Capsule depolymerase overexpression reduces *Bacillus anthracis* virulence

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Capsule depolymerase (CapD) is a \(\gamma\)-glutamyl transpeptidase and a product of the *Bacillus anthracis* capsule biosynthesis operon. In this study, we examined the effect of modulating *capD* expression on *B. anthracis* capsule phenotype, interaction with phagocytic cells and virulence in guinea pigs. Transcriptional fusions of *capD* were made to the genes encoding heat-shock protein 60 (*hsp60*) and elongation factor Tu (*EFTu*), and to *capA*, a *B. anthracis* capsule biosynthesis gene. Translation signals were altered to improve expression of *capD*, including replacing the putative ribosome-binding site with a consensus sequence and the TTG start codon with ATG. CapD was not detected by immunoblotting in lysates from wild-type *B. anthracis* Ames but was detected in strains engineered with a consensus ribosome-binding site for *capD*. Strains overexpressing *capD* at amounts detected by immunoblotting were found to have less surface-associated capsule and released primarily lower-molecular-mass capsule into culture supernatants. Overexpression of *capD* increased susceptibility to neutrophil phagocytic killing and adherence to macrophages and resulted in reduced fitness in a guinea pig model of infection. These data suggest that *B. anthracis* may have evolved weak *capD* expression resulting in optimized capsule-mediated virulence.

INTRODUCTION

*Bacillus anthracis* is the causative agent of anthrax and is considered one of the most important biological warfare threats. The virulence of *B. anthracis* is primarily attributed to genes located on pathogenicity islands of pX01 and pX02. A tripartite toxin composed of protective antigen, lethal factor and oedema factor is encoded on pX01, while genes for the synthesis of an antiphagocytic, poly-\(\gamma\)-D-glutamic acid capsule are carried on pX02 (Mock & Fouet, 2001). The capsule operon is composed of five genes, *capB*, C, A, D and E. Capsule depolymerase, encoded by *capD*, is a \(\gamma\)-glutamyl transpeptidase (GGT)-family protein that autocatalytically forms a heterodimer consisting of 35 kDa and 15 kDa subunits. CapD shares 32 % identity with the *Bacillus subtilis* GGT and 35 % identity with a recently reported *Francisella* CapD homologue (Su *et al.*, 2007). Despite this amino acid sequence similarity, the *B. subtilis* GGT is reported to be secreted (Kimura *et al.*, 2004), while *B. anthracis* CapD is membrane associated (Candela & Fouet, 2005). The individual subunits of CapD associate to form a functional enzyme that cleaves the \(\gamma\)-linked peptide bond of the polyglutamate capsule, transferring glutamate to a water molecule (hydrolysis) or an amino acid (transpeptidation). This mechanism results in release of low-molecular-mass capsule and attachment of the capsule to the peptidoglycan layer (Candela & Fouet, 2005; Makino *et al.*, 2002; Uchida *et al.*, 1993), specifically, to the side-chain amino group of meso-diaminopimelic acid (Richter *et al.*, 2009). We previously demonstrated that the capsule-degrading activity of recombinant CapD can be exploited to enzymically remove the capsule from the surface of bacilli, resulting in enhanced phagocytic killing by human neutrophils and protection in mouse models of anthrax infection (Scorpio *et al.*, 2007, 2008).

