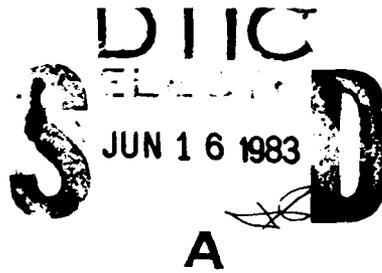


AD A129443

INFECTION AND IMMUNITY, Jan. 1983, p. 371-376  
0019-9567/83/010371-06\$02.00/0



Vol. 39, No. 1

## Evidence for Plasmid-Mediated Toxin Production in *Bacillus anthracis*

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Received 10 June 1982/Accepted 15 October 1982

Large-molecular-weight plasmids were isolated from virulent and avirulent strains of *Bacillus anthracis*. Each strain contained a single plasmid species unique from the others with respect to molecular weight. Bacterial strains were cured of their resident extrachromosomal gene pools by sequential passage of cultures at 42.5°C. Coincidental to the curing of plasmids was a loss of detectable lethal toxin and edema-producing activities and a dramatic decrease in lethal factor and protective antigen serological activities. The involvement of these plasmids in the production of toxin was firmly established by transformation of heat-passaged cells with plasmid DNA purified from the parent strain. The ability to produce parent strain levels of toxin was restored, and the plasmid DNA similar in molecular weight to that isolated from the parent was reisolated in all transformants examined. The exact role these plasmids play in the production of toxin remains to be elucidated. Two additional strains of *B. anthracis*, designated Pasteur vaccine strains, were examined for the ability to produce toxin and for the presence of plasmid DNA. Both strains were found to be nontoxigenic and contained no detectable plasmid elements. It is therefore likely that we, like Pasteur, cured *B. anthracis* strains of temperature-sensitive plasmids which code for toxin structural or regulatory proteins.

*Bacillus anthracis* is the etiological agent of anthrax, a highly infectious disease of considerable economic importance. Domestic livestock such as cattle, sheep, goats, and horses are the most common victims of the disease; however, human cases of anthrax often occur as a result of exposure to infected animals or animal products, such as hides, wool, meat, or bones (8). Most human anthrax cases are cutaneous in nature and respond favorably to the administration of antibiotics. However, gastrointestinal and pulmonary forms of the disease, which represent fewer than 5% of all human cases, are usually fatal (2).

To be considered virulent, the organism must not only be encapsulated but must also produce a tripartite toxin consisting of edema factor, protective antigen, and lethal factor (1, 12). The protective antigen component has been reported to effect transient alterations in neural and cardiovascular function in the challenged host (15), whereas edema factor or lethal factor alone is not biologically active. In combination with edema factor or lethal factor, protective antigen produces edema or death, respectively, in experimental animals (D. C. Fish and R. E. Lincoln, Fed. Proc. 26:1534-1538, 1967). This toxin was first demonstrated in the middle 1950s (11) and since has been the focus of attention of

numerous investigators who have sought to elucidate its role in the disease process. It is believed that an understanding of the mechanism of action of the toxin would contribute to the development of a safe, effective, and long-lasting human vaccine. Although Greenfield may in fact be due the honor (see reference 14), credit for the development of the first livestock vaccine effective against anthrax has historically been given to Louis Pasteur. A century ago at Pouilly-le-Fort, France, Pasteur successfully immunized animals against anthrax with a strain of *B. anthracis* which had been attenuated by repeated subculture at elevated temperature. Concerning this vaccine strain, Pasteur asked, "How is virulence lost during these eight days at 43°C?" (7). To date, virtually nothing has been reported concerning the genetics of anthrax toxin production or the molecular mechanisms involved in Pasteur's attenuation procedures.

It is well established that extrachromosomal genetic elements are responsible for many phenotypic characteristics of bacterial cells, including virulence factors (10). We therefore examined several strains of the anthrax bacillus for plasmid DNA and attempted to correlate the presence of these elements with the production of anthrax toxin. Additionally, by including two Pasteur vaccine strains in these studies it was

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anticipated that we might be able to answer the question asked by Pasteur so many years earlier.

