Published in final edited form as:
Protein Expr Purif. 2011 November ; 80(1): 80–90. doi:10.1016/j.pep.2011.05.016.

A *Bacillus anthracis* strain deleted for six proteases serves as an effective host for production of recombinant proteins

Andrei P. Pomerantsev[1], **Olga M. Pomerantseva**[2], **Mahtab Moayeri**[1], **Rasem Fattah**[1], **Cynthia Tallant**[3], and **Stephen H. Leppla**[1]

[1] National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD, 20892

[2] Biological Defense Research Directorate, Naval Medical Research Center, Rockville, MD, 20852, USA

[3] Proteolysis Lab, Department of Structural Biology, Molecular Biology Institute of Barcelona, CSIC, Barcelona Science Park, 08028 Barcelona, Spain

Abstract

Bacillus anthracis produces a number of extracellular proteases that impact the integrity and yield of other proteins in the *B. anthracis* secretome. In this study we show that anthrolysin O (ALO) and the three anthrax toxin proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), produced from the *B. anthracis* Ames 35 strain ($pXO1^+$, $pXO2^-$), are completely degraded at the onset of stationary phase due to the action of proteases. An improved Cre-*loxP* gene knockout system was used to sequentially delete the genes encoding six proteases (InhA1, InhA2, camelysin, TasA, NprB, and MmpZ). The role of each protease in degradation of the *B. anthracis* toxin components and ALO was demonstrated. Levels of the anthrax toxin components and ALO in the supernatant of the sporulation defective, $pXO1^+$ A35HMS mutant strain deleted for the six proteases were significantly increased and remained stable over 24 h. A pXO1-free variant of this six-protease mutant strain, designated BH460, provides an improved host strain for the preparation of recombinant proteins. As an example, BH460 was used to produce recombinant EF, which previously has been difficult to obtain from *B. anthracis*. The EF protein produced from BH460 had the highest *in vivo* potency of any EF previously purified from *B. anthracis* or *E. coli* hosts. BH460 is recommended as an effective host strain for recombinant protein production, typically yielding greater than 10 mg pure protein per liter of culture.

Keywords

Bacillus anthracis; protease deficient; protein overproduction; edema factor

INTRODUCTION

The Gram-positive bacterial pathogen *Bacillus anthracis* secretes high levels of the three proteins that are collectively termed anthrax toxin: protective antigen (PA), edema factor

Correspondence to: Stephen H. Leppla, Ph.D., Laboratory of Bacterial Diseases, National Institute of Allergy and Infectious Diseases, 33 NORTH DR, BG 33 RM 1W20B MSC 3202, BETHESDA, MD 20892-3202, 301-594-2865 telephone, 301-480-0326 FAX, sleppla@niaid.nih.gov.

(EF), and lethal factor (LF), when grown under conditions thought to mimic those in an infected animal host. PA is a receptor-binding component which acts to deliver LF and EF to the cytosol of eukaryotic cells; EF is a calmodulin-dependent adenylate cyclase and LF is a zinc metalloprotease that cleaves most members of the mitogen-activated protein kinase kinase family for reviews see [1–4]. Because anthrax pathogenesis is highly dependent on the actions of the anthrax toxin proteins, vaccine and therapeutic development efforts have focused on countering toxin action, typically by generating antibodies to PA. The anthrax vaccine currently licensed in the USA, and developed almost 50 years ago [5], consists of a partially purified culture supernatant of a protease-deficient strain (V770-NP1-R). PA is the most abundant protein and the key immunogen in this vaccine. Efforts to produce a recombinant PA vaccine from *B. anthracis* by scale-up of an established process [6] appear to have been hampered by instability of the final product, possibly due to protease contamination.

While the toxin components can be purified as recombinant proteins from *B. anthracis* culture supernatants [6–9], the integrity and yields are limited by the *B. anthracis* proteolytic enzymes that are co-secreted. Two extracellular proteases are reported to be abundant in the *B. anthracis* secretome: NprB (GBAA_0599) – neutral protease B, a thermolysin-like enzyme highly homologous to bacillolysins from other *Bacillus* species, and InhA1 (GBAA_1295) – immune inhibitor A1, a homolog of the immune inhibitors A from other members of the *Bacillus cereus* group [10–12]. These two proteases contain zinc-binding motifs typical for the zincin tribe of metallopeptidases, His-Glu-Xxx-Xxx-His, and belong, respectively, to the M4 and M6 families of metalloproteases according to the **MEROPS** database (http://merops.sanger.ac.uk). A third metalloproptease, camelysin (GBAA_1290), belonging to the M73 family is found in the secretome of several *B. anthracis* strains. This protease is similar to the camelysin of *B. cereus*, a novel surface metalloprotease [13].

B. anthracis also contains a gene encoding the InhA2 metalloprotease (GBAA_0672, M6 family), although it is not known whether this protease is expressed and secreted. This gene is an ortholog of the InhA1 described above (68% amino acid identity). Similarly, the genome of *B. anthracis* also contains genes encoding TasA (GBAA_1288, M73 superfamily), which is an ortholog of camelysin (60% amino acid identity), and MmpZ (GBAA_3159, ZnMc superfamily), which is a putative extracellular zinc-dependent matrix metalloprotease, a member of the metzincin clan of metallopeptidases. This clan is characterized by an extended zinc-binding motif, His-Glue-Xxx-Xxx-His-Xxx-Xxx-Gly/Asn-Xxx-Xxx-His/Asp [14].

We hypothesized that secretion of these and other extracellular proteases might significantly decrease the levels of intact anthrax toxin components in the *B. anthracis* secretome, so that disruption of these protease-encoding genes might result in higher protein yields. We previously inactivated NprB and InhA1 individually and found that NprB deficiency reduced proteolysis of casein [15], while coagulation of human blood by *B. anthracis* required InhA1 for proteolytic activation of prothrombin and factor X [16].

In this report, we describe the adaptation of an improved Cre-*loxP* system for sequentially deleting additional protease-encoding genes of *B. anthracis.* Also, we describe a role of each protease in degradation of *B. anthracis* toxin components and another potential virulence factor, anthrolysin O (ALO) [17]. Our work parallels earlier work to knock out up to eight proteases from *Bacillus subtilis* [18,19] so as to produce an improved expression host. Finally, we suggest that the final *B. anthracis* strain generated, designated BH460, lacking six proteases and being sporulation deficient and free of the virulence plasmids, provides an improved host for production of recombinant proteins. As an example, we show that EF

produced from BH460 is highly active, whereas previous *B. anthracis* host strains produced truncated proteins having low potency.

MATERIALS AND METHODS

Materials

The proteases, oligonucleotide primers, plasmids, and bacterial strains used and analyzed in this study are listed in Tables 1–4.

Bacterial growth conditions and phenotypic characterization

E. coli strains were grown in Luria-Bertani (LB) broth and used as hosts for cloning. LB agar was used for selection of transformants [20]. *B. anthracis* strains were also grown in LB or FA medium [21]. Antibiotics (Sigma-Aldrich, St. Louis, MO) were added to the medium when appropriate to give the following final concentrations: ampicillin (Ap), 100 μg/ml (only for *E. coli*); erythromycin (Em), 400 μg/ml for *E. coli* and 10 μg/ml for *B. anthracis*; spectinomycin (Sp), 150 μg/ml for both *E. coli* and *B. anthracis*; kanamycin (Km), 20 μg/ml (only for *B. anthracis).* SOC medium (Quality Biologicals, Inc., Gaithersburg, MD) was used for outgrowth of transformation mixtures prior to plating on selective medium. *B. anthracis* spores were prepared as previously described [22] after growth on NBY minimal agar (nutrient broth, 8 g/liter; yeast extract, 3 g/liter; $MnSO_4 \cdot H_2O$, 25 mg/liter; agar, 15 g/liter) at 30 °C for 5 days. Spores and vegetative cells were visualized with a Nikon Eclipse E600W light microscope (Nikon Instrument Inc., New York).