We observed by proteomic analysis that CapD, like CapB, C and A, is associated with the membrane during growth in laboratory media and in vivo (Scorpio *et al.*, 2005). An interesting finding from this study was that while CapB, C and A were detected in similar amounts, CapD was only weakly detected in whole-cell and membrane-enriched preparations from laboratory-grown bacilli and was detected in amounts <1 % that of CapB, C and A in bacilli isolated from infected guinea pigs. Nevertheless, a functional CapD has been shown to be required for virulence in mice and guinea pigs (Candela & Fouet, 2005; Makino *et al.*, 2002; Richter *et al.*, 2009). These observations led us to hypothesize that increased expression of *capD* may result in more capsule being released from the...
surface of bacilli, potentially allowing the bacilli to be more readily phagocytosed. To investigate this, in the present study we upregulated capD expression using transcriptional fusions to various promoters and modifications to translation signals. Strains with higher capD expression had less surface-associated capsule, increased susceptibility to phagocytic killing, and reduced virulence in guinea pigs compared with strains with wild-type levels of capD expression. We propose that B. anthracis may have evolved weak capD expression signals resulting in wild-type amounts of cell-associated capsule and a ratio of low- to high-molecular-mass capsule that is optimal for virulence.

METHODS

Bacterial strains and animals. Strains and plasmids used in this study are listed in Table 1. B. anthracis Ames (pX01 + pX02 +) and ΔANR (pX01− pX02−) were cultured in brain heart infusion (BHI) broth with 0.8% sodium bicarbonate and 5% carbon dioxide or on NBY bicarbonate agar plates at 37°C with 5% carbon dioxide (Chabot et al., 2004). Escherichia coli strains DH5α and GM2163 were used for cloning and strain construction. Antibiotics were used at the following concentrations: kanamycin, 50 μg ml⁻¹ for E. coli strains and 20 μg ml⁻¹ for B. anthracis; and spectinomycin, 200 μg ml⁻¹ for B. anthracis. B. anthracis spores were prepared as previously described (Chabot et al., 2004). Female Hartley guinea pigs, 350–400 g, were obtained from the National Cancer Institute.

Construction of a capD knockout strain of B. anthracis Ames. All but the first 180 and last 105 bases of capD were deleted by homologous recombination with plasmid pE03 (Mendelson et al., 2004) containing the promoterless aphA-3 cassette from pUC18k (Menard et al., 1993) flanked by regions upstream and downstream of capD. The upstream flank was made by PCR with a 5′ Ndel site using forward primer 5′-ATAGGAATTCGCGGCGGAAAGCT-TATG-3′ and reverse primer 5′-TCCCCCGGTCGACCCGAGGATGAGGCGAC-3′ and the downstream flank was made by PCR with a 3′ KpnI site with forward primer 5′-TCCCCCGGGGCAAGAATTGCAATGGAATTC-3′ and reverse primer 5′-GGGGTACCCCTGTTTTTAACGCTTGCTTGAC-3′. Flanks were cut with the appropriate enzyme plus XmaI and ligated simultaneously into pE03 after digestion with KpnI and Ndel. The aphA-3 nonpolar mutagenesis cassette from pUC18k was then cloned into the XmaI site. Selection for homologous recombination was performed as described previously (Mendelson et al., 2004). Stable knockouts were selected for by loss of erythromycin resistance (5 μg ml⁻¹ in LB broth) and were confirmed by PCR with internal and external primers. Colonies appeared hypermucoid, compared to wild-type, when grown on NBY bicarbonate plates at 37°C with 5% CO₂.

Construction of capD fusion strains. Transcriptional fusions of capD were created by cloning an approximately 300 bp PCR product encoding the 3′ end of hsp60 (BA0267), EFTu (BA0108) or capA into the shuttle plasmid pASD2 (Day et al., 2007) as an XmaI–XhoI fragment. The ORF of capD including 29 bp upstream from the putative start codon was cloned 3′ to the above fragments as an XhoI–KpnI fragment. The reverse primer used to generate capD for fusion to capA was designed to anneal at the 3′ end of capE. Additional PCR products were generated that included the capD ORF and contained a consensus ribosome-binding site (RBS), AGGAGGT, and a TTG start codon with the forward primer 5′-GGGGCAGGCTGAGAGGAGGAC-3′ and the above capD reverse primers. Additional capD ORFs were generated that contained a consensus RBS and an ATG start codon or the wild-type RBS and a TTG start codon. Primers employed to generate the amplicons are listed in Table 2. The pASD2 plasmid constructs were passed through E. coli GM2163 before electroporation into B. anthracis strains. B. anthracis ΔANR (pX01− pX02−) was transformed with the plasmids containing capD fused to hsp60 or EFTu, and kanamycin/spectinomycin-resistant colonies were isolated on Luria–Bertani (LB) agar containing antibiotics. The plasmids were integrated at the 3′ end of hsp60 or EFTu as previously described (Day et al., 2007) and the linkages transferred to fully virulent B. anthracis Ames by CP-51 phage transduction (Green et al., 1985). This resulted

