2 hr at room temperature or 18 hr at 4°C. After incubation, the membranes were reacted with horseradish peroxidase linked to Staph A protein (500 ng/ml) and the enzyme reaction was visualized with 250 μg/ml tetramethylbenzidine solubilized in 2 mg/ml dioctylsodium sulfosuccinate, 5% methanol, 12.5 mM Hepes, pH 7.5 (Ron Sekura, personal communication).

Electrophoretic Analysis of Proteins

Twenty μl of E. coli lysates, prepared as in the dot blot assay, were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and the proteins were electroblotted onto a nitrocellulose sheet with a transverse current (Towbin et al., 1979). Proteins that reacted with anti-PA antibody were detected by an ELISA as in the dot blot assay.

Cellular Elongation Assay

The Chinese hamster ovary (CHO) cell line and its growth conditions have already been described (Leppla, 1982). Native protein preparations from E. coli were dialyzed against 0.05 M Hepes (pH 7.5), 0.1 M NaCl and were incubated in 24-well plates with various combinations of *PA* and *EF* overlaid on the cell monolayer. The cell monolayer was photomicrographed under 400× magnification 3–5 hr later.

Acknowledgments

We thank Herb Green, Billy Lollar, and Chris Bolt for technical assistance. We are grateful to Perry Mikesell and Tom Dreier for performing the electron microscopy and to Perry Mikesell and Leonard A. Smith for their help in the preparation of this manuscript. M. H. V. is a senior National Research Council fellow.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 30, 1983

References

Beall, F. A., Taylor, M. J., and Thorne, C. B. (1962). Rapid lethal effects in rats of a third component found upon fractionating the toxin of *Bacillus Anthracis*. J. Bacteriol. *83*, 1274–1280.

Bolivar, F., and Backman, K. (1979). Plasmids of *E. coli* as cloning vehicles. Meth. Enzymol. *68*, 252.

Brachman, P. S., Gold, H., Plotkin, S. A., Fekety, F. R., Werrin, M., and Ingraham, N. R. (1962). Field evaluation of a human anthrax vaccine. Am. J. Pub. Hlth. *52*, 632–645.

Covarrubias, L., Cervantes, L., Covarrubias, A., Soberon, X., Vichido, P., Blanco, A., Kupeaztoch-Portney, Y. M., and Bolivar, F. (1981). Construction and characterization of new cloning vehicles V. Mobilization and coding properties of pBR322 and several deletion derivatives including pBR327 and pBR328. Gene *13*, 25–35.

Debnau, D., Ramakrishna, N., Cabane, K., and Smith, I. (1981). Cloning of an early sporulation gene in *Bacillus subtilis*. J. Bacteriol. *147*, 622–632.

Engvall, E. (1980). Enzyme immunoassays, ELISA and EMIT. Meth. Enzymol. *70*, 419–438.

Fish, D. C., Mahlandt, B. G., Dobbs, J. P., and Lincoln, R. E. (1968). Purification and properties of *in vitro*-produced anthrax toxin components. J. Bacteriol. *95*, 907–918.

Foust, A., Kiler, A., and Rapoport, G. (1982). Cloning and expression in *Escherichia coli* of the sucrase gene from *Bacillus subtilis*. Mol. Gen. Genet. *186*, 399–404.

Gladstone, G. P. (1946). Immunity to anthrax: protective antigen present in all cell-free culture filtrates. Br. J. Exp. Pathol. *27*, 394–418.

Gonzalez, J., Jr., Brown, B. J., and Carlton, B. C. (1982). Transfer of *Bacillus thuringiensis* plasmids coding for δ endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc. Nat. Acad. Sci. USA *79*, 6951–6955.

Gray, O., and Chang, S. (1981). Molecular cloning and expression of *Bacillus licheniformis* β-lactamase gene in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. *145*, 422–428.

Held, G. A., Bulla, L. A., Jr., Ferrari, E., Hoch, J., Aronson, A. I., and Minnich, S. A. (1982). Cloning and localization of the lepidopteran protoxin gene of *Bacillus thuringiensis* subsp. *kurstaski*. Proc. Nat. Acad. Sci. USA *79*, 6951–6955.

Hirochika, H., Kobayashi, Y., Kawamura, F., and Saito, H. (1981). Cloning of sporulation gene spoOB of *Bacillus subtilis* and its genetic and biochemical analysis. J. Bacteriol. *146*, 494–506.