(This work was presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, New Orleans, La., April 1982.)

#### MATERIALS AND METHODS

**Bacterial strains and media.** Two toxigenic nonencapsulated strains (Sterne and V770-NP1-R) and a toxigenic encapsulated strain (Vollum 1B) were obtained from the culture collection of the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md. Two Pasteur vaccine strains of *B. anthracis* were obtained from the American Type Culture Collection (ATCC 4229 and ATCC 6602). Bacterial cultures were routinely grown at 37°C in a chemically defined medium (0.8% sodium bicarbonate, 0.25% glucose, salts, amino acids, and vitamins) (J. D. Ristroph and B. E. Ivins, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, E68, p. 71).

**Plasmid isolation.** Cells were harvested by centrifugation from 20 ml of a mid-log phase culture, washed two times in TES buffer (50 mM Tris, [pH 8.0], 5 mM EDTA, 50 mM NaCl), and suspended in 2.0 ml of TES buffer with 20% sucrose. Lysozyme (10.5 mg/ml) and RNase (0.05 µg/ml; heat inactivated at 100°C for 10 min) were added, and the suspension was incubated with shaking for 90 min at 37°C. TES buffer (2.0 ml), 350 µl of 30% Sarkosyl, and 0.2 mg of predigested pronase were added, and incubation was continued for 30 min. The total volume of the lysate was brought to 7.5 ml with TES, and then CsCl (10.35 g) and ethidium bromide (3.0 ml, 4 mg/ml in sodium phosphate buffer [pH 7.0]) were added to the tube. The samples were centrifuged at  $140,000 \times g$  at 10°C for 40 h. Plasmid bands were removed from the tube, extracted with cesium chloride-saturated isopropanol, and dialyzed for 24 h against TES buffer.

**Gel electrophoresis.** Plasmid samples were subjected to electrophoresis in 1.2% agarose (Seakem Marine Colloids Inc., Portland, Maine), using Tris-borate running buffer (89 mM Tris base, 2.5 mM disodium EDTA, 8.9 mM boric acid) and tracking dye (0.7% bromophenol blue, 7% sodium dodecyl sulfate, 16% glycerol in water) (6). Samples were electrophoresed at 40 mA for 18 to 20 h. The gels were stained for 30 min in aqueous ethidium bromide (0.5 µg/ml) and then washed twice for 15 min each in distilled water. DNA bands were visualized on a Transilluminator C-63 (Ultraviolet Products, San Gabriel, Calif.) and photographed with Polaroid type 55 film through no. 9 and no. 23A Wratten gelatin filters.

**Electron microscopy.** Plasmid DNA samples were prepared and spread by using a modified formamide technique (4). The spreading solution contained 0.1 to 1.0 µg of DNA per ml, 0.045 mg of cytochrome *c* per ml, 10 mM EDTA, and 100 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 8.5) in 55% formamide. The hypophase was 39% less than the spreading solution in formamide, 10 mM Tris, and 1 mM disodium EDTA (pH 8.5). Films were allowed to stand for 1 min and then picked up on Parlodion-coated copper grids, rinsed in 80% ethanol, stained in 50 mM uranyl acetate, and rinsed again in 80% etha-

no. The grids were rotary shadowed with platinum-palladium wire (80:20) and examined on a Joel 100B electron microscope at an accelerating voltage of 60 kV. Molecules were measured with a Numonics Graphics Calculator.  $\phi$ X174 RF II DNA (New England Biolabs, Beverly, Mass.) was used as an internal length standard on all electron micrographs.

**Curing.** The strains were cultured and passaged for 10 consecutive days at 42.5°C in the chemically defined broth medium. At each passage, a 0.05-ml culture portion was inoculated into 5.0 ml of fresh medium and incubated further for 24 h. After the 10th passage, individual colonies from these broth cultures were isolated on sheep blood agar and then inoculated into 125 ml of fresh broth media ( $10^3$  colony-forming units per ml) and incubated for 18 h at 37°C. Bacteria-free culture supernatants were tested for edema-producing and lethal toxin activities, and cells were screened for the presence of plasmid DNA.