| Table 1. Bacterial strains and plasmids used in this study |
|---------------------------------|-----------------|------------------|
| **Strain or plasmid** | **Relevant characteristics** | **Source** |
| **B. anthracis** | | |
| Ames | Wild-type | Lab collection |
| ΔANR | pX01− pX02− | Lab collection |
| **E. coli** | | |
| DH5α | dam dcm | New England Biolabs |
| GM2163 | | New England Biolabs |
| **Plasmids** | | Mendelson et al. (2004) |
| pE03 | Integrative plasmid | Day et al. (2007) |
| pASD2 | Integrative plasmid | This work |
| pASD2hsp60capD | Fusion of the 3′ end of hsp60 to capD ORF | This work |
| pASD2hsp60capDRBS | Fusion of the 3′ end of hsp60 to capD ORF with engineered RBS for capD | This work |
| pASD2EFTucapDRBS | Fusion of the 3′ end of EFTu to capD ORF with engineered RBS for capD | This work |
| pASD2capAcapDRBSATG | Fusion of the 3′ end of capA to capD ORF with engineered RBS and ATG capD start codon | This work |
| pASD2capAcapJTTCG | Fusion of the 3′ end of capA to capD ORF with engineered TTG capD start codon | This work |
| pASD2capAcapDRBSTTTCG | Fusion of the 3′ end of capA to capD ORF with engineered RBS and TTG start codon | This work |
| pASD2capAcapDATTCG | Fusion of the 3′ end of capA to capD ORF with engineered ATG capD start codon | This work |
| pASD2capAcapD | Fusion of the 3′ end of capA to capD ORF | This work |
in two copies of capD in the hsp60 and EFTu fusions, one on the chromosome and one on pX02. The fusions of capD to capA were made by integrating the fusion plasmids at the 3' end of capA in the B. anthracis Ames capD mutant. Genetic linkages were confirmed by dye terminator Sanger sequencing.

**Analysis of CapD and capsule expression.** Strains overexpressing capD and wild-type Ames were grown in BHI/bicarbonate broth at 37 °C with shaking. Bacteria were collected by centrifugation and resuspended in PBS, pH 7.4. The cells were disrupted by sonication and the resulting lysates added to an equal volume of 2× SDS protein sample buffer. Samples were heated at 100 °C for 10 min and centrifuged for 2 min. The extracted proteins were separated by SDS-PAGE and stained with Gel Code Blue (Pierce Biotechnology).

Expression of capD was examined by immunoblotting (Invitrogen) and probed with mouse polyclonal anti-CapD antiserum at 1:1000 dilution. Capsule in culture supernatants from *B. anthracis* strains was visualized by electrophoresis in 1% agarose gels followed by staining with 0.3% methylene blue in 20% ethanol and destaining with water. In one experiment 9 μl of the culture supernatant from *EFTucapDRBS* containing low-molecular-mass capsule was treated with 1 μl (0.3 μg) recombinant CapD (prepared as described previously; Scorpio et al., 2007) and 1.1 μl 1 M serine. Mixtures with and without CapD were incubated at 37 °C for 2 h. Capsule degradation by CapD was visualized by electrophoresis in 2% agarose gels followed by staining with methylene blue, as described above.

