

## Contribution of Individual Toxin Components to Virulence of *Bacillus anthracis*

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Three proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF; a calmodulin-dependent adenylate cyclase), compose the lethal (PA+LF) and edema (PA+EF) toxins secreted by *Bacillus anthracis*. Mutant strains, each deficient in the production of one toxin component, were constructed, and their virulence was then studied. A kanamycin resistance cassette was inserted in each *cya* (encoding EF) and *lef* (encoding LF) gene, and the constructs were separately introduced into *B. anthracis* Sterne on a mobilizable shuttle plasmid. An EF<sup>-</sup> strain and an LF<sup>-</sup> strain were then isolated after homologous recombination with the resident toxin-encoding plasmid, pXO1. Spores from these mutants and from a previously constructed PA<sup>-</sup> mutant were used to inoculate mice, and the lethality and local edema formation were monitored. LF<sup>-</sup> or PA<sup>-</sup> mutants were not lethal even at high inocula, whereas the EF<sup>-</sup> mutant induced lethal infections. This indicates that LF in combination with PA is a key virulence factor required for lethality. Skin edema formation was observed with the LF<sup>-</sup> mutant, which produces only the combination of PA and EF. However, EF<sup>-</sup> and LF<sup>-</sup> mutants were significantly less efficient at inducing, respectively, lethality and edema than was the parental Sterne strain. These results suggest that the three toxin components might act synergistically in vivo to cause lethality and edema formation.

*Bacillus anthracis*, the etiological agent of anthrax is a gram-positive bacterial pathogen of humans and animals. It secretes two toxins, the edema and lethal toxins, made up of three different proteins, protective antigen (PA; 85 kDa), lethal factor (LF; 83 kDa), and edema factor (EF; 89 kDa) (8, 18, 28). Both toxins are organized according to the A-B-type model, in which a B domain involved in cellular receptor binding is associated with an A domain displaying toxic and usually enzymatic activities (10). In the case of anthrax toxins, the A and B domains are two separate proteins. The PA factor is the B component in both the edema and lethal toxins. PA is the receptor-binding component mediating entry of either LF or EF into target cells (17). EF has been shown to be a calmodulin-dependent adenylate cyclase (16). No enzymatic activity has yet been identified for LF. Consistent with the A-B model, none of the three individual proteins is toxic for animals (27). However, PA combined with LF forms the lethal toxin, whose intravenous injection leads to death in experimental animals (3, 27). Edema toxin consists of PA and EF. It induces an increase in intracellular cyclic-AMP levels in eukaryotic cells (16) and elicits skin edema after subcutaneous injection (7).

Fully virulent strains of *B. anthracis* are encapsulated and toxigenic. Formation of the capsule, a poly-D-glutamic acid polymer (2), is dependent on the presence of plasmid pXO2 (11, 34). The toxin production is conferred by plasmid pXO1 (21), which carries the structural genes encoding PA, EF, and LF. These three genes, respectively *pag*, *cya*, and *lef*, have been cloned and sequenced (4, 6, 21, 38), thus providing the basis for genetic studies.

The pathogenesis of infection by *B. anthracis*, the role of the two toxins in the disease, and the molecular mechanism of the lethal effect of the toxins remain poorly understood. Most of the available data have been obtained with purified

toxin components (3, 7, 27) or by studying animal susceptibility to *B. anthracis* strains (35, 37). The Sterne strain has been widely used in animal models. This unencapsulated strain, carrying only plasmid pXO1, is toxigenic and is used as a live veterinary vaccine against anthrax (12, 29, 36). Nevertheless, the inoculation of mice with high doses of Sterne strain spores causes a lethal disease that seems to be identical to anthrax caused by a fully virulent encapsulated *B. anthracis* strain (35). The pathogenesis of infection with the Sterne strain is the result of the effects of the three components (PA, EF, and LF) produced by this strain. This paper describes an analysis of the role of individual toxin components accomplished by inactivating each toxin gene encoded by pXO1.