**Biological activity assays.** Bacteria were removed from cultures by centrifugation and filtration through a cellulose acetate membrane. The resulting cell-free supernatants were assayed for lethal toxin activity by intravenous injection into Fischer 344 rats (1, 5) and for edema-producing activity by intradermal injection into guinea pigs (11).

**Serological assay.** Supernatant fluids, concentrated 10-fold from heat-treated and untreated cultures were tested for soluble antigen by an Ouchterlony double diffusion assay (13). The antiserum (diluted 1:4 with 50 mM potassium phosphate buffer, pH 7.2) was obtained from guinea pigs which had received five biweekly intramuscular injections of concentrated Sterne supernatant (60 µg of total protein per injection) in 50% Freund incomplete adjuvant and were bled 9 weeks after the first injection.

**Supernatant protein.** Total supernatant protein of culture supernatants was assayed by using Coomassie blue (9). Each strain was inoculated into the defined liquid medium at a concentration of  $10^3$  colony-forming units per ml and incubated for 18 h with gentle agitation at 37°C. At this time the culture had attained approximately  $10^8$  colony-forming units per ml and was in early stationary phase. The cells were removed as described above, and the protein concentration in the supernatant was assayed.

**Transformation.** Transformation experiments were performed by the method of Chang and Cohen (3). Modifications of this procedure included increasing the lysozyme concentration (10.5 mg/ml) and incubating the cells for 3 to 4 h at 37°C after addition of the lysozyme. Cells obtained from cell wall regeneration media plates were stab inoculated onto immunoassay plates which contained the defined medium, 1% agarose, and 1.5 ml of antiserum from goats (immunized with viable spores of the Sterne strain). Parent and heat-treated isolates were used as controls on all assay plates. Transformants were identified by immunoprecipitin halos which formed around the colonies.

#### RESULTS

A sample agarose gel electrophoresis profile of the plasmid isolates is shown in Fig. 1. It was not possible to size these molecules based on gel migration data. Molecular weight estimates were



FIG. 1. Agarose gel profile of cesium chloride-purified plasmid DNA from strains of *B. anthracis*. Plasmid samples (50  $\mu$ l) were mixed with tracking dye (30  $\mu$ l) and electrophoresed as described in the text. The upper bands represent plasmid DNA and the lower dense bands are chromosomal (chr) DNA. (A) Pasteur vaccine strain (ATCC 4229), (B) Sterne, (C) V770-NP1-R, and (D) Vollum 1B. Lanes B, C, and D are plasmid preparations from parent strains; each contains a single plasmid species. Samples of heat-treated strains resembled the Pasteur strain (lane A) in that no plasmid DNA was detected, whereas plasmid DNA isolated from transformants presented an identical gel migration profile as V770-NP1-R (lane C).

obtained by electron microscopy; a sample electron micrograph of the Vollum 1B plasmid is shown in Fig. 2. The molecular weights of these plasmid species were found to range from  $60 \times 10^6$  to  $130 \times 10^6$  (Fig. 3). A length distribution histogram could not be constructed for Sterne plasmid DNA due to the very low number of intact molecules observed. These plasmid elements may be sensitive to shear forces generated in either the isolation protocol or the formamide spread technique. The few intact molecules measured indicate this plasmid has a molecular weight of approximately  $110 \times 10^6$ . Interestingly, the virulent Vollum 1B strain contained the smallest plasmid isolated from these strains. Plasmid elements were not detected by cesium chloride centrifugation or gel electrophoresis in cultures which had been passaged at  $42.5^\circ\text{C}$  nor in the two Pasteur vaccine strains.