**Neutrophil killing and macrophage adhesion assays.** Neutrophils were isolated and killing assays performed as previously described (Scorpio et al., 2007). Briefly, heat-shocked spores were germinated (2×10⁷ ml⁻¹) in BHI containing 0.8% sodium bicarbonate for 90–120 min until capsule was readily visible by India ink stain. Encapsulated bacilli were resuspended in Dulbecco’s Modified Eagle’s medium containing 10% heat-inactivated fetal calf serum (DF10) and 10% type AB human serum (Sigma Aldrich) as a source of complement. Purified human neutrophils were mixed with a 1:1 ratio. Neutrophils were incubated in the presence or absence of recombinant CapD (Scorpio et al., 2007) in 200 μl PBS for 30 min and centrifuged at 11 000 r.p.m. Neutrophil killing and macrophage adherence comparisons were evaluated by analysis of variance (ANOVA) with Tukey’s post hoc tests. Differences in virulence were evaluated with Student’s *t*-test for competitive index and log rank test for survival curve.

**RESULTS**

**Translational signalling affects capD expression**

To examine the effect of transcriptional and translational signalling on *capD* expression, the *capD* ORF and
translation initiation site were cloned into pASD2 downstream of the 3′ end of hsp60, EFTu or capA and the resulting plasmids integrated via homologous recombination at the respective genes in B. anthracis Ames. Two transcriptional fusions of capD were made to hsp60, one carrying the wild-type capD RBS and one with a consensus RBS (AGGAGGT). One transcriptional fusion was made to EFTu with a consensus RBS for capD translation. Five transcriptional fusions of capD were made to capA consisting of combinations of an ATG or TTG start codon with the wild-type or consensus RBS. According to the most recent B. anthracis genome annotation (Ames ancestor), the stop codon of capA overlaps with the TTG start codon of capD (http://www.ncbi.nlm.gov/nuccore/NC_007323).

Construction of the capAcapD fusions carrying a consensus RBS resulted in a 20 bp separation between the capA stop codon and the capD start codon. The capAcapD fusion strains constructed to have TTG or ATG start codons and wild-type RBS had two possible translation start sites separated by 36 bp. The capAcapD fusions included capE, which has been shown to be necessary for capsule synthesis (Candela et al., 2005), whereas capE was not included in capD fusions to hsp60 and EFTu, which were made in Ames carrying a functional capE on pXO2. Genetic linkages of the capAcapD fusion strains are detailed in Table 3.

Expression of capD was examined by immunoblotting of whole-cell lysates from strains grown for 5 h or overnight (18 h) at 37 °C in BHI/bicarbonate broth. Consistent with our observations that CapD is poorly detected by proteomics analysis, we were unable to detect CapD in lysates of either the parent wild-type Ames strain or a capD mutant at 18 h (Fig. 1a) or 5 h (Fig. 1c). By contrast, CapD was readily detected in lysates of hsp60capDRBS and EFTucapDRBS, in which the predicted capD RBS was replaced with a consensus RBS sequence. CapD was also detected at 5 h and 18 h in capAcapDRBSATG, which contains both a consensus RBS and an ATG start codon (Fig. 1a, c). CapD was not detected in capAcapD, in which the pASD2 capAcapDRBSATG plasmid integrated at capD directly after the engineered ATG start codon and formed a wild-type linkage of capD to capA. This strain behaved identically to wild-type Ames in all experiments. Additionally, CapD was not detected in capAcapDRBSTTG, capAcapDTTGG or capAcapDATG (Fig. 1a), suggesting that both a consensus RBS and an ATG start codon were necessary for detectable capD expression for capAcapD fusions. Interestingly, for all lysate preparations tested, only the large subunit of CapD reacted with the polyclonal antibody, suggesting that the small subunit was poorly immunogenic, consistent with a previous report (Candela & Fouet, 2005).