As previously reported (31), a conjugal transfer system which can be used to transfer DNA into *B. anthracis* is available. We devised a genetic strategy allowing the construction of a mutant strain deficient in PA (5) but producing normal amounts of LF and EF. In the present work, the strategy has been extended to the genes encoding EF and LF. We obtained and characterized Sterne strain mutants producing only one toxin: either lethal toxin (PA+LF) or edema toxin (PA+EF). Analysis of the virulence of these mutants in the mouse provides new insights into the contribution of each toxin to the pathogenesis of *B. anthracis*.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture media.** *Escherichia coli* HB101 harboring the mobilizing plasmid pRK212.1 (31) was used in conjugal transfer experiments. Shuttle plasmid pAT18 (32), conferring erythromycin resistance (Erm<sup>r</sup>), was used as the vector for transferring DNA into *B. anthracis* strains. Recombinant plasmids used in this work are listed in Table 1. *Bacillus subtilis* SMY was used as a control. *E. coli* was grown in L broth or L agar (22), and *B. anthracis* was grown in brain-heart infusion medium (Difco Laboratories,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Plasmid	Relevant characteristics	Source or derivation
<b>Strain</b>			
<i>B. anthracis</i> 7700	None	Nal <sup>r</sup>	7702 derivative cured of pXO1
<i>B. anthracis</i> 7702	pXO1	PA <sup>+</sup> EF <sup>+</sup> LF <sup>+</sup>	Sterne strain from the Pasteur collection
<i>B. anthracis</i> RP8 (PA <sup>-</sup> )	pXO1- <i>pag</i> Δ322 <sup>a</sup>	PA <sup>-</sup> EF <sup>+</sup> LF <sup>+</sup> Erm <sup>r</sup>	7702 derivative (5)
<i>B. anthracis</i> RP9 (EF <sup>-</sup> )	pXO1- <i>cya</i> Δ303	PA <sup>+</sup> EF <sup>-</sup> LF <sup>+</sup> Kan <sup>r</sup>	7702 derivative (this work)
<i>B. anthracis</i> RP10 (LF <sup>-</sup> )	pXO1- <i>lef</i> Δ238	PA <sup>+</sup> EF <sup>+</sup> LF <sup>-</sup> Kan <sup>r</sup>	7702 derivative (this work)
<i>B. subtilis</i> SMY	None		Gift of A. L. Sonenshein
<b>Plasmid</b>			
pAT18		Shuttle plasmid; Mob <sup>+</sup> Erm <sup>r</sup>	32
pCPL110		pAT18 recombinant plasmid carrying inactivated <i>lef</i> gene; Kan <sup>r</sup> Erm <sup>r</sup>	
pMMA110		pAT18 recombinant plasmid carrying inactivated <i>cya</i> gene; Kan <sup>r</sup> Erm <sup>r</sup>	

<sup>a</sup> The number indicates the gene codon at which the deletion starts.

Detroit, Mich.) or in NBY medium for preparation of spores (11). R medium (25) was used for toxin production. Antibiotics were used at the following concentrations: ampicillin, 100 µg/ml in *E. coli* cultures; kanamycin, 50 and 20 µg/ml in *E. coli* and *B. anthracis* cultures, respectively; erythromycin, 180 and 10 µg/ml for *E. coli* and *B. anthracis* cultures, respectively.

**Plasmid preparation and recombinant DNA analysis.** pXO1 was prepared by the method of Green et al. (11). Methods for recombinant DNA manipulations were as described by Maniatis et al. (19). DNA fragments were prepared from agarose gel by using the GeneClean Kit (Bio 101, La Jolla, Calif.). The procedure to subclone the *lef* gene was as follows: a 3.5-kb *SacI*-*Clai* DNA fragment of pXO1 containing the *lef* locus (26) and derived from a 9-kb *SacI* DNA fragment was cloned into pUC18 (39) and pAT18 (32).

**Mating procedure.** The recombinant shuttle plasmids were transferred from *E. coli* to *B. anthracis* 7702 by conjugation. The filter mating system was used as previously described (5, 31). *E. coli* and *B. anthracis* were grown in L and brain heart infusion media, respectively:  $5 \times 10^8$  *E. coli* and  $1 \times 10^8$  *B. anthracis* cells were mixed, washed to eliminate antibiotics, and loaded onto a Millipore 0.45-µm-pore-size filter supported on brain heart infusion agar. After 15 h of incubation at 37°C, cells were resuspended and plated on selective media containing appropriate antibiotics.

**Adenylate cyclase assay.** Adenylate cyclase was assayed in *B. anthracis* R-medium culture supernatants as previously described (14). Enzymatic activity is expressed in units per milliliter. One unit corresponds to 1 nM cyclic AMP formed in 1 min at 30°C.