**Biological and serological assays.** The biological activities of parent and heat-treated supernatants are shown in Table 1. Neither activity was

demonstrable in the culture supernatants from the heat-treated strains, whereas both lethal toxin and edema-producing activities were easily detectable in parent strain supernatants. Supernatant fluids, concentrated 10-fold, from heat-treated and untreated cultures were tested for soluble antigen by the Ouchterlony double-diffusion assay (Fig. 4). With each parent strain supernatant, two strong precipitin lines were consistently seen. The supernatant fluids from heat-treated strains occasionally showed a barely discernible precipitin line. A more sensitive assay was therefore performed by using either anti-protective antigen or anti-lethal factor-specific antiserum. Heat-treated strains were stab inoculated onto plates of solid media containing 1% agarose and the defined medium. After incubation for 48 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ , 1.0-mm-diameter wells were punched in the agar at a distance of 5.0 mm from the area of the growth and filled with specific antiserum, and the plates were incubated for an additional 48 h at  $4^\circ\text{C}$ . Faint single lines of precipitation between the areas of growth and the anti-lethal factor and anti-protective antigen antiserum wells suggested that toxin antigens were being produced at very low levels by the heat-treated strains. When assayed with Coomassie blue, the protein concentrations of the culture supernatants were found to be two to three times greater for the parent than for the heat-treated strains. The Pasteur vaccine strains were also similar to the heat-treated strains with respect to supernatant serological and biological activities and supernatant protein concentrations.

To determine whether the heat-treated strains were still virulent, vegetative cells of the parent Vollum 1B strain and cells of the same strain cured of resident plasmid DNA were injected intramuscularly into guinea pigs. A 50% lethal dose of  $6 \times 10^3$  was obtained with the animals injected with parent cells, whereas none of the guinea pigs injected with a maximum of  $10^7$  heat-treated cells died. No reversion to the parent phenotype of toxin production has been demonstrated in heat-treated strains regrown at  $37^\circ\text{C}$  for 10 daily passages, nor have plasmid elements been reisolated from these cultures.

**Transformation.** It was necessary to demonstrate that the elimination of plasmids was not a coincidental event to heat alteration of chromosomal-borne toxin genes. Transformation experiments were performed with either heat-treated V770-NP1-R or Pasteur strain cells and plasmid DNA purified from the parent V770-NP1-R strain. Five transformants were identified by immunoprecipitin halos which formed around the colonies on the immunoassay plates. The supernatants from broth cultures of these transformants and from cultures of colonies which

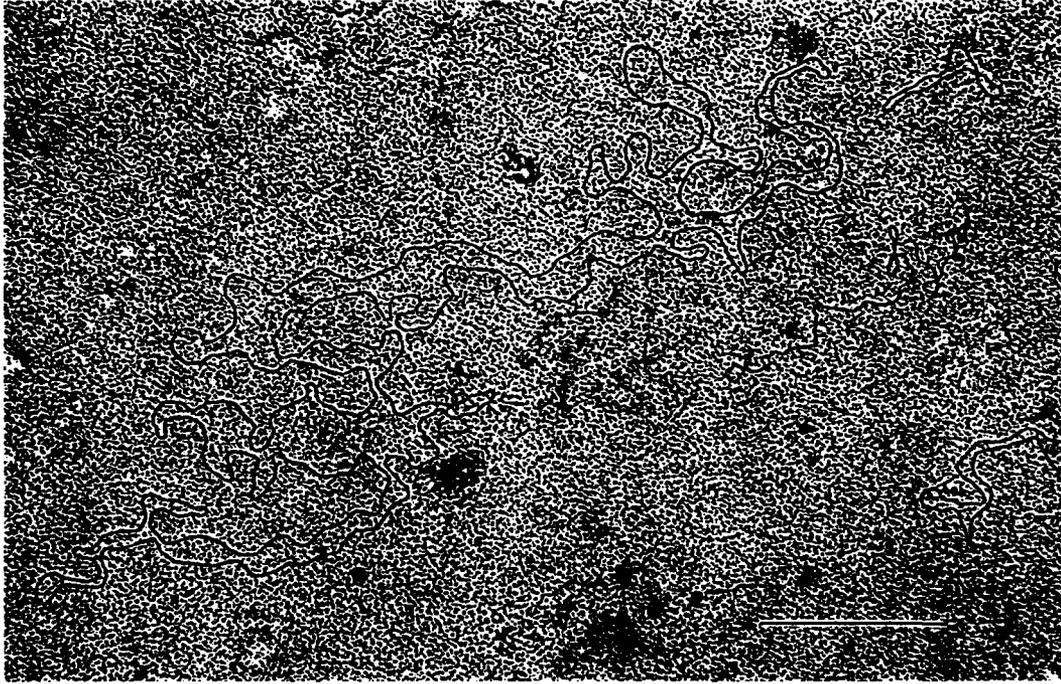


FIG. 2. Electron micrograph of plasmid DNA isolated from parent Vollum 1B.  $\phi$ X174 DNA (3.558 megadalton) internal standard is shown in upper right corner. Bar represents 1  $\mu$ m.

