Immunization of Mice with Formalin-Inactivated Spores from Avirulent Bacillus cereus Strains Provides Significant Protection from Challenge with Bacillus anthracis Ames

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Bacillus anthracis is the infectious form of the organism for humans and animals. However, the approved human vaccine in the United States is derived from a vegetative culture filtrate of a toxigenic, noncapsulated B. anthracis strain that primarily contains protective antigen (PA). Immunization of mice with purified spore proteins and formalin-inactivated spores (FIS) from a noncapsulated, nontoxicogenic B. anthracis strain confers protection against B. anthracis challenge when PA is also administered. To investigate the capacity of the spore particle to act as a vaccine without PA, we immunized mice subcutaneously with FIS from nontoxicogenic, noncapsulated B. cereus strain G9241 pBCXO1−/pBC210− (dcG9241), dcG9241 ΔbclA, or 569-UM20 or with exosporium isolated from dcG9241. FIS vaccination provided significant protection of mice from intraperitoneal or intranasal challenge with spores of the virulent B. anthracis Ames or Ames ΔbclA strain. Immunization with dcG9241 ΔbclA FIS, which are devoid of the immunodominant spore protein BclA, provided greater protection from challenge with either Ames strain than did immunization with FIS from BclA-producing strains. In addition, we used prechallenge immune antisera to probe a panel of recombinant B. anthracis Sterne spore proteins to identify novel immunogenic vaccine candidates. The antisera were variably reactive with BclA and with 10 other proteins, four of which were previously tested as vaccine candidates. Overall our data show that immunization with FIS from nontoxicogenic, noncapsulated B. cereus strains provides moderate to high levels of protection of mice from B. anthracis Ames challenge and that neither PA nor BclA is required for this protection.

Bacillus anthracis is a Gram-positive, spore-forming, rod-shaped bacterium that can cause cutaneous, inhalational, or gastrointestinal anthrax. Anthrax disease, which typically occurs in grazing mammals and incidentally in humans, develops after introduction and subsequent germination of B. anthracis spores within the host. In the United States, human anthrax cases are rare and occur predominantly after exposure to contaminated animal products such as wool or animal hides (1–3). The intentional dissemination of B. anthracis spores through the U.S. postal system in 2001 resulted in 22 cases of anthrax with five fatalities from inhalational anthrax (4). In recent years, Bacillus cereus strains that produce B. anthracis virulence factors have been isolated from humans with severe pulmonary “anthrax-like” infections (5–9). B. anthracis and B. cereus are closely related members of the B. cereus sensu lato group, but only B. anthracis is categorized by the U.S. Centers for Disease Control and Prevention as a Category A bioterrorism agent.

B. anthracis contains the two virulence plasmids, pXO1 and pXO2. Genes that encode the anthrax toxin subunits edema factor (EF), lethal factor (LF), and protective antigen (PA) are found on pXO1, and the genes needed to produce the poly-γ-D-glutamic acid capsule are encoded on pXO2. EF or LF combines with PA to form edema toxin (ET) or lethal toxin (LT), respectively. PA is essential for toxicity because PA binds to target cell receptors and mediates entry of EF or LF into the host cytosol (reviewed in reference 10). ET is a calmodulin-dependent adenylate cyclase that appears to elicit edema at the site of infection (11–13) and also has antiphagocytic effects on neutrophils (14). LT is a zinc-dependent metalloprotease that cleaves and subsequently inactivates mitogen-activated protein kinase kinases 1 and 2 (15, 16). The poly-γ-D-glutamic acid capsule protects vegetative bacilli from phagocytosis and macrophage killing (reviewed in reference 17). B. cereus G9241, which was isolated from a welder with pulmonary anthrax-like disease, contains the pXO1 homolog pBCXO1 and an unrelated megaplasmid, pBC210 (8). B. cereus G9241 produces PA, LF, and EF from the pBCXO1-carried genes pag, lef, and cya, respectively (8, 18). In addition, pBCXO1 encodes an intact and functional operon required for hyaluronic acid capsule synthesis (19), and pBC210 contains an operon that is necessary for production of a putative tetrascarbohydrate capsule (8, 18, 19).

In the United States, the only Food and Drug Administration-approved anthrax vaccine for human use is AVA (anthrax vaccine adsorbed) or Biothrax. AVA is derived from a vegetative culture filtrate of the attenuated B. anthracis V770-NP1-R and contains primarily PA as well as small amounts of LF and EF (20). Anti-PA antibodies generated as a result of vaccination with AVA are the main source of protection against anthrax (21). The current AVA vaccine has the following shortcomings: (i) a slightly variable composition, (ii) an 18-month/5-dose vaccination schedule with required annual boosters (22, 23), and (iii) minor to moderate local reactogenicity (24, 25). Furthermore, the effectiveness of AVA and other PA-based vaccines varies in different animal models. These vaccines provide no protection against toxicogenic, encap-
sulated *B. anthracis* in mice (26–28), confer variable protection against geographically diverse *B. anthracis* isolates in guinea pigs (29, 30), and are highly protective in rabbits and rhesus macaques (29).