DNA isolation and manipulation

Preparation of plasmid DNA from *E. coli*, transformation of *E. coli*, and recombinant DNA techniques were carried out by standard procedures [20]. *E. coli* SCS110 competent cells were purchased from Stratagene (La Jolla, CA) and *E. coli* TOP10 competent cells from Invitrogen (Carlsbad, CA). Recombinant plasmid construction was carried out in *E. coli* TOP10. Plasmid DNA from *B. anthracis* was isolated according to the protocol for the purification of plasmid DNA from *B. subtilis* (Qiagen, Valencia, CA). Chromosomal DNA from *B. anthracis* was isolated with the Wizard genomic purification kit (Promega, Madison, WI). *B. anthracis* was electroporated with unmethylated plasmid DNA isolated from *E. coli* SCS110 (dam^- dcm^-). Electroporation-competent *B. anthracis* cells were prepared and transformed as previously described [23]. Restriction enzymes, T4 ligase, and Antarctic phosphatase were purchased from New England Biolabs (Ipswich, MA). *Taq* polymerase, Platinum PCR SuperMix High Fidelity kit and the TOPO TA cloning kit were from Invitrogen. The pGEM-T Easy Vector system was from Promega. Ready-To-Go PCR Beads were from GE Healthcare Biosciences Corp. (Piscataway, NJ). For routine PCR analysis, a single colony was suspended in 200 μl of TE buffer [20] (pH 8.0), heated to 95 °C for 45 s, and then cooled to room temperature. Cellular debris was removed by centrifugation at 15,000 × *g* for 10 min. Two microliters of the lysate contained sufficient template to support PCR. The GeneRuler DNA Ladder Mix from MBI Fermentas (Glen Burnie, MD) was used to assess DNA fragment length. All constructs were verified by DNA sequencing and/or restriction enzyme digestion.

Construction of vectors for protease gene inactivation

B. anthracis Ames 35 ($pXO1^+$ $pXO2^-$) (A35) was used for genetic manipulations. The GenBank database (GenBank Accession No. for the Ames strain is **NC_003997**) was analyzed for the identification of target genes and for the corresponding primer design. The Cre/Lox genetic modification method was adapted to introduce precise genetic knockouts into *B. anthracis* genes encoding putative proteases. The general schemes for producing *B.*

anthracis mutants using Cre-*loxP* system were described previously [15,23]. The system employs vectors we designate generically as pDC, for *d*ouble-*c*rossover plasmid or pSC, for single-*c*rossover plasmid. These plasmids are derived from the highly temperature-sensitive plasmid pHY304 [24], which has permissive and restrictive temperatures of 30 °C and 37 °C, respectively. The pDC vector was used to inactivate the *spo0A* (GBAA_4394), *nprB* (GBAA_0599) [15], *inhA1* (GBAA_1295) [16], *inhA2* (GBAA_0672) and *calY* (GBAA_1290) genes. The pSC vector was used to inactivate the *tasA* (GBAA_1288) and *mmpZ* (GBAA_3159) genes. Both plasmids were used to produce a genomic deletion of the region from *tasA* (GBAA_1288) to *inhA1* (GBAA_1295). To inactivate the *inhA2* gene we amplified left and right fragments with primer pairs 0672LL/0672LR and 0672RL/0672RR (Table 2) and inserted them into pDC to produce the pInhA2I plasmid (I at the end of the proteases gene number means inactivation). To inactivate *tasA* we amplified left and right fragments with primer pairs 1288LL/1288LR and 1288RL/1288RR and inserted them into pSC to produce the pTasALI and pTasARI plasmids. To inactivate the camelysin gene *calY* we amplified a DNA fragment overlapping the protease gene with primer pairs 1290L/1290R and inserted the fragment into the EcoRI-site of pHY304. The internal *Bgl*II-fragment of the *calY* gene was replaced with a *loxP-Ω-sp-loxP* cassette flanked by two *Bgl*II sites [15] to create the pCamI plasmid for the gene inactivation. To inactivate the *mmpZ* gene, we amplified left and right fragments with primer pairs 3159LL/3159LR and 3159RL/3159RR and inserted them into pSC to produce pMmpZLI and pMmpZRI plasmids. To delete the *tasA-inhA1* gene cluster we transformed the double protease mutant A35DM strain (having a LoxP site in the inhA1 gene) with pTasALI and integrated the plasmid into the genome as described previously [23]. Subsequent transformation of the recombinant strain with the pCrePAS plasmid eliminated the complete *tasA-inhA1* gene cluster and produced the tetra-protease mutant strain A35TM.

The Cre recombinase-expressing plasmids pCrePAS and pCrePA both have permissive and restrictive temperatures of 30 °C and 37 °C, respectively, and differ only in the selectable marker. The pCrePA was used for elimination of DNA regions containing a spectinomycin resistance cassette located between two similarly oriented *loxP* sites [15], while pCrePAS was used in a similar way when the recipient strain did not contain a spectinomycin marker. In that case, the region to be deleted generally contained an erythromycin resistance gene along with backbone vector pSC [23]. In both cases, a single *loxP* site replaced the DNA region targeted for deletion.

To complement the mutation in the *mmpZ* gene (A35ΔMmpZ strain) and to restore the deleted *tasA-inhA1* region in the A35TM strain, the 3159CF/3159CR and deltaCF/deltaCR PCR fragments were inserted into the pSC plasmid. The resulting pMmpZC and pΔTasA-InhA1C plasmids containing, respectively the intact *mmpZ* gene and the whole *tasA-inhA1* region (C at the end of the proteases gene means complementation), were used for complementation. Each plasmid was inserted separately into the corresponding mutant by a single crossover event. During the crossover both the *mmPZ* gene and whole *tasA-inhA1* region were inserted into the genomes of the corresponding mutants. Subsequent elimination of the vector sequences by Cre recombinase left an intact functional copy of the originally mutated gene along with an inactive duplicate copy of a fragment of the gene.

Preparation of mutants

The strains used and their relevant characteristics are listed in Table 4. The *B. anthracis* NprB, InhA2, TasA, camelysin, InhA1, and MmpZ mutants were constructed in the A35 strain by the replacement of coding sequences with the *loxP* element as described in the previous section. The double NprB, InhA1 mutant (A35DM) was created starting from the A35ΔNprB strain (Table 4) as described before [16]. The tetra-protease mutant, A35TM was created by deletion of the *tasA-inhA1* gene region in the A35DM strain. The A35TM was

then used for inactivation of the *inhA2* and *mmpZ* genes with plasmids pInhA2I and pMmpZLI/pMmpZRI. The resulting penta- and hexa-protease mutants were designated A35PM and A35HM, respectively. The mutant strains were checked at each step to ensure they had retained the ability to sporulate [25]. To intentionally produce a sporulation-deficient hexa-protease mutant of A35HM, we inactivated the *spo0A* (GBAA_4394) gene in A35HM (Table 4) using the plasmid pSΩL304 [15], obtaining A35HMS. The final protease-deficient *spo0A*-negative mutant lacking pXO1 was obtained by repeated passage of the A35HMS mutant at elevated temperatures to cure pXO1 as described previously [26]. The final strain was designated *B. anthracis* BH460 (Table 4).

PCR and sequence analysis of chromosomal modifications

PCR fragments containing *loxP* sites within mutated genes were amplified and sequenced using primers listed in Table 2 (0672seqF/0672seqR, 1288seqF/1288seqR, 1290seqF/1290seqR, 3159seqF/3159seqR). All primers for PCR and sequencing were synthesized by Operon Biotechnologies, Inc. (Huntsville, AL) or the FDA core facility (Bethesda, MD). Sequences were determined from both sides of the PCR fragments (Macrogen, Rockville, MD). For verification of the genomic deletion in the *tasA-inhA1* gene area, the region encompassing the start of *tasA* gene and the end of *inhA1* gene was amplified in the mutant strains by PCR using the primer pair deltaF/deltaR. The location of the *loxP* site inside the PCR fragments was determined by sequencing the fragments.

Western blot analyses

The A35 strain and the mutants were grown at 37 °C in LB in air to analyze *B. anthracis* toxin production by Western blot. Overnight cultures were diluted into fresh LB to give A_{600} = 0.002 and growth was measured at 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 17 and 24 h. Supernatant samples (6 ml) from each time point were filtered (0.22 μm Millex syringe-driven filter units, Millipore, Cork, Ireland) and concentrated 10-fold using Amicon Ultra-4 membranes (Millipore). Samples of 5 μl were mixed with 5 μl of 2X Tris-glycine SDS sample loading buffer (126 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.005% bromphenol blue) (Invitrogen), heated (96 °C, 10 min) and separated on 4–12% Bis-Tris NuPAGE gels) using NuPAGE MOPS SDS running buffer (Invitrogen). Precision Plus Protein Standard (All Blue, Bio-Rad, Hercules, CA) was used as molecular weight marker. Proteins were transferred to MagnaCharge 0.45 μm nylon membranes (Osmonics Inc., Minnetonka, MN) using transfer buffer (25 mM Tris, 190 mM glycine, and 20% methanol), blocked in PBS + 3% skim milk (Difco, Lawrence, KS) for 1 h at room temperature, followed by three 10 min washes in PBST (PBS + 0.05% Tween20). Membranes were then incubated with primary antibody diluted in PBS + 1% skim milk overnight at 4 °C. Mouse monoclonal antibody PA-05-A-G1 Lot#071100-02, 5.9 mg/ml (Naval Medical Research Center, Biological Defense Research Directorate), for detection of PA, and mouse monoclonal antibody LF-03-A-G1 Lot# 150900-01, 7.4 mg/ml (NMRC, BDRD), for detection of LF, were both used at 1:2000. Rabbit antisera for development of blots included anti-EF serum #5900 (used at 1:8000), anti-camelysin (used at 1:2000), and anti-recombinant ALO (used at 1:2000). The latter was a kind gift of Richard Rest, Drexel University College of Medicine, Philadelphia, PA. Appropriate HRP-conjugated secondary IgGs (KPL, Gaithersburg, MD) were used at 1:10000 followed by development with TMB (3,3′,5,5′-tetramethylbenzidine) (KPL).