did not evidence halos were reassayed for lethal toxin and edema-producing activities. Both activities were restored, and plasmid DNA, similar in molecular weight to that of the parent strain, was reisolated only from cultures of the five transformants.

#### DISCUSSION

The results of these studies demonstrate that plasmid DNA is involved in the production of toxin by *B. anthracis*; however, the specific role

remains to be elucidated. A considerable effort is being expended in our laboratory to determine whether plasmid toxin genes are structural or regulatory in nature. In addition to the strains of *B. anthracis* described in this study, several other strains of the organism, including bovine isolates, have been examined by the parameters described (data not shown). All strains contain a large-molecular-weight plasmid which is successfully eliminated by growing cultures at elevated temperature. These cured isolates also

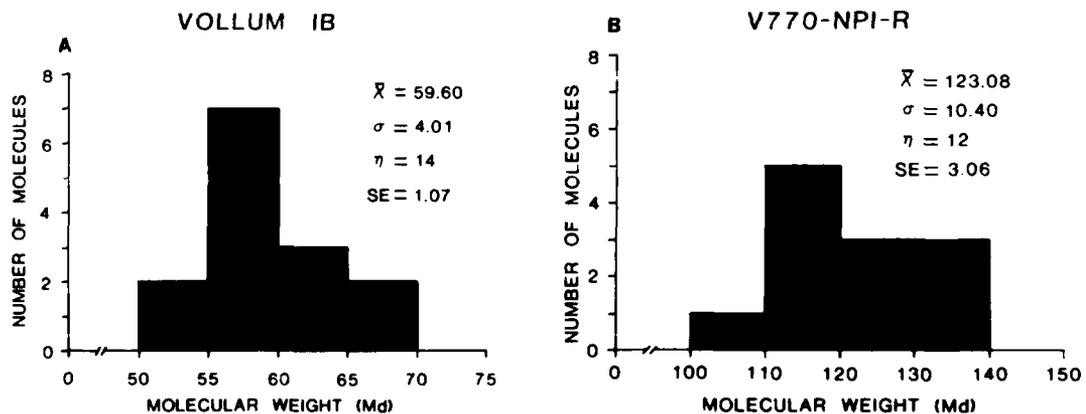


FIG. 3. Length distribution of Vollum 1B (A) and V770-NPI-R (B) plasmid molecules. Statistical parameters are shown for each graph.

TABLE 1. Culture supernatant biological activities in parent and heat-treated strains of *B. anthracis*

Strain	Biological activity	
	Edema-producing <sup>a</sup>	Lethal toxin, (toxic units ml) <sup>b</sup>
Parent		
Sterne	8	60
V770-NP1-R	8	60
Vollum 1B	8	150
Heat-treated		
Sterne	<1 <sup>c</sup>	<2 <sup>d</sup>
V770-NP1-R	<1 <sup>c</sup>	<2 <sup>d</sup>
Vollum 1B	<1 <sup>c</sup>	<2 <sup>d</sup>

<sup>a</sup> Expressed as the reciprocal of the maximum dilution of culture supernatant yielding a positive response when 0.2 ml was injected into guinea pig skin.

<sup>b</sup> Lethal potency of supernatants was determined by the method of Haines et al. (6).

<sup>c</sup> No demonstrable positive response with either diluted or undiluted culture supernatant.

<sup>d</sup> No lethality seen in rats injected intravenously with 8.0 ml of undiluted culture supernatant.

contain no detectable lethal toxin or edema-producing activities. The association of a large plasmid with the production of toxin may be a universal characteristic for this species; however, it is conceivable that toxigenic plasmid-

negative variants may eventually be found. Such might be the case in strains in which the plasmid is integrated into the chromosome.