**Protein analysis.** Sodium dodecyl sulfate-polyacrylamide (8%) gel electrophoresis was performed as described by Laemmli (15). Gels were either stained with Coomassie blue or subjected to immunoblot analysis (30). Western blots (immunoblots) were probed with rabbit sera raised against LF or PA purified from the polyacrylamide gel or against a truncated form of EF (CYA62) (13). Immunodetected proteins were visualized with <sup>125</sup>I-labeled protein A and autoradiography on X-ray films.

**Preparation of spores.** Stocks of *B. anthracis* strains were prepared as follows. Bacteria were streaked on NBY agar slopes and incubated for 7 days at 30°C. Spore formation was monitored by phase microscopy. Five milliliters of sterile distilled water was added to each slope, and the samples were suspended. The spore suspension, in a sterile screw-

cap tube, was incubated at 65°C in a water bath for 30 min to kill any remaining bacilli. The spores were collected by centrifugation and resuspended in water (1/20 [vol/vol] of the original culture) to give samples containing at least 90% spores and 10<sup>8</sup> CFU/ml. These spore stocks were divided into 5-ml aliquots and stored at 4°C. Prior to each infection in mice, aliquots were diluted and viable spore counts were estimated. Spores of *B. subtilis* were obtained in L broth after incubation of bacteria for 17 h at 37°C and were prepared according to the same procedure.

**Infection of mice.** Female 3- to 6-week-old pathogen-free Swiss mice were supplied by Charles River (Saint-Aubin-lez-Elboeuf, France). Animals were fed a sterilized vitamin-supplemented diet and sterile water (pH 3). Mortality was monitored by subcutaneously inoculating groups of 10 mice with appropriate viable spore doses (in a volume of 0.2 ml). The 50% lethal dose (LD<sub>50</sub>) was determined for groups of 10 mice by the probit method (1). Bacterial survival in host tissues was monitored by infecting mice in the right hind footpad with large doses of spore suspensions (in a volume of 0.1 ml). The magnitude of the inflammatory reaction induced in the infected footpad was measured at intervals with dial callipers (Schnelltaster, Hessen, Federal Republic of Germany) and compared with the size of the contralateral noninfected footpad. The results are expressed in units (0.1 mm) of footpad thickness. Groups of five infected mice were sacrificed at intervals, and feet were sectioned, washed with sodium hypochlorite solution (1 min) to kill skin contaminants and then with sterile phosphate-buffered saline (pH 7.2) (1 min), and ground in phosphate-buffered saline. One hundred microliters of serial 10-fold dilutions in phosphate-buffered saline were plated on brain heart infusion agar. Results were expressed as the log<sub>10</sub> bacterial count per foot.

## RESULTS

**Construction and characterization of *B. anthracis* EF<sup>-</sup> or LF<sup>-</sup> strains.** A genetic strategy has previously been devised and used for the construction of a *B. anthracis* strain, RP8, deficient for PA (5). A similar strategy was used for the inactivation of the *lef* and *cya* genes on pXO1, which encode LF and EF, respectively. The details of the constructions are given in Fig. 1. The first step was performed in *E. coli*. Deletions removing part of *cya* and *lef* were followed by insertion of a kanamycin resistance cassette (33). The DNA fragments carrying the inactivated *cya* or *lef* genes were then