The bioterrorism threat associated with *B. anthracis* and the shortcomings of AVA fostered significant research support by government agencies to develop a better and more effective anthrax vaccine. While most of the newer vaccine candidates are still based on PA, additional research on other components of *B. anthracis*, such as the spore and spore components, has yielded promising results in animals (27, 30–35). Live, attenuated spore vaccines are used safely and effectively in humans in Russia and China and in animals worldwide (36, 37). Moreover, immunization with PA and formalin-inactivated spores (FIS) of a nontoxigenic, nonencapsulated *B. cereus* strain conferred protection against virulent *B. anthracis* 9602 but protected against accessible components on the spore surface can protect against virulent *B. anthracis* in a mouse model.

(This work was presented in part at the Bacillus ACT 2011 Meeting, Bruges, Belgium, August 2011, and at the 112th General Meeting of the American Society for Microbiology, San Francisco, CA, June 2012 [38].)

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** All bacterial strains and plasmids used in this work are listed in Table 1. Bacteria were routinely cultured in Luria-Bertani (LB) broth with shaking at 37°C (250 rpm), and selection of transformants was done on LB agar plates at 37°C unless stated otherwise. Where appropriate, antibiotics were used for selection at the following final concentrations: ampicillin (Amp), 100 μg/ml; kanamycin (Kan), 100 μg/ml; and erythromycin (Erm), 5 μg/ml.

**Spore preparation and inactivation.** *B. anthracis* Sterne, *B. cereus*, and *B. anthracis* Ames spores were prepared as previously reported (18, 34). The bioterrorism threat associated with *B. anthracis* and the shortcomings of AVA fostered significant research support by government agencies to develop a better and more effective anthrax vaccine. While most of the newer vaccine candidates are still based on PA, additional research on other components of *B. anthracis*, such as the spore and spore components, has yielded promising results in animals (27, 30–35). Live, attenuated spore vaccines are used safely and effectively in humans in Russia and China and in animals worldwide (36, 37). Moreover, immunization with PA and formalin-inactivated spores (FIS) of a nontoxigenic, nonencapsulated *B. cereus* strain conferred protection against virulent *B. anthracis* 9602 but protected against accessible components on the spore surface can protect against virulent *B. anthracis* in a mouse model.

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B. cereus
G9241 pBCXO1
1-kb downstream fragment obtained by EcoRI and NotI digestion of upstream and downstream PCR fragments were ligated into the pGEM-T restriction enzyme sites are underlined and italicized, respectively. Both AAA TCA TCA ATT TGA GTC ATA GG) primers; the EcoRI and NotI restriction enzyme sites are underlined and italicized, respectively. Both AAA TCA TCA ATT TGA GTC ATA GG) primers; the EcoRI and NotI restriction enzyme sites are underlined and italicized, respectively. Both AAA TCA TCA ATT TGA GTC ATA GG) primers; the EcoRI and NotI restriction enzyme sites are underlined and italicized, respectively. Both AAA TCA TCA ATT TGA GTC ATA GG) primers; the EcoRI and NotI restriction enzyme sites are underlined and italicized, respectively. Both AAA TCA TCA ATT TGA GTC ATA GG) primers; the EcoRI and NotI restriction enzyme sites are underlined and italicized, respectively.

58
= AAC TGA TAT CAG TTT TAC TGC TTT TTC ATT GG) and bclA-down-

= AAC TGA TAT CAG TTT TAC TGC TTT TTC ATT GG) and bclA-down-

= AAC TGA TAT CAG TTT TAC TGC TTT TTC ATT GG) and bclA-down-

= AAC TGA TAT CAG TTT TAC TGC TTT TTC ATT GG) and bclA-down-

saline (PBS) with 0.5 mM EDTA and protease inhibitor cocktail set VII (EMD Millipore Chemicals, Billerica, MA). Spores were disrupted by sonication with a Fisher Scientific model 705 Sonic Dismembrator (Thermo Fisher Scientific, Pittsburgh, PA) on ice for 5 min at 40% power with 15 s on and 30 s off. After this sonication step, the lysed spores were pelleted twice by centrifugation at 15,000 × g for 20 min at 4°C. The supernatants from both centrifugation steps were pooled and filtered through a 0.45-μm polyvinylidene difluoride (PVDF) filter to remove residual spore debris. The filtered supernatant was subjected to centrifugation at 40,000 rpm in a Ti50 rotor. The supernatant was removed, and the exosporium pellet was washed in PBS and stored at −80°C.

Immunization and challenge of BALB/c mice. All B. anthracis Ames work was done under animal biosafety level 3 (ABS3) conditions in accordance with Institutional Animal Care and Use Committee regulation at the U.S. Army Medical Research Institute of Infectious Diseases. On days 1 and 15, 6- to 8-week-old female BALB/c mice (National Cancer Institute, Frederick, MD) were immunized s.c. with 200 μl of either 1 × 10^6 B. cereus FIS in 0.3% alhydrogel (Sigma-Aldrich, St. Louis, MO) or 30 μg purified dcG9241 exosporium in 0.3% alhydrogel. Sera were collected from BALB/c mice on days 14 and 28 and combined into three separate pools for each vaccine group. On day 29, mice were challenged either intraperitoneally (i.p.) with 3 × 10^6 spores (5 to 10 times the median lethal dose [LD_{50}]) or i.n. with 5 × 10^6 spores (70 to 120 times the LD_{50}) of B. anthracis Ames or B. anthracis Ames ΔbclA (39, 48, 49). The mice were monitored for morbidity and mortality for 14 days postchallenge. Significant differences in survival after challenge were determined by Fisher’s exact test, Kaplan-Meier survival analysis, and log rank tests with SAS version 8.2 (SAS Institute Inc., Cary, NC). GraphPad Prism version 5.03 (GraphPad Software Inc., La Jolla, CA) was used to calculate the median time to death (MTTD) and to test for significant differences among the groups with a nonparametric Kruskal-Wallis test and Dunn’s multiple comparison test. P values of <0.05 were considered statistically significant.

