Construction of *Bacillus anthracis* mutant strains producing a single toxin component

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The two protein exotoxins secreted by *Bacillus anthracis* are composed of three distinct components: protective antigen (PA), lethal factor (LF), and (o)edema factor (EF). We have developed a genetic strategy that permits us selectively to inactivate each of the genes coding for PA, EF or LF. This strategy involved the deletion of a portion of the structural gene and the insertion of an antibiotic resistance cassette. With this technique, double mutant strains of *B. anthracis* producing only one toxin component have been constructed. Characterization of the mutant strains indicated that they produced the expected single toxin protein. Using a simple, two-step protocol, we have purified PA, LF and EF to homogeneity from culture supernatants. These three mutant strains are potentially powerful tools for studying the individual effect of each toxin component *in vitro* and *in vivo*.

Introduction

Bacillus anthracis, the aetiological agent of anthrax, secretes three separate proteins – protective antigen (PA; 85 kDa), (o)edema factor (EF; 89 kDa) and lethal factor (LF; 83 kDa) – that make up the two anthrax toxins (Stanley & Smith, 1961; Fish *et al.*, 1968*b*; Leppla, 1984). Individually, none of the three toxin components has any biological effect (Smith & Stoner, 1967). PA combined with LF forms the lethal toxin whereas oedema toxin consists of PA and EF (Beall *et al.*, 1962; Smith & Stoner, 1967). PA is the common receptor-binding component mediating entry of either LF or EF into target cells (Leppla, 1984). EF has been shown to be a calmodulin-dependent adenylate cyclase (Leppla, 1982). The mode of action of LF remains unknown.

Fully virulent strains of *B. anthracis* possess two large plasmids, pXO1 and pXO2. pXO2 codes for the formation of a poly-D-glutamic acid capsule (Avakyan *et al.*, 1965; Uchida *et al.*, 1985), whereas toxin production is conferred by pXO1 (Mikesell *et al.*, 1983) which carries the toxin structural genes. These three genes, *pag*, *cya* and *lef*, coding for PA, EF and LF, respectively, have been cloned and sequenced (Bragg & Robertson, 1989;

Escuyer *et al.*, 1988; Mock *et al.*, 1988; Welkos *et al.*, 1988), thus providing the basis for genetic studies.

Most of the available data on toxin mode of action have been obtained with toxin components purified from culture supernatants of the Sterne strain (Beall *et al.*, 1962; Fish *et al.*, 1968*a*; Smith & Stoner, 1967). Because of the difficulty in separating the three proteins from each other, several strategies have been developed in an attempt to increase purity. These approaches include multi-step chromatographic procedures (Leppla, 1988; Quinn *et al.*, 1988) or expression in different bacterial systems (Vodkin & Leppla, 1983; Ivins & Welkos, 1986; Iacono-Connors *et al.*, 1990).

We have previously devised a genetic strategy allowing the construction of *B. anthracis* mutant strains deficient in one toxin component (Cataldi *et al.*, 1990; Pezard *et al.*, 1991). We have extended this genetic approach to the construction of double mutant strains with the aim of producing PA, EF and LF as single toxin components from *B. anthracis*. Use of these mutant strains in toxin preparation precludes the possibility of cross-contamination by individual toxin components, and should prove to be a useful and convenient tool for purifying each protein to homogeneity.

Methods

Bacterial strains, plasmids and culture media. *Escherichia coli* HB101 harbouring the mobilizing plasmid pRK212.1 (Trieu-Cuot *et al.*, 1987) was used in conjugal transfer experiments. Shuttle plasmid

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Abbreviations: EF, (o)edema factor; LF, lethal factor; PA, protective antigen.

Table 1. *Bacterial strains and plasmids*

Strain/plasmid	Plasmid*	Relevant characteristics	Derivation/reference
<i>B. anthracis</i> strains			
7702	pXO1	PA ⁺ EF ⁺ LF ⁺	Sterne strain (Pasteur collection)
RP8	pXO1- <i>pag</i> Δ322	PA ⁻ EF ⁺ LF ⁺ Erm ^r	7702 (Pezard <i>et al.</i> , 1991)
RP9	pXO1- <i>cya</i> Δ303 (K ^r)	PA ⁺ EF ⁻ LF ⁺ Kan ^r	7702 (Pezard <i>et al.</i> , 1991)
RP10	pXO1- <i>lef</i> Δ238	PA ⁺ EF ⁺ LF ⁻ Kan ^r	7702 (Pezard <i>et al.</i> , 1991)
RP42	pXO1- <i>lef</i> Δ238- <i>cya</i> Δ303 (E ^r)	PA ⁺ EF ⁻ LF ⁻ Kan ^r Erm ^r	RP10 (this work)
RP31	pXO1- <i>lef</i> Δ238- <i>pag</i> Δ652†	PA ⁻ EF ⁺ LF ⁻ Kan ^r Erm ^r	RP10 (this work)
RP4	pXO1- <i>cya</i> Δ303 (K ^r)- <i>pag</i> Δ652	PA ⁻ EF ⁻ LF ⁺ Kan ^r Erm ^r	RP9 (this work)
Other plasmids			
pAT28		Shuttle plasmid Mob ⁺ , Spc ^r	Trieu-Cout <i>et al.</i> (1990a)
pCPA110		pAT28 recombinant plasmid carrying the inactivated <i>pag</i> Δ652 gene (Spc ^r Erm ^r)	
pMMA111		pAT28 recombinant plasmid carrying the inactivated <i>cya</i> Δ303 gene (Spc ^r Erm ^r)	