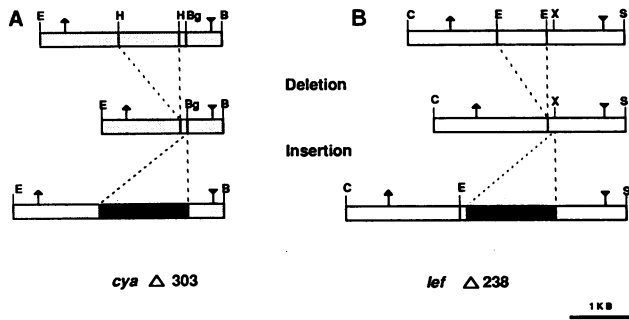


FIG. 1. Schematic representation of the construction of interrupted *cya* (A) and *lef* (B) genes. DNA fragments of pXO1 carrying the *cya* and *lef* genes are represented by shadowed (A) and empty (B) boxes (↑ and ▾ indicate the start and stop codons of the genes, respectively). The 1.5-kb *Kan<sup>r</sup>* cassette is symbolized by a black box. Constructions were as follows. (A) Plasmid pMMA861 (13), carrying the 3.2-kb *EcoRI*-*Bam*HI DNA fragment of pXO1, was used. A 1-kb *Hind*III deletion ( $\Delta$ 303) starting at codon 303 was created, and the *Kan<sup>r</sup>* cassette was inserted at the *Bgl*II site (6). The resulting DNA fragment was cloned into pAT18, yielding shuttle plasmid pMMA110. (B) The 3.5-kb *Clal*-*Sac*I DNA fragment was cloned directly into pAT18 prior to construction of the interrupted genes. A 0.7-kb *Eco*RI deletion ( $\Delta$ 238) starting at codon 238 was created, and the *Kan<sup>r</sup>* cassette was inserted at the *Xho*I site (4). The resulting shuttle plasmid was called pCPL110. Restriction sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; C, *Clal*; E, *Eco*RI; H, *Hind*III; S, *Sac*I; X, *Xho*I.

cloned into pAT18 (32). The resulting recombinant shuttle plasmid pCPL110 (*lef* $\Delta$ 238) or pMMA110 (*cya* $\Delta$ 303) was transferred by conjugation into *B. anthracis* 7702, yielding *Erm<sup>r</sup>* *Kan<sup>r</sup>* transconjugants which harboured both pXO1 and one recombinant plasmid (pCPL110 or pMMA110). These transconjugants were cultured in the absence of erythromycin (selective antibiotic for pMMA110 or pCPL110) but in the presence of kanamycin (selecting for the maintenance of the cassette). *Kan<sup>r</sup>* *Erm<sup>s</sup>* clones which presumably had lost the recombinant plasmid and integrated the *Kan<sup>r</sup>* cassette into pXO1 by homologous recombination were isolated (5). Two clones called RP9 and RP10, which originated from transconjugants carrying pMMA110 or pCPL110, respectively, were thus obtained and used for subsequent experiments.

For both strains, replacement of the wild-type *lef* or *cya* gene on pXO1 by the corresponding deleted copy carrying the *Kan<sup>r</sup>* cassette was verified. In plasmid pXO1, the *lef* gene is located on a 9-kb *Sac*I fragment (26) and the *cya* gene is located on a 7-kb *Bam*HI fragment (6, 23). *Bam*HI or *Sac*I digests of recombinant pXO1 plasmids originating from strains RP9 and RP10, respectively, were cloned into pUC19. Recombinant pUC derivative plasmids, able to confer *Kan<sup>r</sup>* on *E. coli*, were obtained in both cases. Restriction enzyme analysis of these plasmids indicated that they contained DNA fragments carrying the expected constructions (Fig. 1). These results therefore confirmed that the inactivated genes *cya* $\Delta$ 303 and *lef* $\Delta$ 238 were present on pXO1, yielding *EF<sup>-</sup>* (RP9) and *LF<sup>-</sup>* (RP10) strains.

**Toxin production in mutant strains of *B. anthracis*.** The three anthrax toxin components PA, EF, and LF are produced by *B. anthracis* 7702 when bacteria are grown in R medium (25). These three proteins were easily detected in supernatants by Western blot analysis with specific antisera raised against EF, LF, or PA (Fig. 2, 7702). EF has adenylate cyclase activity. The adenylate cyclase activity in the

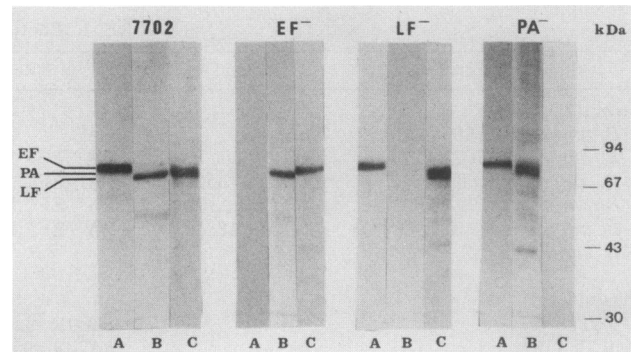


FIG. 2. Proteins contained in 1-ml culture supernatants of *B. anthracis* Sterne 7702 or mutants *EF<sup>-</sup>* (RP9), *LF<sup>-</sup>* (RP10), and *PA<sup>-</sup>* (RP8) were analyzed by immunoblot and probed with anti-EF (A), anti-LF (B), or anti-PA (C) immune serum.