ELISA to detect anti-spore antibodies. The direct binding of antibodies in immune mouse sera to B. anthracis Ames or B. anthracis Ames ΔbclA spores was assayed by enzyme-linked immunosorbent assay (ELISA) as previously described (34, 50, 51). Specifically, irradiation-sterilized spores (1 × 10^7 spores/well) were added to an Immulon II HB microplate (Thermo Fisher Scientific) and allowed to incubate O/N at 4°C. The plate was washed three times with PBS containing 0.1% Tween 20 (PBS-T) and then blocked with PBS containing 0.5% Tween 20 and 5% milk for 24 to 48 h at 4°C. The three serum pools from each vaccine group were diluted into the blocking buffer and serially diluted in triplicate. After incubation at 37°C for 1 h, the plate was washed as before and goat anti-mouse horseradish peroxidase-conjugated IgG (KPL, Inc., Gaithersburg, MD) diluted 1:1,000 was added. After 1 h of incubation at 37°C, the plate was washed with PBS-T six times and the horseradish peroxidase substrate was added [2,2’-azino-bis(3-ethylbenzthiazolin-sulfonic) acid (ABTS) 2-component microwell peroxidase substrate kit; KPL, Inc., ]. The plate was incubated at 37°C for 30 min and the absorbance at 405 nm (A_{405}) read on a microplate reader (BioTek Instruments, Winooski, VT). The reported average A_{405} value was the mean A_{405} value from triplicate samples from each pool. ELISA data were analyzed with SPSS version 20.0.0 (IBM Inc., Armonk, NY). Overall differences in antispoar antibody titers among immunization groups were compared by two-way analysis of variance (ANOVA), adjusting for differences among dilutions, followed by Tukey pairwise post hoc comparisons. Differences at a given dilution were analyzed by one-way ANOVA followed by Tukey pairwise post hoc comparisons.

Expression and purification of exosporium proteins. All recombinant exosporium proteins used in this study (Table 2) were described
The protein was eluted from the resin in 4 resin volumes of 7 M urea, 20 mM Tris (pH 7.5), 500 mM NaCl, and 10 mM imidazole. Cells grown overnight at 30°C in autoinducing LB medium (ForMedium; E. coli previously (34). The proteins were expressed in

Table 2 B. anthracis genes expressed in this study

| Locus tag | Sterne locus tag | Protein name |
|-----------|-----------------|--------------|
| BA0108    | BAS0108         | Translation elongation factor Tu |
| BA0252    | BAS0238         | Alanine racemase |
| BA0355    | BAS0340         | CotB homolog |
| BA0803    | BAS0766         | CotC |
| BA0804    | BAS0767         | CotB |
| BA0805    | BAS0768         | CotA |
| BA1222    | BAS1130         | BdA |
| BA1234    | BAS1141         | CotZ/ExsY |
| BA1237    | BAS1144         | BxpB/ExsFA |
| BA1238    | BAS1145         | CotZ/ExsY |
| BA1489    | BAS1378         | Fe-Mn superoxide dismutase (SOD15) |
| BA1786    | BAS1655         | ExsE |
| BA2150    | NA              | ExsG |
| BA2162    | BAS2008         | BxpA |
| BA2292    | BAS2138         | Hypothetical protein (p2138) |
| BA2332    | BAS2174         | BxpC |
| BA2554    | BAS2377         | ExsK |
| BA2617    | BAS2439         | ExsD |
| BA2888    | BAS2693         | Inosine-uridine-prefering nucleoside hydrolase |
| BA3211    | BAS2986         | Hypothetical protein (p2986) |
| BA3668    | BAS3402         | Glycosyl hydrolase, family 18 |
| BA3906    | BAS3619         | CotE |
| BA4266    | BAS3957         | Hypothetical protein (p3957) |
| BA4499    | BAS4177         | Mn superoxide dismutase (SOD1A) |
| BA4722    | BAS4383         | Thi/PfpI family protein |
| BA4898    | BAS4544         | Small, acid-soluble spore protein B |
| BA5640    | BAS5241         | Cell wall hydrolase |
| BA5641    | BAS5242         | Ywdl |
| BA5699    | BAS5303         | Hypothetical protein (p5303) |

See reference 34. NA, not applicable.