Isolation and purification of EF protein

EF protein with an N-terminal six-histidine tag was expressed from plasmid pProEx-H6-EF (provided by Wei-Jen Tang) in *E. coli* BL21(DE3) (Promega) as previously described [27]. EF was expressed in *B. anthracis* host strains from plasmid pSJ136EFOS, which contains the EF structural gene in plasmid pYS5 under the control of the PA promoter and signal

sequence [21]. Host strains BH450 and BH460 containing pSJ136EFOS were grown in FA medium containing 15 μg/ml of kanamycin at 37 °C for 14 h, largely following procedures previously used for production of LF [9]. The cultures were cooled, supplemented with 2 μg/ml of AEBSF [4-(2-Aminoethyl)-benzenesulfonylfluoride·HCl] (US Biological, Swampscott, MA) and centrifuged at 4550 × g for 30 min. All subsequent steps were performed at 4 °C. The supernatants were filter sterilized and supplemented with 5 mM EDTA. Solid ammonium sulfate was added to the supernatants to obtain 40% saturation. Phenyl-Sepharose Fast Flow (low substitution, GE Healthcare Biosciences Corp.) was added and supernatants gently mixed in the cold for 1.5 h. The resins were collected on porous plastic funnels (BelArt Plastics, Pequannock, NJ) and washed with buffer containing 1.5 M ammonium sulfate, 10 mM Tris-HCl, and 1 mM EDTA (pH 8.0). The EF proteins were eluted with 0.3 M ammonium sulfate, 10 mM Tris-HCl, and 1 mM EDTA (pH 8.0), precipitated by adding an additional 30 g ammonium sulfate per 100 ml eluate, and centrifuged at 18,370 × *g* for 20 min. The proteins were dissolved and dialyzed against 5 mM HEPES, 0.5 mM EDTA (pH 7.5). The dialyzed samples were applied to a Q-Sepharose Fast Flow column (GE Healthcare Bio-sciences Corp.) and eluted with a 0–0.5 M NaCl gradient in 20 mM Tris-HCl, 0.5 mM EDTA (pH 8.0). The EF-containing fractions identified by SDS-PhastGel analysis were purified on a column of ceramic hydroxyapatite (Bio-Rad Laboratories, Hercules, CA) with a gradient of 0.02–1.0 M potassium phosphate containing 0.1 M NaCl (pH 7.0). The fractions containing EF were dialyzed overnight against 5 mM HEPES and 0.5 mM EDTA, pH 7.5, concentrated as necessary, frozen, and stored at −80 °C. The molecular mass of purified EF was estimated by liquid chromatogram-electrospray mass spectrometry using an HP/Agilent 1100 MSD instrument (Hewlett Packard, Palo Alto, CA) at the NIDDK core facility, Bethesda, MD).

Analysis of EF activity *in vitro* and *in vivo*

EF activity was measured by analysis of cAMP production in the RAW264.7 macrophage cell line. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 2 mM Glutamax, 2 mM HEPES and 50 μg/ml gentamicin (all from Invitrogen) at 37 °C in 5% CO_2. Cells were seeded in 96-well plates 24 h prior to assays. EF preparations were serially diluted in a constant PA concentration (250 ng/ml) prior to addition to cells and incubation for 1 h at 37 °C. Total cAMP levels were assessed using the BioTRAK cAMP enzyme immunoassay kit (GE Healthcare Biosciences Corp.) according to the manufacturer's protocol. For analysis of EF potency, groups of five 8-week old female Balb/cJ mice (Jackson Laboratories, Bar Harbor, ME) were injected via tail vein with EF preparations combined with an equal dose of PA. Toxin was prepared in sterile PBS. Mice were monitored for survival for 168 h. All experiments involving animals were performed under protocols approved by the Animal Care and Use Committee of the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

RESULTS AND DISCUSSION

Insertion of *loxP* into protease genes results in truncation of the corresponding proteins

A number of protease genes on the *B. anthracis* chromosome as well as the *spo0A* gene were targeted for inactivation in this study. They are identified here with the gene numbers assigned by The Institute for Genomic Research (Rockville, Maryland; http://www.tigr.org) for the "Ames ancestor" strain chromosome (GenBank Accession No. **NC_007530**, gene designation GBAA_gene number) [28]. The gene numbers are coincident with the previous "Ames" strain chromosome (GenBank Accession No. **NC_003997**, gene designation BA_gene number) [29]. All genes and proteins inactivated or analyzed in this study are listed in Table 1 together with corresponding locus tags.

The inactivation of the *nprB* and *inhA1*genes has been previously described [15,16]. In this study we inactivated the *inhA2*, *tasA*, *calY*, and *mmpZ* genes. The *B. anthracis* InhA2 protein is 96% identical in sequence to the InhA2 protease of *Bacillus thuringiensis*, which is an essential virulence factor in that insect pathogen [30]. The *tasA* gene (GBAA_1288) located downstream of the putative signal protease gene *sipW* (GBAA_1287) is only 5 genes upstream of the *InhA1* gene (GBAA_1295). The intervening genes include the *calY* protease gene, and the two regulatory genes *sinI* and *sinR* (Table 4). The SinI and SinR proteins play important roles in *B. subtilis* biofilm formation, acting through protease-dependent processes [31], while in *B. anthracis* these proteins regulate secreted proteases [32]. It is interesting that the genes corresponding to *calY* and *inhA1* are not found in the *sinI*, *sinR* region of the *B. subtilis* genome. Only the gene for the TasA protease, *tasA*, is located downstream of *sipW* and upstream of *sinR* and *sinI* [32]. Both TasA and camelysin are similar to the *B. subtilis* TasA protease (36% and 34% sequence identities, respectively). The final gene selected for inactivation, *mmpZ*, is reported to form an operon with the downstream gene (GBAA_3160) [33]. The latter gene encodes a hypothetical secreted protein that is overproduced in *B. anthracis* [10]. The absence of the MmpZ protease in the *B. anthracis* secretome indicates that this protease could be a target for proteolytic degradation by other proteases during the stationary phase of growth [10].

The protease genes were inactivated as described in the Materials and Methods section, followed by sequencing to locate the *loxP* insertions and infer the corresponding amino acid changes in the mutated proteins. These are shown in Fig. 1. Typically, the 34-bp *loxP* sequence will generate a frameshift and early downstream occurrence of a stop codon in an alternative reading frame either within the *loxP* sequence or soon thereafter. Thus, all four inactivated protease genes encode greatly shortened proteins.

Degradation of proteins in *B. anthracis* mutants compared with the A35 strain

The levels of the three *B. anthracis* toxin components, ALO, and camelysin produced by the *B. anthracis* mutants were compared to those of the parental A35 strain by Western blot (Fig. 2). The ten strains were grown in LB at 37 °C over a 24-h period. All strains grew similarly except the six-protease mutant BH460, which appeared to have a slight lag before reaching exponential growth phase.

Expression of PA by Ames 35 was detectable starting at 5 h of growth. However, full length PA (83 kDa) disappeared by the 9th hour of growth due to proteolytic degradation. Inactivation of the InhA2 protease did not influence production of PA while inactivation of Spo0A or camelysin actually reduced the half-life of PA by 1–2 h. Inactivation of NprB, TasA, or MmpZ resulted in increased PA stability up to 10 h. However, PA produced by all these strains was completely degraded after 17 h. The most stable production of PA among the single knockout mutants was found in the InhA1 strain. PA was present even at 24 h of growth with this strain, although some degradation occurred. Surprisingly, the A35DM double mutant did not demonstrate enhanced PA production. PA degradation generally began at 6–7 h of growth and continued through 24 h. Strikingly, the A35HMS strain with six inactivated proteases produced PA with minimal to no degradation during the full 24 h of growth. Similar analyses were performed to follow production of the other *B. anthracis* toxin components, EF and LF. Full length EF (89 kDa) was found to be more vulnerable to degradation than PA, while LF (90 kDa) was quite stable when produced from most mutant strains. The levels of full length EF and LF, and the timing of their production, paralleled what was seen for PA for each mutant strain.