supernatants of cultures of the *EF<sup>-</sup>* strain and parental strain 7702 was therefore determined. In *EF<sup>-</sup>* strain supernatants, no adenylate cyclase activity was found, whereas in strain 7702 supernatants, 200 U of activity per ml was found. Consistently, supernatants of RP9 cultures contained no EF-related protein, as determined by Western blot analysis (Fig. 2, *EF<sup>-</sup>*, lane A), thus confirming the inactivation of the *cya* gene in this strain. No LF-related protein was detected by Western blot of supernatants of RP10 cultures (Fig. 2, *LF<sup>-</sup>*, lane B), but the adenylate cyclase activity was identical to that of strain 7702 (200 U/ml). As previously described (5), *PA<sup>-</sup>* mutant RP8 was deficient in the production of PA (Fig. 2, *PA<sup>-</sup>*, lane C) but still produced adenylate cyclase (300 U/ml) and LF in supernatants. These results clearly show that each of the three mutants, *PA<sup>-</sup>*, *EF<sup>-</sup>*, and *LF<sup>-</sup>*, are specifically defective for the production of only one toxin component and can therefore be used in an experimental model to study the contribution of these proteins to the pathogenesis of *B. anthracis* infection.

**Virulence of *B. anthracis* mutants in mice.** The virulence of the three *B. anthracis* mutants was studied in mice by using parental strain 7702, pXO1-free strain 7700, and an unrelated avirulent *B. subtilis* strain, SMY, as controls. The *LD<sub>50</sub>* of each strain was first estimated by subcutaneously inoculating groups of 10 mice with a series of doses of spores ( $10^4$  to  $10^9$ ) from these strains, and mortality was monitored for 15 days. The results are reported in Table 2. Parental strain 7702, which produces the three toxin components, induced a rapidly lethal infection (3 to 4 days). The *LD<sub>50</sub>* was estimated to be  $10^6$  spores per mouse. As expected, pXO1-free strain 7700, the *PA<sup>-</sup>* mutant, and *B. subtilis* were totally avirulent,

TABLE 2. Virulence in mice of different strains

Bacterial strain	Presence of virulence factor:			<i>LD<sub>50</sub></i> <sup>a</sup>
	EF	PA	LF	
<i>B. anthracis</i>				
Sterne 7702	+	+	+	$10^6$
7700	-	-	-	$>10^9$
Mutant <i>EF<sup>-</sup></i>	-	+	+	$10^7$
Mutant <i>PA<sup>-</sup></i>	+	-	+	$>10^9$
Mutant <i>LF<sup>-</sup></i>	+	+	-	$>10^9$
<i>B. subtilis</i> SMY	-	-	-	$>10^9$

<sup>a</sup> Determined by subcutaneously inoculating groups of 10 mice with viable spore doses. Mortality was monitored for 2 weeks.