RESULTS

Immunization with inactivated B. cereus spores protects BALB/c mice from B. anthracis Ames or Ames ΔbclA challenge. We previously reported that live spores of dcG9241, a plasmid-cured derivative of B. cereus G9241, is avirulent in mice (18). In a preliminary study, we found that vaccination with live dcG9241 spores completely protected A/J mice against i.n. and s.c. challenge with 10 times the LD₅₀ of B. anthracis Sterne spores (data not shown). Since dcG9241 does not produce toxins or capsule, we concluded that antibodies against spore components or vegetative antigens were sufficient to protect the mice from infection with the attenuated B. anthracis strain. Here we asked whether spores from nontoxicogenic, nonencapsulated B. cereus strains could protect mice from challenge with the fully virulent B. anthracis Ames strain. We used formalin to inactivate spores of dcG9241 and 569-UM20, a derivative of the laboratory isolate B. cereus 569 (52) produced by UV mutagenesis (40); spores were inactivated to prevent outgrowth of the vegetative form and thus to allow attribution of any protection to the spore components themselves. We vaccinated BALB/c mice twice, 2 weeks apart, with 569-UM20 or dcG9241 FIS or with exosporium isolated from dcG9241 and then challenged the mice with a lethal dose of B. anthracis Ames spores on day 29. As shown in Table 3, s.c. immunization with 569-UM20 or dcG9241 FIS protected 40 to 60% of mice challenged by i.n. or i.p. inoculation. In addition, the MTTD for those mice that succumbed to infection after immunization with FIS from either strain was significantly longer (14 and 11 days for dcG9241 and 569-UM20, respectively) than that for mice given the adjutant control (3 days) (Table 3). In contrast, vaccination with the isolated exosporium conferred no protection against B. anthracis Ames challenge. These results demonstrate that immunization with FIS alone from two nontoxicogenic, nonencapsulated B. cereus strains can partially protect mice from challenge with the virulent B. anthracis Ames.
Previous reports showed that immunization with the immunodominant spore protein BclA can enhance protection from challenge with B. anthracis, especially when BclA is administered as part of a vaccine regimen that includes PA (27, 31, 33). To determine whether BclA contributed to the protection we observed after immunization with intact spores, we immunized BALB/c mice on days 1 and 15 with dcG9241 ΔbclA FIS and challenged them on day 29 with a lethal dose of B. anthracis Ames. We observed 80% and 100% survival after i.n. and i.p. challenge, respectively, with B. anthracis Ames (Table 3). The percent survival afforded by immunization with dcG9241 ΔbclA FIS compared to immunization with dcG9241 FIS (P < 0.029) or 569-UM20 FIS (P < 0.0016) was statistically significant for the i.p. challenge route but only suggestive for the i.n. challenge route. The increased survival trend for the dcG9241 ΔbclA FIS-immunized mice suggests that removal of the immunodominant protein BclA from the vaccine may provide a beneficial effect. These findings parallel previous observations that the presence of BclA on the spore surface occludes immunogenic antigens and that the removal of BclA makes these antigens more available to the host immune system (33, 34, 45, 53, 54).

To determine whether the presence of BclA on the spore surface of the challenge strain hinders the protective capacity of antibodies generated against other, less abundant or less accessible spore proteins in response to the vaccination regimen, we challenged immunized mice with B. anthracis Ames ΔbclA spores. Deletion of bclA from B. anthracis Sterne or Ames does not reduce the virulence of either strain in mice (39, 55). Immunization with FIS from any of the strains tested provided significant protection from lethal i.n. or i.p. challenge with B. anthracis Ames ΔbclA; however, purified exosporium failed to confer protection (Table 3). As with B. anthracis Ames challenge, we observed a trend toward increased protection from B. anthracis Ames ΔbclA challenge in mice that were immunized with dcG9241 ΔbclA FIS compared to mice immunized with dcG9241 FIS or 569-UM20 FIS. Overall, these results show that immunization with B. cereus FIS partially or completely protects mice from B. anthracis Ames and Ames ΔbclA i.p. and i.n. challenge and that BclA is not a major contributor to the protection afforded by FIS; in fact, the presence of BclA may actually impede the capacity of the host immune system to recognize and respond to other immunogenic spore proteins.

**Antisera from B. cereus FIS-immunized mice are reactive with intact B. anthracis spores.** To assess the serum antibody response against inactivated B. anthracis Ames or B. anthracis Ames ΔbclA spores, we used an ELISA to analyze pooled antisera collected from each vaccination group on days 14 and 28. The day 14 antisera reacted only minimally with spores (data not shown), but the day 28 antisera exhibited a strong antisporo response (Fig. 1). The day 28 antisera from mice immunized with dcG9241 or 569-UM20 FIS were significantly more reactive with irradiated B. anthracis Ames and Ames ΔbclA spores than were antisera from the PBS/alhydrogel group. Despite the strong protection from B. anthracis Ames and B. anthracis Ames ΔbclA challenge that we observed in dcG9241 ΔbclA FIS-immunized mice, the overall antisporo titer in the antisera from mice immunized with dcG9241 ΔbclA FIS differed significantly (P < 0.001) only from those for the PBS/alhydrogel group when tested against B. anthracis Ames ΔbclA spores. However, the reactivity of antisera from mice immunized with dcG9241 ΔbclA FIS against both B. anthracis Ames spores and B. anthracis Ames ΔbclA spores differed significantly from the antibody response generated by mice immunized with dcG9241 FIS or 569-UM20 FIS (P < 0.001). Furthermore, immunization with dcG9241 ΔbclA FIS elicited a significantly lower antibody response against Ames spores and a higher response against Ames ΔbclA spores than did immuniza-