The enhanced breakdown of PA and EF found in the A35DM strain may be explained by increased production of camelysin in A35DM compared with A35 or the two corresponding single protease knockouts. Although both the NprB and InhA1 knockout strains had

increased levels of camelysin, the double mutation produced what seems to be a greater than additive effect. The camelysin levels produced by the A35DM strain were similar to those by the Spo0A mutant strain, A35ΔSpo0A. Both strains produced camelysin (19 kDa) that remained stable over the 24-h period. These observations on post-translational regulation of camelysin production by several proteases support and expand recently published data demonstrating that the concentration of InhA1 in culture supernatants is inversely proportional to the concentration of camelysin [32]. Another interesting finding of our work was that the global transcriptional regulator Spo0A inhibited camelysin production. Pflughoeft et al. [32] recently demonstrated that *B. anthracis sinR* (which was deleted in those mutants containing the *tasA-ihhA1* deletion) also negatively regulates transcription of camelysin and InhA1, both of which have been suggested to be associated with virulence [12,34].

Knocking out TasA also increased production of camelysin but to a lesser extent than elimination of NprB and InhA1. The InhA2 knockout did not result in any change in camelysin degradation or production when compared to A35, while MmpZ elimination was actually detrimental to camelysin production. We conclude that inactivation of six proteases in *B. anthracis* leads to increased production of full-length toxin proteins in culture supernatants relative to A35, single, or double protease mutants.

Very low levels of ALO were produced from the A35 strain compared to all the protease knockouts, with the exception of the InhA2 mutant. The ALO produced by the A35 strain completely disappeared after 9 hours of growth. Every protease knockout strain showed increased levels of production or greater stability of ALO (53-kDa band). It is interesting that ALO breakdown started in A35DM (less in A35ΔInhA1) only after 17 h of growth. A similar effect was not seen in the A35HMS strain, which allowed accumulation of full length ALO throughout the 24 h of growth. The ALO gene is under control of a PlcR-dependent promoter, so that the truncation of the PlcR protein in *B. anthracis* is expected to greatly limit ALO synthesis, along with all the other PlcR-dependent proteins [35]. The fact that ALO can be observed in several of the protease-deficient mutants implies that previous reports of low ALO production may be attributed, at least in part, to its degradation rather than to low expression. Camelysin overexpression did not influence ALO production, indicating that this toxin is not a target for camelysin. Taken together, the results demonstrate the involvement of multiple proteases in controlling the accumulation of extracellular proteins in *B. anthracis*.

Complementation of mutations restores proteolytic activity

To verify that the changes observed above were due to the intended gene knockouts rather than to unrecognized second-site mutations, we complemented several mutants. To complement the *mmpZ* mutation, the A35ΔMmpZ strain was transformed with pMmpZC. This plasmid undergoes a single crossover to insert the full length, wild type *mmpZ* gene next to the mutated one. The pSC vector was eliminated by Cre-recombinase treatment as described previously [23]. The presence of the intact *mmpZ* gene in A35MmpZC was confirmed by PCR and sequencing. Western blot analysis of LF production from A35ΔMmpZC (Fig. 3A) over 24 h indicated that proteolytic activity was restored to levels similar to that of A35 (Fig. 2). To restore the large *tasA-inhA1*deletion in the genome of the A35TM strain, we transformed with the plasmid pΔTasA-InhA1C (containing the entire *tasA-inhA1* region) and followed steps similar to what was described above. Analysis of ALO production from the complemented strain verified restoration of proteolytic activity (Fig. 3B). Complementation of the A35ΔInhA2 strain, and restoration of the InhA2 protease in the same manner, however, did not result in any difference in secreted proteins (data not shown).

All the studies described above were done in derivatives of Ames 35, where the toxin proteins are encoded on the large virulence plasmid pXO1. Production of the toxin proteins and secreted proteases in these strains is highly dependent on the growth medium. In particular, the production of the three toxin proteins and certain proteases is greatly enhanced by the addition of bicarbonate [11,33,36]. Thus, it is likely that growth in certain media could produce higher concentrations of both proteases and substrates (e.g., PA, LF, EF, etc.) and this could lead to even greater degradation than observed here.

Bacillus host strains are widely used in biotechnological processes, and avirulent *B. anthracis* strains have been used in this laboratory for a number of years to produce PA and LF [7,9,37]. However, these strains have not been useful as generic hosts for recombinant protein production due to the secreted proteases demonstrated by the work presented above. Our successful elimination of many of the most abundant proteases suggested that the resulting strains could have value as protein expression hosts. To create an optimal host, we further modified the A35HMS strain by curing it of plasmid pXO1. The resulting strain, designated BH460, is non-toxigenic, and can be considered innocuous since it lacks the major virulence factors of *B. anthracis*. The permanent deletion of the *spoOA* gene assures that the strain dies rapidly at the end of exponential growth, eliminating concerns regarding laboratory contamination.

B. anthracis strain BH460 produces full-length EF protein

Production of EF from *B. anthracis* hosts has previously been difficult because this protein is more susceptible to proteolytic degradation than are PA and LF. The plasmid pSJ136EFOS encodes the mature EF protein with its native N-terminus (thus, the “OS” for original sequence) fused to the PA signal sequence and under the control of the PA promoter. This plasmid is otherwise similar to the plasmids pYS5 and pSJ115 that are routinely used in this laboratory to produce PA and LF, respectively. Protein purified from the transformant BH460(pSJ136EFOS) was compared to a preparation made in a similar way from the single protease mutant host BH450, and to a His6-tagged EF protein purified from *E. coli* [27], the latter being the type of material used in previous toxicity analyses reported by our lab [38]. SDS-PAGE profiles of the recombinant EF proteins are shown in Fig. 4A. The EF produced from BH460 appeared to be slightly less degraded than that isolated from BH450. This finding was confirmed by mass-spectrometry analyses (Fig. 4B). The molecular mass of the recombinant protein isolated from BH460 (88,820 Da) compared well with the theoretical molecular weight for EF (88,822 Da), differing by 2 Da, which is within the instrumental error. A second species was found that had a lower mass (88,687 Da) consistent with loss of the N-terminal methionine. These two protein species were present in about equal amounts (47% for the larger, 53% for the smaller). Mass spectra of the EF produced from BH450 showed degradation as indicated by losses of 1495.7 and 2455.7 Da, resulting in proteins of 87,326 and 86,366 Da, found with similar abundances of 54% and 46%, respectively (Fig. 4C). EF purified from *E. coli* was monomorphic, with a mass of 89,995 Da, differing by only 6 Da from the theoretical molecular weight (89,989 Da, Fig. 4C). These results clearly demonstrate production of full length EF from BH460 compared to the truncated proteins made by BH450.

Activity of EF produced from *B. anthracis* protease mutants

As noted above, recombinant EF has previously been difficult to produce from *B. anthracis* host strains transduced with plasmids such as pSJ136. Furthermore, the EF that was obtained either from *B. anthracis* Sterne strain culture supernatant [39] or *E. coli* [27] consistently had higher potency in inducing cAMP production in cultured cells or lethality to mice than EF produced from *B. anthracis* (data not shown). In fact, no previous recombinant EF preparations from *B. anthracis* have been lethal to mice even when injected in doses as high

as 100 μg (combined with equimolar PA) (data not shown). The ES-MS analyses shown in Fig. 4 suggest that the low potency of previous *B. anthracis*-derived EF preparations could be due to degradation. Consistent with prior results, the BH450-derived EF displayed an extremely low level of specific activity and was not lethal for mice (Fig. 5A and 5B). However, the recombinant EF purified from the BH460 culture supernatant had a specific activity exceeding that of highly active *E. coli* BL21(DE3)-derived EF (Fig. 5A). Similarly, when injected with equal doses of PA, recombinant EF prepared from BH460 was lethal to animals at the 25 μg dose, whereas this dose of *E. coli*-derived EF had minimal effect (Fig. 5B). EF purified from BH450 was not lethal at 50 μg (Fig. 5B) and even at doses up to 100 μg (data not shown). Thus, the BH460 strain, which produces 5–7 mg of EF per liter of culture, allows for the first time the purification of substantial amounts of highly active EF from *B. anthracis*. Because other proteins purified in the same way from *B. anthracis* have consistently been free of endotoxin, use of these EF preparations also eliminates concerns regarding endotoxin-mediated cAMP co-signaling associated with *E. coli*-derived preparations. The BH460 strain has also proven very useful for expression of a variety of other proteins, typically yielding in excess of 10 mg of final pure protein per liter of culture (data not shown), as will be reported in separate reports on proteins produced in this system.

Acknowledgments

We thank Richard Rest (Drexel University College of Medicine, Philadelphia, PA) for supplying rabbit antibody against recombinant ALO, Wei-Jen Tang (University of Chicago) for providing plasmid pPro-EX-H6-EF, and Aaron Firoved and Pradeep K. Gupta for constructing pSJ136EFOS. We thank Lisa Yeh for assistance with some experiments, D. Eric Anderson (NIDDK) for assistance with ESI-MS, Devorah Crown for help with animal studies, and Xavier Gomis Ruth for his support and encouragement. This work was supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, MD, USA.