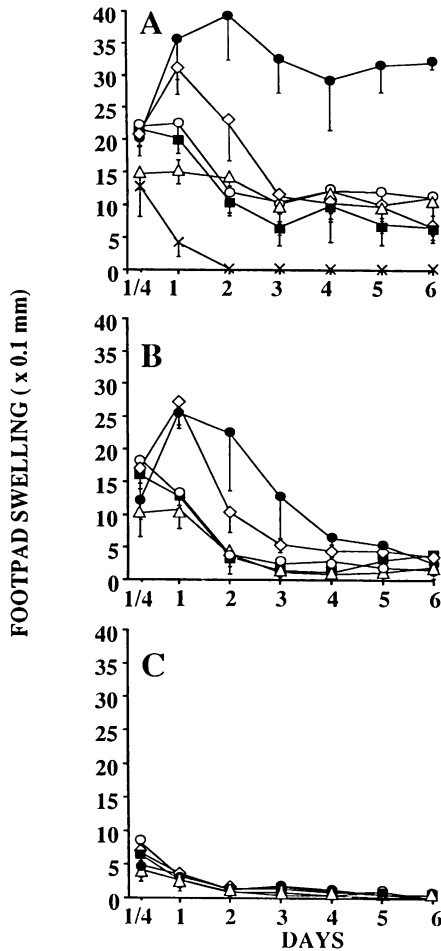


FIG. 3. Inflammatory reaction induced in footpads of infected mice. Groups of 10 mice were inoculated with different *B. anthracis* strains: Sterne 7702 (●), 7700 (○), EF<sup>-</sup> mutant (△), LF<sup>-</sup> mutant (◇), PA<sup>-</sup> mutant (■), and *B. subtilis* SMY (×). Mice were infected with 10<sup>8</sup> (A), 10<sup>7</sup> (B), or 10<sup>6</sup> (C) spores. One of the mice infected with 10<sup>8</sup> spores of strain 7702 died by day 5. The size of the edema was measured as described in Materials and Methods.

with a LD<sub>50</sub> of >10<sup>9</sup> spores. The LF<sup>-</sup> mutant totally abolished the lethality of *B. anthracis* regardless of the production of adenylate cyclase (EF). In contrast, the EF<sup>-</sup> mutant caused mortality in mice within 3 to 4 days. The estimated LD<sub>50</sub> was 10<sup>7</sup> spores, which is 1 log<sub>10</sub> unit higher than that of the wild type. These results clearly indicate that adenylate cyclase contributes to the expression of virulence in mice.

The magnitude of the inflammatory reaction elicited by the various strains was then estimated by inoculating spores intradermally into the right hind footpads of mice. The kinetics of footpad edema was monitored with a dial calliper. The results are illustrated in Fig. 3. The inoculation of 10<sup>8</sup> spores induced a rapid swelling (13 to 22 U, with 1 U corresponding to 0.1 mm) after 6 h. The subsequent kinetics of the inflammatory reaction was dependent on the virulence factors expressed by the strains. Strain 7700 and EF<sup>-</sup> and PA<sup>-</sup> mutants behaved similarly in that they produced a weak inflammatory reaction. Strain 7702 and LF<sup>-</sup> mutants induced edema formation that reached a peak of 30 to 40 U after 24 to 48 h. This strong inflammatory reaction further

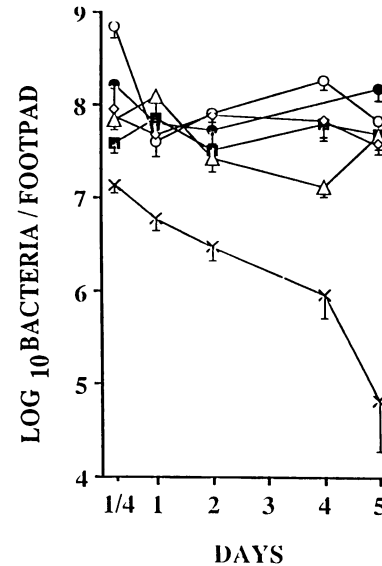


FIG. 4. Growth curves of *B. anthracis* strains and *B. subtilis* SMY. Mice were inoculated in the right hind footpad with 10<sup>8</sup> spores of Sterne 7702 (●), 7700 (○), EF<sup>-</sup> mutant (△), LF<sup>-</sup> mutant (◇), PA<sup>-</sup> mutant (■), or *B. subtilis* SMY (×). Strain Sterne 7702 induced mortality by days 3 and 4 (three mice died by day 3 and two more died by day 4; numbers of mice were sufficient to have groups of five mice per time point). Bacterial growth was monitored as described in Materials and Methods.

persisted only with strain 7702. As shown in Fig. 3B and C, edema formation was dose dependent, with almost no effect from an inoculum of 10<sup>6</sup> spores. To determine the effect of differential local bacterial survival in host tissues on the inflammatory reaction, bacteria were counted in the footpads of mice inoculated with high challenges (10<sup>8</sup> spores). As shown on Fig. 4, bacterial growth curves were remarkably similar for all strains of *B. anthracis*, with a long-lasting plateau of around 10<sup>8</sup> bacteria per footpad for 5 days. *B. subtilis* wild-type strain SMY was eliminated from host tissues, with a 3-log decrease by day 5 of infection (Fig. 4). These data indicate that the differences observed in the magnitudes of inflammatory reactions caused by *B. anthracis* mutants are not related to the survival of bacteria in the footpads but presumably to the local production of toxins. That all strains of *B. anthracis*, including 7700, survived significantly longer than *B. subtilis* in the mouse means that bacterial survival is not related to toxin production but might be due to intrinsic properties of *B. anthracis*.