**Table 3** Survival and times to death for vaccination studies

| B. anthracis challenge strain | Challenge route | Vaccine | Survivala| MTTR (days)b |
|------------------------------|----------------|---------|----------|-------------|
| Ames                        | Intranasal     | PBS/alhydrogel | 0/20 (0) | 3           |
|                             |                | dcG9241 exosporium | 0/10 (0) | 3.5         |
|                             |                | 569-UM20 FIS | 10/20 (50)** | 11**        |
|                             |                | dcG9241 FIS | 11/20 (55)** | 14***       |
|                             |                | dcG9241 ΔbclA FIS | 8/10 (80)**** | 14***       |
|                             | Intraperitoneal | PBS/alhydrogel | 2/20 (10) | 2           |
|                             |                | dcG9241 exosporium | 1/10 (10) | 3           |
|                             |                | 569-UM20 FIS | 8/20 (40) | 3.5         |
|                             |                | dcG9241 FIS | 12/20 (60)** | 14***       |
|                             |                | dcG9241 ΔbclA FIS | 10/10 (100)**** | 14***       |
| Ames ΔbclA                  | Intranasal     | PBS/alhydrogel | 0/10 (0) | 3           |
|                             |                | dcG9241 exosporium | 0/10 (0) | 3           |
|                             |                | 569-UM20 FIS | 4/10 (40) | 7.5*        |
|                             |                | dcG9241 FIS | 5/10 (50)* | 9.5*        |
|                             |                | dcG9241 ΔbclA FIS | 8/10 (80)** | 14***       |
|                             | Intraperitoneal | PBS/alhydrogel | 0/10 (0) | 2           |
|                             |                | dcG9241 exosporium | 0/10 (0) | 2.5         |
|                             |                | 569-UM20 FIS | 7/10 (70)** | 14***       |
|                             |                | dcG9241 FIS | 9/10 (90)** | 14***       |
|                             |                | dcG9241 ΔbclA FIS | 9/10 (90)** | 14***       |

a Number of survivors/number challenged (percent survival).

b MTTD, median time to death.

c Values that are statistically different from that for the PBS/alhydrogel group are indicated by asterisks (****, P < 0.0001; ***, P < 0.001; **, P < 0.01; and *, P < 0.05).
tion with dcG9241 FIS or 569-UM20 FIS. Consistent with the survival data, the antispore titers generated in response to vaccination with isolated exosporium were similar to those in response to the PBS/algohydrol control. Taken together, these data demonstrate that vaccination followed by one boost with FIS elicits a robust antispore antibody response in mice at 28 days after the initial immunization. In addition, these findings strongly suggest that the presence of BclA alters the immune response to other spore surface antigens and that other spore proteins contribute to protection.

**Antisera from *B. cereus* FIS-immunized mice are reactive with potentially novel spore surface immunogens.** To identify specific *B. anthracis* proteins to which antibodies were raised in response to vaccination with *B. cereus* FIS, we tested the reactivity of the day 28 mouse antisera pooled from each vaccination group with a previously reported panel of recombinant spore surface proteins (34) (Fig. 2A). We observed the highest reactivity of the antisera to the cell wall hydrolase (BA_5640) for all of the vaccine groups, including the PBS/algohydrol group, so we used the cell wall hydrolase signal intensity for the 100% data normalization value. As predicted by the survival and ELISA data, the reactivity of the exosporium vaccine group antisera was significantly different (P < 0.05) from the same dcG9241 ΔbclA FIS dilution.

**DISCUSSION**

The consensus from the literature is that immunization with *B. anthracis* live spores, inactivated spores, or spore components requires PA to protect mice against challenge with virulent *B. anthracis* (27, 31–35, 56, 57). Despite this requirement for PA, all of these examples demonstrate the capacity of spore antigens to contribute to vaccine efficacy. In this study, we not only provided further evidence for the contribution of spore components toward vaccine efficacy but also showed that immunization with FIS from nontoxigenic, nonencapsulated *B. cereus* strains 569-UM20, dcG9241, and dcG9241 ΔbclA protected mice from the highly virulent *B. anthracis* Ames strain without the addition of exogenous PA to the vaccine regimen. Furthermore, to our knowledge, inactivated *B. cereus* spores have never been tested as an anthrax vaccine in mice, animals that are inherently difficult to protect from a virulent *B. anthracis* challenge (28, 58). Thus, the novelty of our protection experiments is in the use of *B. cereus* inactivated spores and in the absence of any form of PA.