References

1. Leppla, SH. Anthrax Toxin. In: Aktories, K.; Just, I., editors. Bacterial Protein Toxins. Springer; Berlin: 2000. p. 445-472.
2. Moayeri, M.; Leppla, SH. Anthrax toxins. In: Bergman, NH., editor. Bacillus anthracis and anthrax. John Wiley & Sons, Inc; Hoboken, NJ: 2011. p. 121-156.
3. Moayeri M, Leppla SH. Cellular and systemic effects of anthrax lethal toxin and edema toxin. Mol Aspects Med. 2009; 30:439–455. [PubMed: 19638283]
4. Young JA, Collier RJ. Anthrax toxin: receptor-binding, internalization, pore formation, and translocation. Annu Rev Biochem. 2007; 76:243–265. [PubMed: 17335404]
5. Puziss M, Wright GG. Studies on immunity in anthrax. X. Gel-adsorbed protective antigen for immunization of man. J Bacteriol. 1963; 85:230–236. [PubMed: 13972632]
6. Farchaus JW, Ribot WJ, Jendrek S, Little SF. Fermentation, purification, and characterization of protective antigen from a recombinant, avirulent strain of *Bacillus anthracis*. Appl Environ Microbiol. 1998; 64:982–991. [PubMed: 9501438]
7. Varughese M, Teixeira AV, Liu S, Leppla SH. Identification of a receptor-binding region within domain 4 of the protective antigen component of anthrax toxin. Infect Immun. 1999; 67:1860–1865. [PubMed: 10085028]
8. Singh Y, Klimpel KR, Quinn CP, Chaudhary VK, Leppla SH. The carboxyl-terminal end of protective antigen is required for receptor binding and anthrax toxin activity. J Biol Chem. 1991; 266:15493–15497. [PubMed: 1651334]
9. Park S, Leppla SH. Optimized production and purification of *Bacillus anthracis* lethal factor. Protein Expr Purif. 2000; 18:293–302. [PubMed: 10733882]
10. Antelmann H, Williams RC, Miethke M, Wipat A, Albrecht D, Harwood CR, Hecker M. The extracellular and cytoplasmic proteomes of the non-virulent *Bacillus anthracis* strain UM23C1-2. Proteomics. 2005; 5:3684–3695. [PubMed: 16121336]

11. Chitlaru T, Gat O, Gozlan Y, Ariel N, Shafferman A. Differential proteomic analysis of the *Bacillus anthracis* secretome: distinct plasmid and chromosome CO2-dependent cross talk mechanisms modulate extracellular proteolytic activities. J Bacteriol. 2006; 188:3551–3571. [PubMed: 16672610]
12. Chung MC, Popova TG, Millis BA, Mukherjee DV, Zhou W, Liotta LA, Petricoin EF, Chandhoke V, Bailey C, Popov SG. Secreted neutral metalloproteases of *Bacillus anthracis* as candidate pathogenic factors. J Biol Chem. 2006; 281:31408–31418. [PubMed: 16926147]
13. Grass G, Schierhorn A, Sorkau E, Muller H, Rucknagel P, Nies DH, Fricke B. Camelysin is a novel surface metalloproteinase from *Bacillus cereus*. Infect Immun. 2004; 72:219–228. [PubMed: 14688099]
14. Gomis-Ruth FX. Catalytic domain architecture of metzincin metalloproteases. J Biol Chem. 2009; 284:15353–15357. [PubMed: 19201757]
15. Pomerantsev AP, Sitaraman R, Galloway CR, Kivovich V, Leppla SH. Genome engineering in *Bacillus anthracis* using Cre recombinase. Infect Immun. 2006; 74:682–693. [PubMed: 16369025]
16. Kastrup CJ, Boedicker JQ, Pomerantsev AP, Moayeri M, Bian Y, Pompano RR, Kline TR, Sylvestre P, Shen F, Leppla SH, Tang WJ, Ismagilov RF. Spatial localization of bacteria controls coagulation of human blood by 'quorum acting'. Nat Chem Biol. 2008; 4:742–750. [PubMed: 19031531]
17. Shannon JG, Ross CL, Koehler TM, Rest RF. Characterization of anthrolysin O, the *Bacillus anthracis* cholesterol-dependent cytolysin. Infect Immun. 2003; 71:3183–3189. [PubMed: 12761097]
18. Wu XC, Lee W, Tran L, Wong SL. Engineering a *Bacillus subtilis* expression-secretion system with a strain deficient in six extracellular proteases. J Bacteriol. 1991; 173:4952–4958. [PubMed: 1907264]
19. Murashima K, Chen CL, Kosugi A, Tamaru Y, Doi RH, Wong SL. Heterologous production of *Clostridium cellulovorans* engB, using protease-deficient *Bacillus subtilis*, and preparation of active recombinant cellulosomes. J Bacteriol. 2002; 184:76–81. [PubMed: 11741846]
20. Sambrook, J.; Russell, DW. Molecular Cloning; A Laboratory Manual; Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 2001.
21. Singh Y, Chaudhary VK, Leppla SH. A deleted variant of *Bacillus anthracis* protective antigen is non-toxic and blocks anthrax toxin action in vivo. J Biol Chem. 1989; 264:19103–19107. [PubMed: 2509473]
22. Thorne, CB. Bacillus anthracis. In: Sonenshein, AB.; Hoch, JA.; Losick, R., editors. Bacillus subtilis and other Gram-Positive Bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology; Washington, D.C: 1993. p. 113-124.
23. Pomerantsev AP, Camp A, Leppla SH. A new minimal replicon of *Bacillus anthracis* plasmid pXO1. J Bacteriol. 2009; 191:5134–5146. [PubMed: 19502400]
24. Pritzlaff CA, Chang JC, Kuo SP, Tamura GS, Rubens CE, Nizet V. Genetic basis for the beta-haemolytic/cytolytic activity of group B *Streptococcus*. Mol Microbiol. 2001; 39:236–247. [PubMed: 11136446]
25. Sastalla I, Rosovitz MJ, Leppla SH. Accidental selection and intentional restoration of sporulation-deficient mutants of *Bacillus anthracis*. Appl Environ Microbiol. 2010; 76:6318–6321. [PubMed: 20639373]
26. Pomerantsev AP, Kalnin KV, Osorio M, Leppla SH. Phosphatidylcholine-specific phospholipase C and sphingomyelinase activities in bacteria of the *Bacillus cereus* group. Infect Immun. 2003; 71:6591–6606. [PubMed: 14573681]
27. Soelaiman S, Wei BQ, Bergson P, Lee YS, Shen Y, Mrksich M, Shoichet BK, Tang WJ. Structure-based inhibitor discovery against adenylyl cyclase toxins from pathogenic bacteria that cause anthrax and whooping cough. J Biol Chem. 2003; 278:25990–25997. [PubMed: 12676933]
28. Ravel J, Jiang L, Stanley ST, Wilson MR, Decker RS, Read TD, Worsham P, Keim PS, Salzberg SL, Fraser-Liggett CM, Rasko DA. The complete genome sequence of *Bacillus anthracis* Ames "Ancestor". J Bacteriol. 2009; 191:445–446. [PubMed: 18952800]
29. Read TD, Peterson SN, Tourasse N, Baillie LW, Paulsen IT, Nelson KE, Tettelin H, Fouts DE, Eisen JA, Gill SR, Holtzapple EK, Okstad OA, Helgason E, Rilstone J, Wu M, Kolonay JF,

Beanan MJ, Dodson RJ, Brinkac LM, Gwinn M, DeBoy RT, Madpu R, Daugherty SC, Durkin AS, Haft DH, Nelson WC, Peterson JD, Pop M, Khouri HM, Radune D, Benton JL, Mahamoud Y, Jiang L, Hance IR, Weidman JF, Berry KJ, Plaut RD, Wolf AM, Watkins KL, Nierman WC, Hazen A, Cline R, Redmond C, Thwaite JE, White O, Salzberg SL, Thomason B, Friedlander AM, Koehler TM, Hanna PC, Kolsto AB, Fraser CM. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. Nature. 2003; 423:81–86. [PubMed: 12721629]