capD expression affects B. anthracis phenotype

The effect of capD overexpression on the phenotype of B. anthracis was examined by germinating spores in BHI broth containing 0.8% bicarbonate under 5% CO2 and growing them for 5 h or overnight at 37 °C. Bacilli were visualized by India ink staining and phase-contrast microscopy. When grown in BHI broth, fusions of capD to hsp60 and EFTu in which a consensus RBS was present appeared significantly less encapsulated than the parent wild-type Ames strain, while the capAcapDRBSATG fusion appeared devoid of capsule (Fig. 2). By contrast, capAcapD, capAcapDTTGG and capAcapDATG bacilli were encapsulated and appeared similar to wild-type Ames by India ink staining, while capAcapDRBSSTTG was slightly less encapsulated (data not shown).

Agarose gel electrophoresis of BHI/bicarbonate culture supernatants revealed that high-molecular-mass capsule was readily visible in the supernatant from the parent wild-type Ames strain and from capAcapD but was not visible in any of the capD-overexpressing strains, capAcapDRBSATG, EFTucapDRBS and hsp60capDRBS (Fig. 1b). Intermediate-sized capsule was observed in culture supernatants from hsp60capD, while almost entirely low-molecular-mass capsule was observed from capAcapDRBSATG, EFTucapDRBS and hsp60capDRBS. Strain capAcapDTTGG appeared to produce capsule of a slightly higher molecular mass than wild-type Ames,
suggesting a lower level of capD expression, while unexpectedly, capAcapDRBSTTG appeared to produce less total capsule than wild-type Ames (Fig. 1b). The poly-d-glutamic acid nature of the low-molecular-mass capsule was confirmed by reacting it with recombinant CapD and observing degradation on agarose gels (Fig. 3) as described previously for high-molecular-mass capsule (Scorpio et al., 2007).
Overexpression of capD reduces *B. anthracis* virulence

The effect of overexpressing capD on *B. anthracis* virulence was examined in cell culture and in a guinea pig infection model. Bacilli from two strains which overexpressed capD, *capAcapD* RBSATG and *EFTucapD* RBS, strongly adhered to RAW264.7 murine macrophages, while wild-type Ames failed to adhere (Table 4, *P*<0.001, ANOVA with Tukey’s post hoc tests). In addition, bacilli from strains *capAcapD* RBSATG and *EFTucapD* RBS were susceptible to neutrophil killing, with >99% reduction in viability compared to wild-type Ames bacilli, which were resistant, showing slight growth during the incubation (Table 4, *P*<0.001, ANOVA with Tukey’s post hoc tests) as previously reported (Scorpio et al., 2007). Wet-mount microscopy of bacilli and neutrophil mixtures revealed bacilli from *capD*-overexpressing strains engulfed in phagocytic vacuoles while bacilli from wild-type Ames were almost entirely extracellular (data not shown).

Virulence in guinea pigs was measured by intramuscular injection of wild-type Ames and *capAcapD* RBSATG spores in a 1:1 ratio (1000 spores of each strain). Animals were euthanized when moribund and the amount of each strain was measured by plating serial dilutions of spleen homogenate onto LB agar with and without antibiotics. Differences in the mean c.f.u. per g spleen between the two strains revealed that *capAcapD* RBSATG was significantly under-represented relative to wild-type Ames (Fig. 4a, *P*=0.011, Student’s *t*-test, *n*=4). To examine virulence in a non-mixed infection, guinea pigs were infected with 2000 spores of either *capAcapD* or *capAcapD* RBSATG and monitored for mortality. Strain *capAcapD* was chosen for comparison as it contains the pASD2 plasmid integrated at *capD* but is phenotypically identical to wild-type Ames, suggesting that the integrated plasmid does not have polar effects downstream that may inhibit capsule synthesis. All animals succumbed or were euthanized after infection with both strains. Infection with *capAcapD* RBSATG resulted in a mean time to death of 54.4±3.6 h (*n*=5) compared with 40.5±1.0 h (*n*=4) for *capAcapD*, representing a significant increase in survival (Fig. 4b, *P*=0.0027, log rank test). By comparison, previous experiments performed with a 2000

Fig. 2. India ink stain of bacilli from *B. anthracis* Ames and *capAcapD* RBSATG grown overnight in BHI/bicarbonate and examined by phase microscopy (magnification ×700).