Brossier et al. showed that administration of FIS in conjunction with PA elicits a response that completely protects mice and guinea pigs from s.c. challenge with virulent *B. anthracis* 9602, an encapsulated, toxigenic strain of *B. anthracis* with virulence similar to that of Ames; either FIS or PA alone provide only minor protection in guinea pigs and no protection in mice (32). A follow-up study by Gauthier et al. showed that vaccination with FIS plus PA protects guinea pigs from i.n. challenge with *B. anthracis* 9602 but fails to protect mice (35). In those experiments, the FIS vaccine strain was *B. anthracis* RPLC2, a nontoxigenic Sterne derivative (32, 35). We hypothesize that differences in *B. cereus* spore composition or antigen presentation compared to *B. anthracis* RPLC2 spores contributed to the efficacy of *B. cereus* FIS in our experiments. However, we cannot rule out that experimental differences between our FIS vaccination studies and those previously reported could have contributed to our increased FIS vaccine efficacy. These experimental differences include (i) the use of Swiss outbred mice versus BALB/c mice, (ii) challenge with *B. anthracis* 9602 versus *B. anthracis* Ames, (iii) the number of spores administered, and (iv) the time between vaccination and challenge. The use of different mouse strains probably makes little difference.
from a virulence viewpoint because the LD$_{50}$ values for the related strain B. anthracis Vollum 1B vary by at most only 10-fold between Swiss outbred and BALB/c mice (58). The LD$_{50}$ values for B. anthracis Ames and 9602 are similar for i.p. inoculation (32, 49), but B. anthracis 9602 is 10- to 100-fold less virulent than Ames by i.n. administration (35, 48, 59). Since the challenge doses in each experiment were based on LD$_{50}$ values for each bacterial challenge strain in a given mouse strain, the actual number of spores administered also should not contribute to the different FIS vaccination outcomes between our study and previous reports. Lastly, in our experiments, we challenged the mice 2 weeks after the booster injection, while the challenge occurred 3 weeks after the boost in the previously reported FIS studies. Since our mice were challenged 1 week earlier than mice in the other study, our mice actually had less time to mount an immune response. Therefore, the time between vaccination and challenge most likely did not contribute to the different outcomes. We contend that the most significant difference between our experiments and those of others was the choice of vaccine strain. The increased survival rates in the dcG9241 and dcG9241/H9004 bclA FIS-vaccinated mice were all statistically significant compared to those for the PBS/alhydrogel controls, while the increased protection afforded by 569-UM20 FIS vaccination was only variably significant (Table 3). This trend suggests that vaccination with either dcG9241 or dcG9241 ΔbclA FIS is more protective, a finding that highlights the importance of the strain chosen for the FIS vaccine.

BclA is highly immunogenic and is the major immunogen on the spore surface (47). However, here we demonstrated that the presence of BclA on FIS did not contribute to better protection against Ames challenge; in fact, the increased survival trend observed in the dcG9241 ΔbclA FIS vaccination group suggests that removal of BclA from the spore surface is beneficial to the FIS vaccine. Thus, mice vaccinated with dcG9241 ΔbclA FIS were better protected than those vaccinated with the BclA-positive strain dcG9241 or 569-UM20 FIS, although this enhanced protection was statistically significant only for the i.p. challenge route. The relative lack of importance of BclA in protection is supported by the observations that B. anthracis Sterne FIS and Sterne/H9004 bclA FIS induce similar gamma interferon responses in mouse splenocytes (60) and that BclA can occlude other spore surface antigens from the host (34, 39, 53). Thus, removal of BclA from the spore surface, as we did genetically, would allow these potential antigens to

FIG 2 Antisera from FIS-immunized mice react with purified spore proteins. (A) Twenty-nine spore proteins were purified, blotted to a nitrocellulose membrane in triplicate, and probed with the day 28 prechallenge antisera from the different immunization groups. The format for each dot blot is shown at the top left. (B) Normalized signal intensities for the 569-UM20 FIS, dcG9241 FIS, or dcG9241 ΔbclA FIS antisera that reacted with proteins from the spore protein panel. The error bars represent one standard error of the mean.
be better recognized and presented to the host immune system. The *B. anthracis* Ames and Ames Δ*bclA* ELISAs further support this hypothesis. When Ames spores were probed with antisera from FIS-immunized mice, the BclA-positive FIS groups displayed the highest reactivity and were statistically significantly different from the PBS/alhydrogel and dcG9241 Δ*bclA* FIS groups; however, when Ames Δ*bclA* spores were probed, the antiseraum pool from the dcG9241 Δ*bclA* FIS group displayed the highest reactivity and was statistically significantly different from the PBS/alhydrogel and the BclA-positive groups. The survival data combined with ELISA data suggest that protective antigens other than BclA are present on the spore surface and that the lack of BclA on the dcG9241 Δ*bclA* FIS surface is the reason that this group was protected most effectively from *B. anthracis* Ames challenge.