30. Fedhila S, Gohar M, Slamti L, Nel P, Lereclus D. The *Bacillus thuringiensis* PlcR-regulated gene *inhA2* is necessary, but not sufficient, for virulence. J Bacteriol. 2003; 185:2820–2825. [PubMed: 12700261]
31. Chai Y, Kolter R, Losick R. Reversal of an epigenetic switch governing cell chaining in *Bacillus subtilis* by protein instability. Mol Microbiol. 2010; 78:218–229. [PubMed: 20923420]
32. Pflughoeft KJ, Sumby P, Koehler TM. The *Bacillus anthracis sin* locus and regulation of secreted proteases. J Bacteriol. 2010; 193:631–639. [PubMed: 21131488]
33. Passalacqua KD, Varadarajan A, Ondov BD, Okou DT, Zwick ME, Bergman NH. The structure and complexity of a bacterial transcriptome. J Bacteriol. 2009; 191:3203–3211. [PubMed: 19304856]
34. Liu YT, Lin SB, Huang CP, Huang CM. A novel immunogenic spore coat-associated protein in *Bacillus anthracis*: Characterization via proteomics approaches and a vector-based vaccine system. Protein Expr Purif. 2008; 57:72–80. [PubMed: 18029197]
35. Sastalla I, Maltese L, Pomerantseva OM, Pomerantsev AP, Keane-Myers A, Leppla SH. Activation of the latent PlcR regulon in *Bacillus anthracis*. Microbiology. 2010; 156:2982–2993. [PubMed: 20688829]
36. Bartkus JM, Leppla SH. Transcriptional regulation of the protective antigen gene of *Bacillus anthracis*. Infect Immun. 1989; 57:2295–2300. [PubMed: 2501216]
37. Gupta PK, Moayeri M, Crown D, Fattah RJ, Leppla SH. Role of N-terminal amino acids in the potency of anthrax lethal factor. PLoS ONE. 2008; 3:e3130. [PubMed: 18769623]
38. Firoved AM, Miller GF, Moayeri M, Kakkar R, Shen Y, Wiggins JF, McNally EM, Tang WJ, Leppla SH. *Bacillus anthracis* edema toxin causes extensive tissue lesions and rapid lethality in mice. Am J Pathol. 2005; 167:1309–1320. [PubMed: 16251415]
39. Leppla SH. Purification and characterization of adenylyl cyclase from *Bacillus anthracis*. Methods Enzymol. 1991; 195:153–168. [PubMed: 1903483]

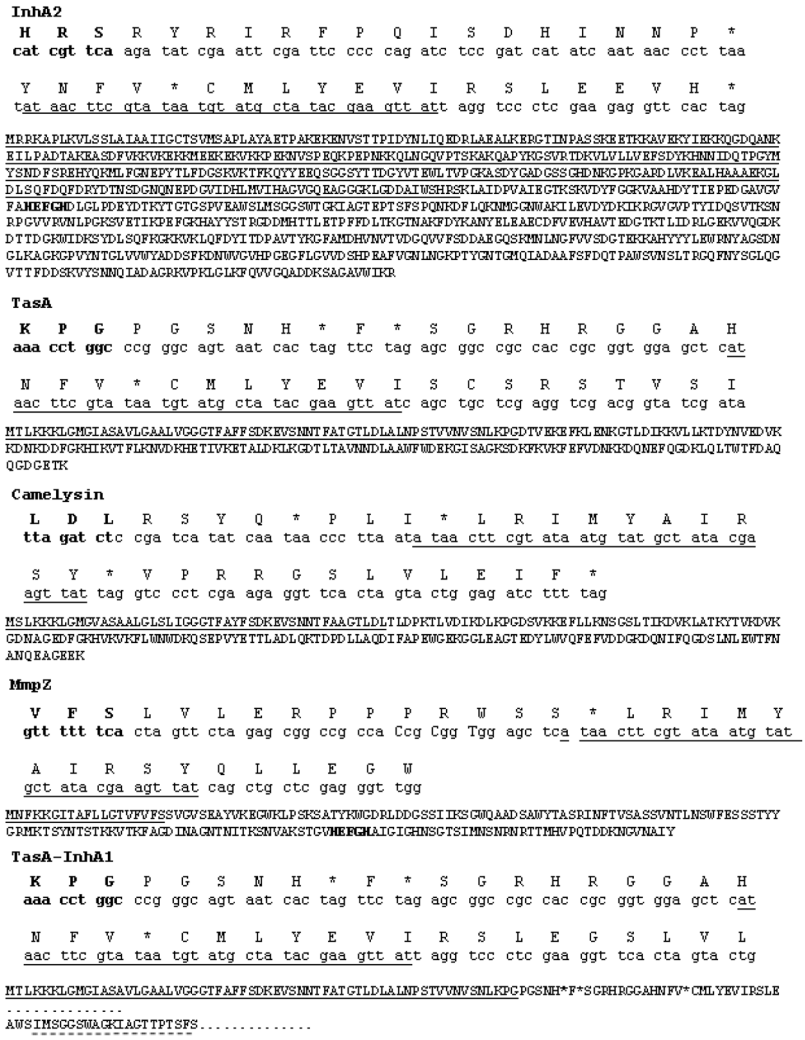


Fig. 1. Sequences of truncated and inactivated *B. anthracis* proteases

Protease genes were inactivated using a procedure that results in the insertion of the 34-bp pair *loxP* sequence (underlined in the nucleotide sequences) flanked by several endonuclease restriction sites (not indicated). The large region between the TasA and InhA1 genes was replaced by a *loxP* sequence using the same procedure. For each protease, the amino acid and nucleotide sequences shown in bold in the upper section identify the final three amino acids retained from the original protease. The amino acid sequences following this are nonsense and/or out of frame translations encoded by the restriction sites and *loxP* sequences; translation terminates at the codons indicated by asterisks. The bottom sections for each gene show the entire amino acid sequences (including signal sequences) for each of the first four proteases. The frame shifts that occur following the three amino acids shown in the upper sections cause only the underlined portions of the original amino acid sequences to be translated. In the InhA2 and MmpZ amino acid sequences, the two identical zinc-binding active site sequences (HEFGH) are shown in bold. For the large TasA-InhA1 deletion, truncation of the TasA protein occurs as in the single TasA deletion shown above. The DNA sequence deleted extends to and includes that encoding the first 402 amino acids of InhA1 (not indicated). The remaining DNA begins with sequences that correspond to the last 393 amino acids of InhA1, which begins with the sequence IMSGGS (dashed underlining).

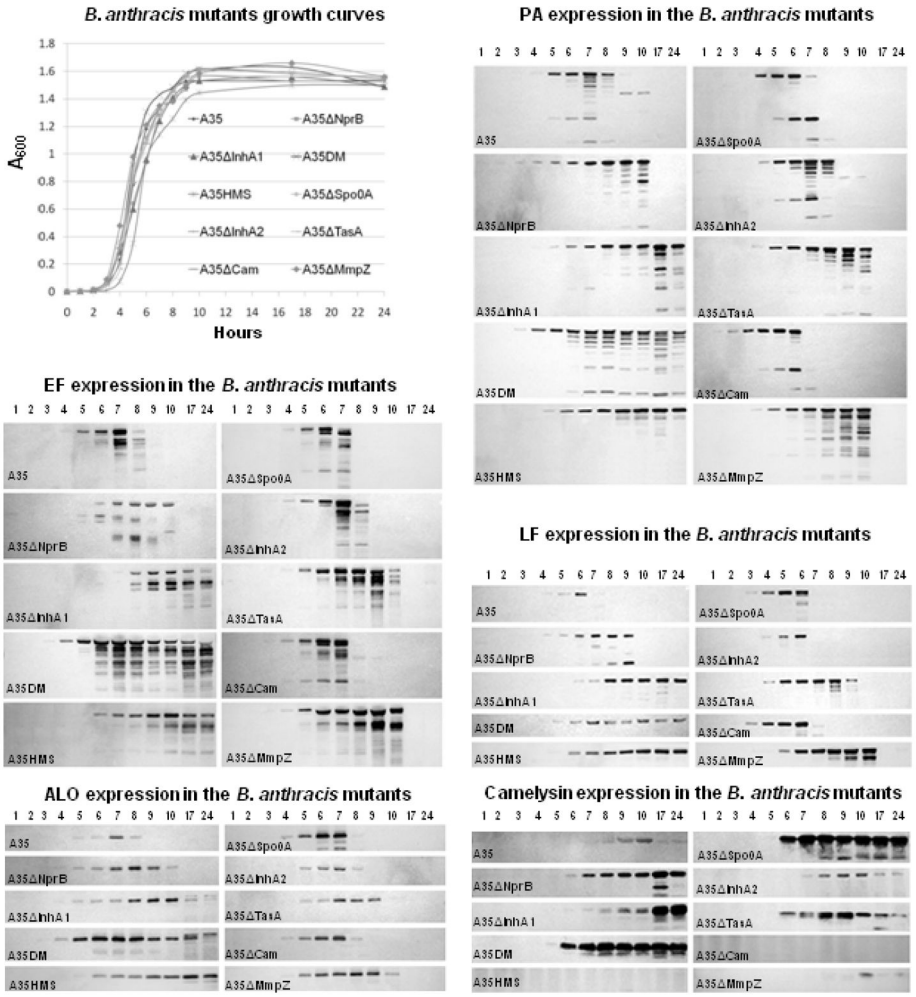


Fig. 2. Growth curves and PA, EF, LF, ALO, and camelysin production analyses

Growth curves in LB medium are shown for ten *B. anthracis* strains over 24 h. Western blot analyses of PA, LF, EF, ALO, and camelysin at various time points are shown for each strain. The most slowly migrating band in each set of blots corresponds to the full-length proteins: PA (83 kDa), EF (89 kDa), LF (90 kDa), ALO (53 kDa), and camelysin (19 kDa).