Fig. 3. Low-molecular-mass capsule can be degraded by CapD. *EFTucapD* RBS supernatant was digested for 2 h at 37 °C with 0.3 μg CapD in PBS with 0.1 M serine (lane 2). Undigested capsule was treated in the same way, except that CapD was not included (lane 1). Samples were electrophoresed in 2% agarose and the gel stained with methylene blue.

Table 4. Macrophage adherence and neutrophil killing of *capD*-overexpressing strains

Bacilli from the different strains were assayed for macrophage adherence and neutrophil killing as described in Methods.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Macrophage adherence (no. of bacilli per cell, mean ± SEM)</th>
<th>Neutrophil killing (% viable, mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ames</td>
<td>0.04 ± 0.03</td>
<td>181.3 ± 86</td>
</tr>
<tr>
<td><em>EFTucapD</em></td>
<td>3.9 ± 1.4*</td>
<td>0.04 ± 0.01*</td>
</tr>
<tr>
<td><em>capAcapD</em> RBSATG</td>
<td>1.9 ± 0.4*</td>
<td>0.01 ± 0.001*</td>
</tr>
</tbody>
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*P*<0.001 vs wild-type Ames.
spore Ames challenge resulted in a mean time to death of 43.7 ± 3.3 h (unpublished observations).

**DISCUSSION**

*B. anthracis* grows to high numbers in the blood and organs of infected animals. This high-density growth is largely a result of resistance to innate immune defence mechanisms conferred in part by the antiphagocytic polyglutamate capsule. CapD has been demonstrated to be a *B. anthracis* virulence factor, contributing to virulence by anchoring the capsule to the peptidoglycan (Candela & Fouet, 2005) and by releasing low-molecular-mass capsule fragments that may interfere with innate immune function (Makino et al., 2002). We previously showed that exogenous recombinant CapD efficiently degrades the capsule, resulting in increased susceptibility to phagocytic killing. Here we demonstrate that overexpression of capD in *B. anthracis* also results in increased susceptibility to phagocytic killing and decreased virulence in *vivo*, presumably due to cleavage of capsule from the bacillus surface by excess CapD, exposing the organism to innate immune defence mechanisms.

Despite the fact that capD resides on a single transcript with capB,C and A, its expression appears to be significantly lower than that of the other genes in the operon (Scorpio et al., 2005). A previous report indicated that CapD was detected by immunoblots of crude extracts from *B. anthracis* RPG1 (Candela & Fouet, 2005). However, we were unable to detect CapD in *B. anthracis* Ames lysates with our polyclonal mouse anti-CapD antibody. The most recent *B. anthracis* genome annotation assigns the capD start codon as part of the capA stop codon, resulting in an overlap of the capD RBS sequence with the 3′ end of capA. Thus, translation of capD may be coupled with capA by a programmed shift of the ORF (Oppenheim & Yanofsky, 1980) in which the ribosome, upon completion of capA translation, is shifted back along the mRNA to reinitiate translation at the capD RBS sequence. The mechanism that suppresses capD expression may involve a combination of factors. For example, cistron terminal overlap (Shcherbakov & Garber, 2000) may affect capD translation efficiency due to ribosome competition between capA termination and capD initiation at the overlapping region (Gesteland & Atkins, 1996). Alternatively, capD expression may be regulated by a translational coupling mechanism in which the mRNA secondary structure inhibits ribosome priming (Rex et al., 1994) at the capD translation initiation region. Finally, a weak RBS and TTG start codon may affect capD expression, although TTG start codons are common in Gram-positive bacteria and are reported to have minimal effect on translation efficiency (Ozbudak et al., 2002). We created genetic fusions of capD to the 3′ end of the highly expressed genes hsp60 and EF-Tu and integrated these into the chromosome of *B. anthracis*. Additionally, we created fusions of capD to the 3′ end of capA in which the TTG start codon was replaced with ATG and the putative RBS was replaced with the consensus sequence, AGGAGGT. The recombinant methods used to construct these strains resulted in a 6 bp separation (the XhoI cloning site) between the capA stop codon and the engineered capD RBS compared with the parent Ames strain, in which the capD RBS overlaps with the 3′ end of capA and is separated by 8 bp from the capD start codon. The engineered sequence changes resulted in higher expression of capD as measured by immunoblotting (Fig. 1a). In particular, adding a consensus RBS to capD when fused to hsp60 resulted in detectable CapD compared with no detection observed with the hsp60capD fusion having the wild-type RBS, suggesting that the RBS significantly affects capD translation, similar to the effect of RBS when comparing capAcapDRBSATG with capAcapDATG (Fig. 1a).