* Plasmid designations: the number after Δ indicates the gene codon at which the deletion starts; E^r, K^r indicate the insertion of an erythromycin (E) or kanamycin (K) resistance cassette into *cya*Δ303 (Trieu-Cuot *et al.*, 1990b).

† *pag*Δ652: deletion begins at nucleotide 1272 and ends at nucleotide 3759 (codon 652) using the numbering from the published sequence of *pag* (Welkos *et al.*, 1988).

pAT28 (Trieu-Cuot *et al.*, 1990a), conferring spectinomycin resistance (Spc^r) was used as the vector for transferring DNA into *Bacillus anthracis* strains. The mating procedure used for conjugal transfer of recombinant plasmids from *E. coli* to *B. anthracis* was as previously described (Pezard *et al.*, 1991).

E. coli was grown in L broth or L agar (Miller, 1972), and *B. anthracis* was grown in brain-heart infusion (BHI) medium (Difco). R-medium (Ristroph & Ivins, 1983) was used for toxin production. Antibiotics were used at the following concentrations: ampicillin, 100 µg ml⁻¹ for *E. coli* cultures; spectinomycin, 100 and 60 µg ml⁻¹ for *E. coli* and *B. anthracis* cultures, respectively; erythromycin, 180 and 10 µg ml⁻¹ for *E. coli* and *B. anthracis* cultures, respectively.

Spores of *B. anthracis* strains were prepared as previously described (Pezard *et al.*, 1991).

DNA techniques. pXO1 was prepared by the method of Green *et al.* (1985). Methods for recombinant DNA manipulations were as described by Sambrook *et al.* (1989). DNA fragments were prepared from agarose gels using the GeneClean Kit (Bio 101). Recombinant plasmid pACP41, a pUC8 derivative which contains the 3.4 kb *Xho*I-*Bam*HI fragment carrying *pag* was used for construction of pCPA110. A 2.5 kb *Hinc*II deletion starting at position 1272 and stopping at 3759 (from the previously published *pag* nucleotide sequence, Welkos *et al.*, 1988) was created in pACP41 and the Erm^r cassette was inserted into the *Hinc*II site. The resulting DNA fragment carrying *pag*Δ652 was then subcloned in the shuttle plasmid pAT28 yielding pCPA110.

Southern blot DNA hybridizations were performed with Hybond-N⁺ nylon membranes (Amersham). DNA probes were labelled using the ECL labelling system (kit RPN3000, Amersham).

Protein analysis and adenylate cyclase assay. SDS-PAGE (8%, w/v, acrylamide) was performed as described by Laemmli (1970). Gels were stained with Coomassie blue or subjected to immunoblot analysis (Towbin *et al.*, 1979). Western blots (immunoblots) were probed with rabbit sera raised against LF or PA purified from polyacrylamide gels or against a truncated form of EF (CYA 62) (Labruyère *et al.*, 1990).

Adenylate cyclase was assayed in *B. anthracis* R-medium culture supernatants as previously described (Ladant, 1988). Enzymic activity

is expressed in units ml⁻¹. One unit corresponds to 1 nM-cAMP formed in 1 min at 30 °C.

Purification of toxin components. Toxin components were prepared from *B. anthracis* mutant strains as follows. Colonies freshly grown on BHI/5% (v/v) blood agar were used to inoculate 500 ml R-medium (Ristroph & Ivins, 1983) in a 1 litre flask. Cultures were grown at 37 °C for 15 h without shaking in an atmosphere of 5% (v/v) CO₂. Culture supernatants (4 l) were filtered through a 0.45 µm pore-size membrane (Nalgene). The cell-free filtrate was concentrated 40-fold by ultrafiltration through PTGC membranes (molecular mass cut-off, 10000 Da) in a Minitan system (Millipore). Protein was precipitated overnight at 4 °C by the addition of (NH₄)₂SO₄ to 70% saturation. The precipitate was collected by centrifugation at 35000 g for 30 min and resuspended in 2 ml 10 mM-Tris/HCl pH 8.0. Crude toxin preparations were desalted by dialysis, and further purified by anion-exchange chromatography over a Mono Q HR 5/5 column (FPLC, Pharmacia LKB), with 10 mM-Tris/HCl buffer pH 8.0 and a linear NaCl gradient as described by Quinn *et al.* (1988).