The toxin antigens used to produce the specific antisera employed in these studies were purified by alternate methodologies in separate laboratories (S. H. Leppla and K. W. Hedlund, personal communication). The faint lines of precipitation on immunoassay plates with specific antisera, therefore, suggest that the cured strains are producing low levels of toxin antigens and that the lines of identity do not represent the recognition of other nonspecific supernatant proteins. We have never detected the presence of an additional plasmid species in any of the strains examined; however, we cannot rule out the existence of a temperature-stable plasmid which may encode for toxin proteins and is not resolved by our DNA isolation protocol.

Parent and heat-treated strains have been examined for other phenotypic markers which may be plasmid encoded. There are no indications that plasmid DNA is involved in the processes of encapsulation, sporulation, or metabolism. Furthermore we have not been able to identify drug or heavy metal resistance factors which may be carried by these extrachromosomal elements.

In assessing Pasteur's experimental regimen by utilizing modern analytical techniques, we are able to offer a reasonable explanation for a

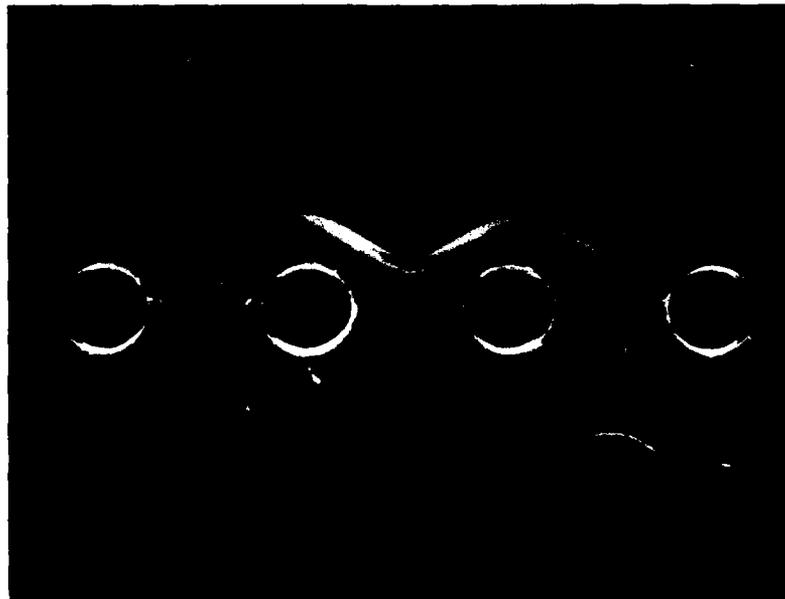


FIG. 4. Ouchterlony double-diffusion assay of antigens in supernatants of parent and heat-treated strains of *B. anthracis*. The top row of wells contained 10× concentrated supernatants from stationary phase liquid cultures of *B. anthracis* strains (left to right) Vollum 1B, Sterne, and V770-NP1-R. The bottom row of wells contained 10× concentrated supernatants from stationary phase liquid cultures of heat-treated *B. anthracis* strains. The middle row of four wells contained antiserum. The faint precipitin line described in the text is located by the arrow.

century-old molecular event which has had such a significant impact in the field of medical microbiology: it is very likely that his attenuation of the anthrax bacillus occurred as a result of curing the strain of a plasmid component which encoded for toxin structural or regulatory proteins.

The primary goal in our research with the anthrax bacillus is to develop a safe and more effective human vaccine. It is anticipated that the use of recombinant DNA technology will significantly influence our future work with this goal in mind. It is conceivable that these studies may also facilitate not only a better understanding of the molecular interactions of each toxin moiety but also of the mechanism of action of the anthrax holotoxin.

#### ACKNOWLEDGMENTS

We thank Guy E. Campbell for excellent technical assistance. We thank S. H. Leppla and K. W. Hedlund for the specific antisera used in these studies. We appreciate the clerical assistance of Diane C. Beall.

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