Imanka, T., Tanaka, T., Tsunekawa, H., and Aiba, S. (1981). Cloning of the genes for penicillinase, *pen P* and *pen I*, of *Bacillus licheniformis* in some vector plasmids and their expression in *Escherichia coli*, *Bacillus subtilis*, and *Bacillus licheniformis*. J. Bacteriol. *147*, 776–786.

Kiler, A., Fargett, F., Ribier, J., and Rapoport, G. (1982). Cloning and expression of the crystal protein genes of *Bacillus thuringiensis* strain *berliner*. EMBO J. *1*, 791–799.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature *227*, 680–685.

Leppla, S. H. (1982). Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations in eukaryotic cells. Proc. Nat. Acad. Sci. USA *79*, 3162–3166.

Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982). Molecular Cloning—a Laboratory Manual. (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory), pp. 93–94 and 150–162.

Mikesell, P., Ivins, B. E., Ristroph, J. D., and Dreier, T. M. (1983). Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. *39*, 371–376.

Miura, K. (1967). Preparation of bacterial DNA by the phenol-pH9-RNases method. Meth. Enzymol. *12*, 543–545.

Molnar, D. M., and Altenbern, R. A. (1963). Alterations in the biological activity of protective antigen of *Bacillus anthracis* toxin. Proc. Soc. Exp. Biol. Med. *114*, 294–297.

Ollington, J. F., Halderwang, W. G., Huynh, T. V., and Losick, R. (1981). Developmentally regulated transcription in a cloned segment of the *Bacillus subtilis* chromosome. J. Bacteriol. *147*, 432–442.

Puzies, M., Manning, L. C., Lynch, J. W., Barclay, E. Abelow, I., and Wright, G. G. (1963). Large-scale production of protective antigen of *Bacillus anthracis* in anaerobic cultures. Appl. Microbiol. *11*, 330–334.

Ristroph, J. H., and Ivins, B. E. (1983). Production of high levels of *Bacillus anthracis* toxin antigens in a new, defined culture medium. Infect. Immun. *39*, 483–486.

Rosenblath, A., Banner, C. D. B., Losick, R., and Fitz-James, P. C. (1981). Identification of a new developmental locus in *Bacillus subtilis* by construction of a deletion mutation in a cloned gene under sporulation control. J. Bacteriol. *148*, 341–351.

Schleif, R. F., and Wensink, P. C. (1981). Practical Methods in Molecular Biology. (New York: Springer-Verlag), p. 16.

Schnepf, H. E., and Whiteley, H. R. (1981). Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proc. Nat. Acad. Sci. USA *78*, 2893–2897.

Stanley, J. L., and Smith, H. (1963). The three factors of anthrax toxin: their immunogenicity and lack of demonstrable enzymic activity. J. Gen. Microbiol. *31*, 329–337.

Stanley, J. L., Sargeant, K., and Smith, H. (1960). Purification of anthrax toxin factors I and II of the anthrax toxin produced *in vivo*. J. Gen. Microbiol. *22*, 206–218.

Surolia, A., and Pain, D. (1981). Preparation of protein A monoconjugate and its use as a reagent in enzyme immunoassays. Meth. Enzymol. *73*, 176–191.

Thorne, C. B., Molnar, D. M., and Strange, R. E. (1960). Production of toxin *in vitro* by *Bacillus anthracis* and its separation into two components. J. Bacteriol. *79*, 450–455.

Towbin, H., Staehlin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Nat. Acad. Sci. USA *76*, 4350–4354.

Vasantha, N., Uratani, B., Ramaley, R., and Freese, E. (1983). Isolation of a developmental gene of Bacillus subtilis and its expression in *Escherichia coli*. *Proc. Nat. Acad. Sci. USA 80*, 183–189.

Voller, A., Bidwell, D. C., and Bartlett, A. (1979). The Enzyme Linked Immunoeorbent Assay (ELISA). (Alexandria, Virginia: Dynatech Laboratories, Inc.), pp. 14–15.

Willemot, K., and Cornelis, P. (1983). Growth defects of *Escherichia coli* cells which contain the gene of an α-amylase from *Bacillus coagulans* on a multicopy plasmid. J. Gen. Microbiol. *129*, 311–319.

Yang, M., Galizzi, A., and Henner, D. (1983). Nucleotide sequence of the amylase gene from *Bacillus subtilis*. Nucl. Acids Res. *11*, 237–249.