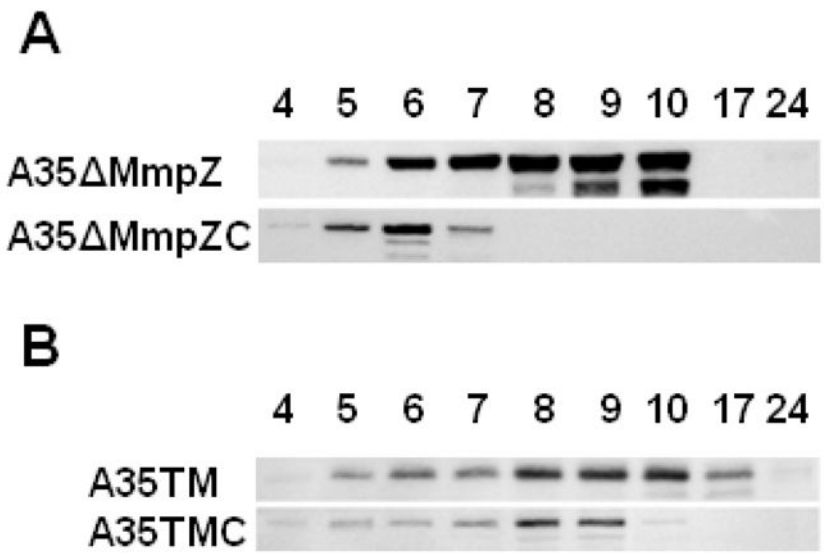
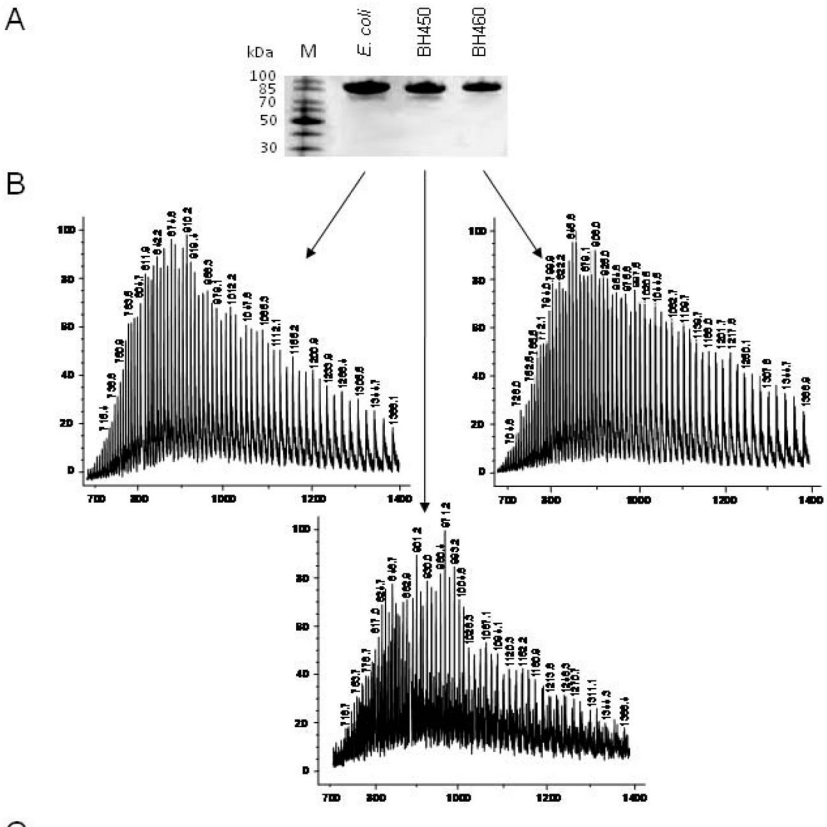


Fig. 3. LF and ALO production by mutated and complemented strains

Western blot analyses are shown of (A) LF production by A35ΔMmpZ compared to A35ΔMmpZC and (B) ALO production by A35TM compared to A35TMC. Numbers on the top of each lane indicate time in hours at which samples were taken.



Strain	*E. coli*	BH450	BH460
Experimental molecular masses (Da) Relative abundances (%) of components	89995 (100%)	87326 86366 (54%) (46%)	88687 88820 (53%) (47%)
Mass (Da) predicted from sequence	89989	88822	88822
Experimental vs. predicted mass (Da)	6	-1496 -2456	-135 2

Fig. 4. Comparison of EF proteins purified from either *E. coli* BL21(DE3) or *B. anthracis* BH450 and BH460 strains

A) SDS-PAGE analysis of the EF proteins. M – molecular mass markers (Fermentas, Page Ruler Unstained Protein Ladder). (B) Electron Spray Ionization – Mass Spectra (ESI-MS) of the EF samples from panel A. Y axis represents relative abundance and X axis represents mass/charge ratio (m/z). C) Comparison of the experimental molecular masses of the EF samples extrapolated from ESI-MS data to the molecular masses calculated from the amino acid sequences. Relative abundances of the resulting components from the ESI-MS data are shown in parentheses. Final line shows differences between experimental and calculated masses.

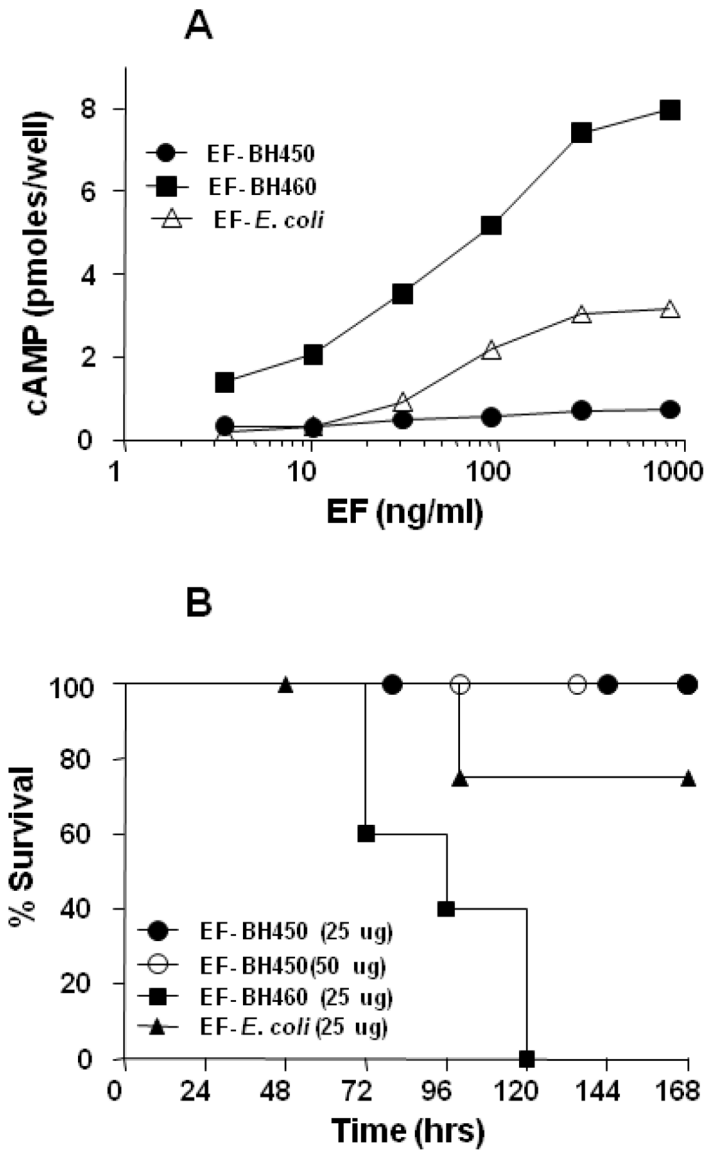


Fig. 5. EF activity analyses

A) cAMP production by different EF preparations was measured following treatment of RAW264.7 cells for 1 h with a range of EF concentrations and a set PA concentration (250 ng/ml). B) Potency of EF prepared from BH460 was compared to EF prepared from BH450 or from *E. coli* BL21(DE3) in Balb/cJ mice challenged with either 25 μg EF + 25 μg PA (for EF made from BH450, BH460, or *E. coli*) or 50 μg EF+ 50 μg PA (for EF made from BH450 only).