The immuno-dot blots show the postvaccination, prechallenge antisera reactivity with 11 spore proteins from the recombinant spore protein panel and also demonstrate the contribution of BclA toward antigen availability. In addition to BclA (BA_1222), the proteins ExsK (BA_2554) and p3957 (BA_4266) reacted less with the dcG9241 Δ*bclA* FIS group’s antisera than with the dcG9241 FIS group’s antisera. BclA is required for ExsK to form high-molecular-weight complexes on the surface of the exosporium (54), and therefore, removal of BclA from the spore would inhibit localization of ExsK on the spore surface. The possible requirement of BclA to localize the hypothetical protein p3957 to the spore surface is currently unknown. Six proteins were more reactive with antisera raised by immunization with BclA-negative FIS: CotZ1/ExsY (BA_1234), CotZ2/CotY (BA_1238), p2138 (BA_2292), inosine-uridine-prefering nucleoside hydrolase (BA_2888), glycosyl hydrolase family 18 (BA_3668), and CotE (BA_3906). Both CotZ1/ExsY and CotZ2/CotY form multimeric complexes with BclA and BxpB/ExsFA (46, 61), and this complex may prevent recognition of CotZ1/ExsY and CotZ2/CotY by the host immune system. There are no reports of direct interactions between BclA and any of the remaining four proteins. The increased signal of the dcG9241 Δ*bclA* FIS antisera versus dcG9241 FIS antisera with the remaining four proteins may be due to the proximity of these proteins to BclA on the spore surface. Of note, the 569-UM20 FIS antisera and dcG9241 Δ*bclA* FIS antisera had similar reactivities with three of these six proteins, CotZ1/ExsY, CotZ2/CotY, and glycosyl hydrolase, an observation that could suggest that the accessible spore surface of 569-UM20 is sufficiently different from the dcG9241 spore surface to permit generation of antibodies toward these proteins. A BLAST query (62, 63) of the *B. cereus* G9241 BclA amino acid sequence against the nonredundant protein sequences database limited to *B. cereus* 569 (*B. cereus* ATCC 10876) yielded two possible BclA homologs present in 569-UM20, BCERE0002_43750 and BCERE0002_21830. The 569-UM20 homologs are 69 and 77 amino acids larger than the G9241 BclA homolog, and they have a relatively low amino acid sequence identity (~37% as calculated by a global alignment with ALIGN [64, 65]) (data not shown). Regardless of which gene product is the true BclA homolog, both proteins are sufficiently different from the BclA protein encoded by dcG9241 to alter spore surface accessibility of 569-UM20 toward CotZ1/ExsY, CotZ2/CotY, and glycosyl hydrolase. The increased reactivity of the 569-UM20 FIS antisera toward the two remaining proteins, the CotB homolog (BA_0355) and ExsD (BA_2617), may also be explained by the probability of a different spore surface morphology between these two bacterial strains. The differences in reactivity between dcG9241 and 569-UM20 FIS antisera could also be a result of amino acid sequence variations between the spore protein homologs of these two species, which, in turn, could provide them with different antigenic properties.

Of the 11 antisera-reactive proteins, six have been previously identified as vaccine candidates. Rabbit antisera generated against live *B. anthracis* Sterne spores was previously found to be reactive with glycosyl hydrolase in a screen to find novel vaccine candidates (66). BclA, CotZ1/ExsY, ExsK, ExsD, and p3957 were detected by rabbit polyclonal antisera 311001-01 generated against inactivated whole spores (34). Of these six proteins, all but ExsD and glycosyl hydrolase were previously evaluated as vaccine candidates in conjunction with PA (27, 31, 33, 34); only vaccination with BclA provided some protection to mice from either *B. anthracis* Sterne (31) or Ames (27) challenge. It should be noted that all of the previously tested vaccine proteins were cloned from *B. anthracis* Sterne and that amino acid sequence differences exist between *B. anthracis* Sterne and the *B. cereus* G9241 protein homologs. The amino acid sequence identities between the G9241 and Sterne proteins are as follows: BclA, 66.5%; CotZ1/ExsY, 82.2%; ExsK, 54.8%; and p3957, 85.6% (global alignment and percent identity for each pair of protein sequences was calculated with ALIGN [64, 65]) (data not shown). It is possible that the G9241 variants of these previously tested proteins may perform better as vaccine components than the Sterne counterparts. In addition, there are likely other spore proteins that have yet to be recognized.

The complete lack of efficacy of vaccination with purified dcG9241 exosporium was unexpected. There are several possible explanations for this finding. First, the lack of a significant response to exosporium as indicated by the ELISA data suggests that insufficient amounts of the exosporium were used for immunization. Steichen et al. reported the generation of antibodies directed toward purified exosporium in which they injected BALB/c mice with 50 μg of purified exosporium initially in complete Freund’s adjuvant and then 4 more times in saline every 3 days (47). In our experiments we used 30 μg of exosporium injected with an alhydrogel adjuvant only twice, 2 weeks apart. The difference between 30 μg and 50 μg is probably not significant and most likely would not explain the lack of an immune response. However, the increased time between boosters, the decreased injection frequency, and the choice of adjuvant used in our experiments all could have contributed to the lack of a robust host immune response from purified exosporium. Furthermore, it is also possible that purified exosporium has a short half-life in the mouse, which could reduce its immunogenicity. While we could have increased the frequency of the immunizations, we wanted the immunization schedule to be the same for all groups. It is also possible that the method we used to isolate exosporium resulted in the loss of protective spore antigens. The procedure incorporated sonication to disrupt the spore particle, a low-speed centrifugation step to remove nonlysed spores and debris, and a high-speed ultracentrifugation step to pellet the exosporium-containing outer spore membrane fraction. With such a procedure, any proteins not tightly associated with or integral to the outer spore membrane could have been lost during the purification process. Another possible explanation for the ineffectiveness of the exosporium as a vaccine is that the spore particle itself may act as a scaffold for antigen presentation, may protect the antigens from degradation, or may act as an additional adjuvant. The approximate size of spores may alter the immune response or antigen presentation, as observed with the use of synthetic microparticles in other vaccine platforms (67). A require-
ment for a scaffold could explain the lack of protection afforded by immunization with recombinant spore proteins alone in previous studies; however, it is also possible that antibodies against these spore proteins simply were not protective in the absence of PA and that an effective vaccine comprised solely of spore proteins remains to be discovered.