Infection of mice. Female, six-week-old, pathogen-free Swiss mice were supplied by Charles River (Saint-Aubin-les Elbeuf, France) and used for determination of the lethal dose (LD₅₀) of each strain, as described previously (Pezard *et al.*, 1991).

Results and Discussion

Construction and characterization of double mutant B. anthracis strains

We have described previously a genetic strategy for the construction of *B. anthracis* mutant strains deficient in the production of PA (RP8), EF (RP9), or LF (RP10). The toxin genes *pag*, *cya* and *lef* were inactivated by intragenic deletion and insertion of an antibiotic resistance cassette (Erm^r or Kan^r), allowing selection of recombinant strains carrying mutated genes on pXO1

(Cataldi *et al.*, 1990; Pezard *et al.*, 1991). We have now extended this approach and constructed double mutant strains capable of producing only a single toxin component.

For this work, two recombinant plasmids derived from pAT28 were constructed. pCPA110 carries the *pag* gene inactivated through deletion and insertion of a DNA cassette conferring erythromycin resistance (*Erm*^r). pMMA111 carries the *cya* gene inactivated in the same manner (*cya*Δ303) as that described previously for pMMA110 (Pezard *et al.*, 1991), except that an *Erm*^r cassette was inserted in the gene. Mutants RP9 and RP10 were used as recipient strains for subsequent inactivation of a second toxin gene (Table 1).

pCPA110 was transferred by conjugation to RP9 or RP10. The resulting transconjugants were cultured in the absence of spectinomycin (selective antibiotic for the vector part of pCPA110) and in the presence of erythromycin (selecting for the maintenance of the cassette), to select *Erm*^r*Spc*^s clones which presumably had lost pCPA110 but integrated the inactivated *pag* gene into pXO1 by homologous recombination. Two strains, RP4 and RP31, which originated from recipient strains RP9 and RP10, respectively, were isolated. A third *Erm*^r*Spc*^s strain, RP42, was isolated from an RP10-derived transconjugant carrying recombinant plasmid pMMA111. Strains RP4, RP31 and RP42 were expected to produce only LF, EF or PA, respectively, after replacement of wild-type *pag* (RP4, RP31) or *cya* (RP42). The recombinant pXO1 from the three mutant strains was subjected to restriction analysis.

The *cya* gene is located on a 7.0 kb *Bam*HI fragment of pXO1 (Mock *et al.*, 1988). A *Bam*HI digest of pXO1 originating from RP42 was cloned into pUC18 and recombinant plasmids able to confer *Erm*^r in *E. coli* were isolated. These plasmids contained a 7.0 kb *Bam*HI fragment and restriction enzyme analysis indicated that the *Bam*HI fragment carried inactivated *cya*Δ303 *E*^r (Table 1).

The *pag* gene is located on a 6.0 kb *Bam*HI fragment of pXO1 (Vodkin & Leppler, 1983). Since the *Erm*^r cassette inserted in the gene was flanked by two *Bam*HI sites, this enzyme could not be used for cloning inactivated *pag*Δ652. We expected *pag*Δ652 to be located on a *Hind*III fragment of pXO1 larger than 3.0 kb. Therefore, we cloned *Hind*III digests of recombinant pXO1 originating from RP4 and RP31 into pUC18. In both cases, *Erm*^r *E. coli* transformants were isolated. Their respective recombinant pUC derivative plasmids carried a 4.0 kb *Hind*III fragment containing the expected *pag*Δ652 construction as determined by restriction enzyme analysis.

The location of the *Erm*^r cassette on the three recombinant pXO1 plasmids was further characterized

Fig. 1. Characterization of mutants by southern hybridization. A 1.2 kb *Erm*^r DNA fragment was used as probe. Plasmid DNA was digested as follows: lane 1, recombinant pUC18 plasmid containing the 7.2 kb *Bam*HI fragment of pXO1 carrying the inactivated *cya* gene digested with *Bam*HI; lane 2, pXO1 of strain RP42 digested with *Bam*HI; lane 3, λ DNA digested with *Hind*III; lane 4, pXO1 of strain RP31 digested with *Hind*III; lane 5, pXO1 of strain RP4 digested with *Hind*III; lane 6, *Hind*III digested recombinant pUC18 plasmid containing the 4.0 kb *Hind*III fragment of pXO1 carrying the inactivated *pag* gene.