## DISCUSSION

In this work, we constructed and studied *B. anthracis* mutant strains derived from the parental toxigenic 7702 Sterne strain and producing both lethal (PA+LF) and edema (PA+EF) toxins. EF<sup>-</sup> and LF<sup>-</sup> mutants were obtained by well-characterized deletions within the *cya* and *lef* genes, respectively. In both mutants, the mutations appear to abolish gene expression, since no truncated gene products (Fig. 2) related to either EF or LF could be detected. The EF<sup>-</sup> mutant did not produce any adenylate cyclase, secreted normal amounts of PA+LF, and thus synthesized only lethal toxin. The LF<sup>-</sup> mutant produced normal amounts of PA+EF and thus of edema toxin.

EF<sup>-</sup> and LF<sup>-</sup> mutants obtained according to the strategy

previously used for constructing the PA<sup>-</sup> mutant strain (5) complete the series of genetic constructs required for studying the role of each toxin component in *B. anthracis* pathogenesis. PA<sup>-</sup>, EF<sup>-</sup>, and LF<sup>-</sup> mutants carry well-defined mutations, which were introduced into pXO1 by exchanging the wild-type *pag*, *cya*, and *lef* genes with their mutated alleles. The mutant strains are therefore specifically deficient for PA, EF, and LF, and as a consequence of the procedure used, reversion to wild type cannot occur. The precise genetic characterization and the stability of these strains make them highly reliable for pathophysiological studies.

The virulence of these three well-defined mutants was then studied in mice by assessing two parameters characteristic of anthrax: lethality and local edema. The results of LD<sub>50</sub> determinations for the different strains (Table 2) strongly suggest that LF in combination with PA is the key virulence factor of *B. anthracis*: the production of LF is absolutely required for lethal infection (Table 2). Skin edema formation can unambiguously be attributed to edema toxin (PA+EF) expression on the basis of the kinetics of the inflammatory responses produced in the footpads by the various strains (Fig. 3). Our results are also consistent with previous reports showing that PA is needed for the toxic effects of EF and LF (5, 17), since the PA<sup>-</sup> mutant (RP8) failed to induce either edema or a lethal infection in mice.

EF<sup>-</sup> and LF<sup>-</sup> mutants were significantly less efficient at inducing lethality and edema than the Sterne strain. It appears, therefore, that adenylate cyclase also plays a role during lethal infection. Twenty-four years ago, Smith and Stoner (27) reported that the three toxin components together were more lethal and caused more serious edemas than either PA+LF or PA+EF. Although these data were obtained with proteins presumably difficult to purify from each other, our data fully confirm this finding. It is possible that lethal and edema toxins are synergistic and can cooperate at the cellular level or modify the interaction of bacteria with the host.

However, the mechanisms whereby lethal factor and adenylate cyclase of *B. anthracis* act as virulence factors during lethal infection are not understood. Purified PA+LF exhibits a lytic effect on macrophages in vitro (9), but the mode of action and the target cells of LF remain unknown. It has been proposed that *B. anthracis* adenylate cyclase might inhibit phagocytosis of polymorphonuclear neutrophils (24), as described for *Bordetella pertussis* adenylate cyclase (20). Nevertheless, EF<sup>+</sup> strains did not appear to survive longer in the host than EF<sup>-</sup> strains.

Surprisingly, we found in this study that the survival of all the strains of *B. anthracis* at the inoculum site was similar during the initial period of infection regardless of the expression of toxins (Fig. 4). This means that pXO1-encoded virulence factors play a minor role, if any, in local bacterial survival. They are presumably involved in the lethal effect and in bacterial dissemination from the inoculum site. This finding also implies that there are as-yet-unidentified chromosomal genes required for survival in host tissues.

This study provides the first in vivo analysis of the role of each toxin component in the pathogenesis of *B. anthracis* infection. These results are in agreement with those obtained with purified toxin components injected into animals (3, 7, 27) and with recent data from cell tissue cultures (9, 16, 24) which have been used to construct models for anthrax toxin action (17). The mutant strains described in this work will be powerful tools for investigations of the role of these toxins during the process of infection. Further studies will also

indicate whether these strains could be valuable as live vaccines against anthrax.

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