Increased capD expression correlated with smaller amounts of surface-associated capsule, presumably due to degradation by CapD. Additionally, the culture supernatant capsule from capD-overexpressing strains in which CapD was detected by immunoblotting was found to be almost

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**Fig. 4.** Effect of capD expression on virulence in guinea pigs. (a) Competitive index of capAcapDRBSATG vs Ames. Guinea pigs were challenged with a mixture containing equal numbers of heat-shocked spores derived from Ames or capAcapDRBSATG. The number of c.f.u. g⁻¹ from the spleens was counted for each strain by plating on antibiotic-selective medium (*P*=0.011). Data are presented as log c.f.u. per g spleen. (b) Non-mixed infection. Guinea pigs were infected with 2000 spores of either capAcapD (■) or capAcapDRBSATG (●) and monitored for mortality. Analysis of Kaplan–Meier survival curves showed that the probability of survival was significantly different (*P*=0.0027).
entirely of low molecular mass. These phenotypic changes were associated with increased phagocytosis of bacilli by human neutrophils and murine macrophages, probably due to reduced surface-associated capsule. Overexpression of capD also reduced virulence in guinea pigs as measured by an increase in the mean survival time in a single-strain challenge and reduced fitness in a competitive index experiment comparing the wild-type Ames strain with capAcapD-DRBSATG. In the single-strain challenge with capAcapD-DRBSATG, bacilli isolated from the blood and spleen had a capsule that was not detectable by India ink but was visible by FITC-labelled monoclonal anti-capsule antibody stain, suggesting that only minimal surface capsule was required for dissemination from the site of infection for this strain (data not shown). The dissemination and residual virulence of the capAcapD-DRBSATG strain also supports the hypothesis that CapD-generated low-molecular-mass capsule contributes to the virulence of B. anthracis (Makino et al., 2002). The higher amounts of low-molecular-mass capsule released from capD-overexpressing strains did not interfere with phagocytosis by neutrophils, as efficient killing of capD-overexpressing strains could be observed even at a 1:1 ratio of neutrophils to bacilli in which 10% of the assay medium was replaced by supernatant capsule from the capAcapD-DRBSATG strain (data not shown). This suggests that the capsule associated with the bacillus surface is the primary mechanism for resistance to phagocytosis. It is possible that low-molecular-mass capsule may be important for resistance to other innate immune defence mechanisms such as cationic antimicrobial peptides and complement.

These data demonstrate that overexpression of capD resulted in significant capsule degradation during growth in BHI/bicarbonate broth and probably in vivo, resulting in smaller amounts of surface-associated capsule, an increase in released, low-molecular-mass capsule and reduced virulence. Taken together, the results suggest that fully virulent B. anthracis has evolved finely tuned expression of capD and that maximum virulence may require both wild-type levels of surface-associated capsule and released low-molecular-mass capsule.

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