by Southern blot analysis. As shown in Fig. 1, a 7.0 kb fragment from a *Bam*HI digest of pXO1 from RP42 (lane 2) and a 4.0 kb fragment from a *Hind*III digest of pXO1 from RP31 or RP4 (lanes 4 and 5) hybridized with the *Erm*^r cassette when it was used as a probe. These results confirmed that for all three *B. anthracis* recombinants the *Erm*^r cassette was located on pXO1, within the *pag* coding sequence for RP4 and RP31 and within the *cya* coding sequence for RP42. We therefore concluded that we had constructed three mutant strains in which two of the three toxin components genes were inactivated. These strains were therefore expected to produce only one toxin component: EF from RP31, LF from RP4, and PA from RP42.

Production of toxin proteins in mutant strains of B. anthracis

The anthrax toxin components PA, EF and LF are produced by *B. anthracis* when bacteria are grown in R-medium (Ristroph & Ivins, 1983) and constitute the most abundant extracellular proteins (Fig. 2, lane 1). Analysis of the culture supernatants of each mutant strain, RP42, RP31 and RP4, indicated the presence of a major species of protein (Fig. 2, lanes 2, 3 and 4); these proteins were further characterized by Western blot analysis using specific antisera raised against PA, EF or LF. The supernatant proteins of strains RP42, RP31 and RP4

Fig. 2. SDS-PAGE analysis of culture supernatants from *B. anthracis* strains. Lane 1, parental strain 7702 (20 µg protein); lane 2, strain RP31 (15 µg protein); lane 3, strain RP42 (15 µg protein); lane 4, strain RP4 (20 µg protein); lane 5, molecular mass markers (Pharmacia).

were exclusively immunodetected with anti-PA, anti-EF, and anti-LF sera respectively (data not shown). These results confirmed that, as expected, each strain produced only one of the three toxin components.

It is known that EF is an adenylate cyclase (Leppla, 1982). To verify inactivation of the *cya* gene we determined adenylate cyclase activity in the culture supernatant of the three mutant strains and of parental strain 7702. No enzymic activity was detected in the supernatant of strain RP42 (PA⁺), thus confirming inactivation of the *cya* gene. As expected, strain RP4 (LF⁺) derived from RP9, was also devoid of this activity. The adenylate cyclase activity found in the culture supernatant of RP31 (EF⁺) was similar to that of wild-type strain 7701 (200 U ml⁻¹) under the same experimental conditions.

Growth of RP4, RP31 and RP42 in large culture volumes allowed us to develop a purification procedure for recovery of each toxin component. Individual toxin components are produced by the corresponding mutant strain in quantities similar to those produced by the parental strain, 7702. Each protein was thus readily available at a high degree of purity with a simple two-step purification protocol (see Methods).

Virulence of *B. anthracis* mutant strains in mice

The virulence of the three *B. anthracis* mutants was studied in mice. The strains constructed in this work are derived from RP9 and RP10, whose virulence properties have been described previously (Pezard *et al.*, 1991).

Strain RP9 producing lethal toxin (PA+LF) causes death in mice at an LD₅₀ of 10⁷. The inactivation of *pag* in mutant RP4 abolished the lethal effect of RP9, and inoculation of mice with 10⁹ spores of RP4 failed to provoke mortality. Strain RP10, producing oedema toxin (PA+EF) has no lethal effect but still induces oedema in mice after subcutaneous injection. The inactivation of *pag* and *cya* in RP31 and RP42, respectively, abolished oedema formation after subcutaneous injection of 10⁹ spores. Therefore, the three mutant strains we have constructed, RP4, RP42 and RP31, producing only LF, PA, or EF, respectively, are avirulent and totally devoid of biological effect. These results demonstrate that individually none of the three proteins secreted by the bacteria is toxic for animals, and they are consistent with previous observation obtained after injection of the proteins into animals (Smith & Stoner, 1967).

The Sterne strain, which carries only pXO1, is toxigenic and uncapsulated and is currently used as the veterinary vaccine against anthrax (Hambleton *et al.*, 1984; Sterne, 1939; Welkos & Friedlander, 1988b). However, since this strain still produces both lethal and oedema toxins it is pathogenic to animals at high doses. Mutant strains totally devoid of lethality might contribute to an improved live vaccine. Further studies with our mutant strains should provide new insights into *B. anthracis* pathogenesis as well as into immunization against anthrax. While it is well known that PA is the main toxin component required for protection of animals and humans (Hambleton *et al.*, 1984; Welkos & Friedlander, 1988a), the contribution of EF and LF to the immune response remains unclear. Investigation of the protective efficacy of these strains should help to clarify the respective roles of the individual toxin components in protection against anthrax.

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