TABLE 1

B. anthracis Ames ancestor strain genes inactivated or analyzed in this study.

Protein	Gene	Function/Name	Locus Tag
NprB	*nprB*	metallopeptidase	GBAA_0599
InhA2	*inhA2*	metallopeptidase	GBAA_0672
TasA	*tasA*	metallopeptidase	GBAA_1288
Camelysin	*calY*	metallopeptidase	GBAA_1290
SinI	*sinI*	regulatory protein	GBAA_1292
SinR	*sinR*	regulatory protein	GBAA_1293
InhA1	*inhA1*	metallopeptidase	GBAA_1295
MmpZ	*mmpZ*	metallopeptidase	GBAA_3159
ALO	*alo*	Thiol-activated cytolysin (anthrolysin)	GBAA_3355
Spo0A	*spo0A*	sporulation regulator	GBAA_4394
EF	*cya*	edema factor	GBAA_pXO1_0142
PA	*pag*	protective antigen	GBAA_pXO1_0164
LF	*lef*	lethal factor	GBAA_pXO1_0172

TABLE 2

Primers used in this study

Primer	Sequence [a] (5′-3′) (location)	Relevant property	Site
0672LL	GCTCGAGCGGATGTACATCTGTAATGAGT	Primer pair to amplify left fragment of *inhA2*gene to clone it into pDC	XhoI
0672LR	GGATATCTTGAACGATGTGACCAAATG		EcoRV
0672RL	GCCCGGGCCTGTCGAAGCTTGGTCATT	Primer pair to amplify right fragment of *inhA2* gene to clone it into pDC	SmaI
0672RR	GCCGCGGTTTGCATACCTGTGTTACCG		SacII
0672seqF	GGTCAAGAAGCTGGTGGAGGTA	Primer pair to verify *inhA2*gene disruption	
0672seqR	TCTGTTCCTGCAATTTTCCC		
1288LL	GCTCGAGTAATTTGGAAGGTGATTAGC	Primer pair to amplify left fragment of *tasA* gene to clone it into pSC	XhoI
1288LR	GCCCGGGTTCACATCTTCTACATTGTAAT		SmaI
1288RL	GACTAGTAACAATCGTAAAAGAAACAGCG	Primer pair to amplify right fragment of *tasA* gene to clone it into pSC	SpeI
1288RR	GGAGCTCTATCGATCGCCTGTAAATTC		SacI
1288seqF	GGGGATATGGACATGACTTT	Primer pair to verify *tasA* gene disruption	
1288seqR	CAGTAAGTGTGTCACCCTTC		
1290L	GAGAAGATAGCTGCTGAGAG	Primer pair to amplify *calY* gene to clone it into pDC	
1290R	TAGAGGGAGTTTAATGGGGA		
1290seqF	GAAATTGCGCAAAAAGAT	Primer pair to verify *calY* gene disruption	
1290seqR	AGAGCCATTCCAGAACGC		
3159LL	GCTCGAGGGGTAATAACTTTCAATTAATAC	Primer pair to amplify left fragment of *mmpZ* gene to clone it into pSC	XhoI
3159LR	GGATATCGAAAAAACAAACACAGTACC		EcoRV
3159RL	GCCCGGGGGTTGGCAAGCTGCCGATTC	Primer pair to amplify right fragment of *mmpZ* gene to clone it into pSC	SmaI
3159RR	GCCGCGGCGAATGGTTCAATTGCTCCG		SacII
3159seqF	GGTACTGTGTTTGTTTTTTC	Primer pair to verify *mmpZ* gene disruption	
3159seqR	GAATCGGCAGCTTGCCAACC		
3159CF	CCTCGAGTTTCATTTTTGAAGTCTTCTTC	Primer pair to complement *mmpZ* gene disruption	XhoI
3159CR	CACTAGTCAGCGAAACGATGATTGATTTT		SpeI
deltaF	TCCGATTAGGAAGTTGACAA	Primer pair to verify deletion of *tasA-inhA1* region	
deltaR	CAGTTACCACCAATTGTTTT		
deltaCF	CCTCGAGTTTTCTTATTGCATTTCTAATGTGTTCG	Primer pair to complement *tasA-inhA1* region deletion	XhoI
deltaCR	CCCGCGGTTAGCGATATAAGCGAACAG		SacII

[a]Restriction enzyme recognition sites are underlined.

TABLE 3

Plasmids used in this study

Plasmid	Relevant characteristic(s)[a]	Source or reference
pHY304	Contains Em^R gene and strongly temperature-sensitive replicon for both *E. coli* and gram-positive bacteria; Em^R in both *E. coli* and *B. anthracis*	[24]
pDC	*Ω-sp* cassette flanked by two similarly oriented *loxP* sites and two external multiple restriction sites (single *Xho*I, *Sal*I, and *Eco*RV upstream of first *loxP* and single *Pst*I, *Xma*I and *Sac*II downstream of second *loxP*) inserted into pHY304	[15]
pSC	Plasmid used for single crossovers in *B. anthracis.* Ap^R in *E. coli*; Em^R both in *E. coli* and *B. anthracis*	[23]
pInhA2I	pDC with *loxP-Ω-sp-loxP* flanked 3′ and 5′ by *inhA2* gene sequences	This work
pTasALI	pSC containing *tasA* fragment amplified with primer pair 1288LL/1288LR	This work
pTasARI	pSC containing *tasA* fragment amplified with primer pair 1288RL/1288RR	This work
pCamI	pHY304 with *loxP-Ω-sp-loxP* flanked 3′ and 5′ by *calY* gene sequences	This work
pMmpZLI	pSC containing *mmpZ* fragment amplified with primer pair 3159LL/3159LR	This work
pMmpZRI	pSC containing *mmpZ* fragment amplified with primer pair 3159RL/3159RR	This work
pCrePA	Contains *cre* gene and strongly temperature-sensitive replicon for both *E. coli* and gram-positive bacteria; Em^R in both *E. coli* and *B. anthracis*	[15]
pCrePAS	Contains *cre* gene and strongly temperature-sensitive replicon for both *E. coli* and gram-positive bacteria; Sp^R in both *E. coli* and *B. anthracis*	[23]
pMmpZC	pSC with 3159F/3159R PCR fragment containing entire *mmpZ* gene	This work
pΔTasA-InhA1C	pSC with deltaCF/deltaCR fragment containing entire *tasA-inhA1* DNA region	This work
pS L304	pDC with *loxP-Ω-sp-loxP* flanked 3′ and 5′ by *spo0A* sequences	[15]
pSJ136EFOS	Contains *B. anthracis cya* gene instead of the *lef* gene in pSJ115 [9]	This work

TABLE 4

Bacterial strains used in this study.

Strain	Relevant characteristic(s)[a]	Source or reference
A33	*B. anthracis* Ames 33 strain (pXO1^{-} pXO2^{-})	[26]
A35	*B. anthracis* Ames 35 strain (pXO1^{+} pXO2^{-})	[15]
A35ΔSpo0A	*spo0A* knockout containing one *loxP* site, previous name of this strain is SΩL35	[15]
A35ΔNprB	*nprB* knockout containing one *loxP* site	[15]
A35ΔInhA2	*inhA2* knockout containing one *loxP* site	This work
A35ΔTasA	*tasA* knockout containing one *loxP* site	This work
A35ΔCam	*calY* knockout containing one *loxP* site	This work
A35ΔInhA1	*inhA1* knockout containing one *loxP* site	[16]
A35ΔMmpZ	*mmpZ* knockout containing one *loxP* site	This work
A35ΔmmpZC	A35ΔMmpZ complemented *in situ* by insertion of native *mmpZ* gene	This work
A35DM	Ames 35 double mutant with *nprB* and *inhA1* knockouts; each gene contains one *loxP* site	This work
A35TM	Ames 35 tetra-protease mutant; has *nprB* knockout containing one *loxP* site and deleted DNA region including *tasA, calY, sinI, sinR,* and *inhA1* genes	This work
A35TMC	A35TM with *tasA-inhA1* deletion restored *in situ* by insertion of native DNA region including *tasA, calY, sinI, sinR,* and *inhA1* genes	This work
A35PM	Ames 35 penta-protease mutant; 35TM with *inhA2* knockout containing one *loxP* site; total of 3 *loxP* sites in chromosome	This work
A35HM	Ames 35 hexa-protease mutant: A35PM with *mmpZ* knockout containing one *loxP* site; total of 4 *loxP* sites in chromosome	This work
A35HMS	A35HM with *spo0A* knockout containing one *loxP* site; total of 5 *loxP* sites in chromosome	This work
BH460	A35HMS cured of pXO1	This work
BH450	A33; *nprB* and *spo0A* knockouts, residual *loxP* sites in both *nprB* and *spo0A* genes; strain was previously named MSLL33	[15]