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REFERENCES
1. Centers for Disease Control and Prevention. 2008. Cutaneous anthrax associated with drum making using goat hides from West Africa—Connecticut, 2007. 57:828–831.
2. Centers for Disease Control and Prevention. 2010. Gastrointestinal anthrax after an animal-hide drumming event—New Hampshire and Massachusetts, 2009. 59:872–877.
3. Guh A, Heyman MI, Barden D, Fontana J, Hadler JL. 2010. Lessons learned from the investigation of a cluster of cutaneous anthrax cases in Connecticut. J. Public Health Manag. Pract. 16:201–210.
4. Jernigan DB, Raghunathan PL, Bell BP, Brechner R, Bresnitz EA, Butler JC, Cetron M, Cohen M, Doyle T, Fischer M, Greene C, Griffith KS, Guernier J, Hadler JL, Havlir D, Meyer R, Petersen LR, Phillips M, Pinner R, Popovic T, Quin CP, Reefhus JS, Reissman D, Rosenstein N, Schuchat A, Shieh WJ, Siegal L, Swerdlow DL, Tenover FC, Traeger M, Woude GF. 1998. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280:734–737.
5. Vitale G, Pelliøttazzi R, Recchi C, Napoliøtto G, Mock M, Montecucco C. 1999. Anthrax lethal factor cleaves the N-terminus of MAPKKS and induces tyrosine/threonine phosphorylation of MAPKKS in cultured macrophages. J. Appl. Microbiol. 87:288.
6. Fouet A. 2009. The surface of Bacillus anthracis. Mol. Aspects Med. 30:374–385.
7. Wilson MK, Vergis MJ, Alem F, Palmer JR, Keane-Myers AM, Brahmbhatt TN, Ventura CL, O’Brien AD. 2011. Bacillus cereus G9241 makes anthrax toxin and capsule like highly virulent B. anthracis Ames but behaves like attenuated toxigenic nonencapsulated B. anthracis Sterne in rabbits and mice. Infect. Immun. 79:3012–3019.
8. Oh SY, Budzik JM, Garufi G, Schneewind O. 2011. Two capsular polysaccharides enable Bacillus cereus G9241 to cause anthrax-like disease. Mol. Microbiol. 80:455–470.
9. Lombard PC. 1991. Anthrax vaccines: past, present and future. Vaccine 9:533–539.
10. Little SF, Ivins BE, Fellows PF, Friedlander AM. 1997. Passive protection by polyclonal antibodies against Bacillus anthracis infection in guinea pigs. Infect. Immun. 65:5171–5175.
11. Friedlander AM, Little SF. 2009. Advances in the development of next-generation anthrax vaccines. Vaccine 27:D28–D32.
12. Wright JG, Quinn CP, Shadomy S, Messonnier N, Centers for Disease Control and Prevention. 2010. Use of anthrax vaccine in the United States: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. MMWR Recomm. Rep. 59:1–30.
13. Marano N, Plikaytis BD, Martin SW, Rose C, Semenova VA, Martin SK, Freeman AE, Li H, Mulligan MJ, Parker SD, Babcock J, Keitel W, El Sahly H, Poland GA, Jacobson RM, Keyserling HL, Soroka SD, Fox SP, Stamper JL, McNeil MM, Perkins BA, Messonnier N, Quinn CP, Anthrax Vaccine Research Program Working Group. 2008. Effects of a reduced dose schedule and intramuscular administration of anthrax vaccine adsorbed on immunogenicity and safety at 7 months: a randomized trial. JAMA 300:1532–1543.
14. Niu M, Ball R. 2009. Adverse events after anthrax vaccination reported to the Vaccine Adverse Event Reporting System (VAERS), 1990–2007. Vaccine 27:290–297.
15. Flick-Smith HC, Waters EL, Walker NJ, Miller J, Stagg AJ, Green M, Williamson ED. 2003. Mouse model characterisation for anthrax vaccine development: comparison of one inbred and one outbred mouse strain. Microb. Pathog. 38:33–40.
16. Hahn UK, Boehm R, Beyer W. 2006. DNA vaccination against anthrax in mice—combination of anti-spoore and anti-toxin components. Vaccine 24:4569–4571.
17. Welkos SL, Friedlander AM. 1988. Comparative safety and efficacy against Bacillus anthracis of protective antigen and live vaccines in mice. Microb. Pathog. 5:127–139.
18. Fellows PF, Linscott MK, Ivins BE, Pitt MLM, Rossi CA, Gibbs PH, Friedlander AM. 2001. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by Bacillus anthracis isolates of diverse geographical origin. Vaccine 19:3241–3247.
19. Little SF, Knudsen GB. 1986. Comparative efficacy of Bacillus anthracis
live spore vaccine and protective antigen vaccine against anthrax in the guinea pig. Infect. Immun. 52:509–512.

31. Brahmbhatt TN, Darnell SC, Carvalho HM, Sanz P, Kang TJ, Bull RL, Rasmussen SB, Cross AS, O’Brien AD. 2007. Recombinant exosporium protein BclA of Bacillus anthracis is effective as a booster for mice primed with suboptimal amounts of protective antigen. Infect. Immun. 75:5240–5247.

32. Brossier F, Levy M, Mock M. 2002. Anthrax spores make an essential contribution to vaccine efficacy. Infect. Immun. 70:661–664.

33. Cote CK, Kaatz L, Reinhardt J, Bozue J, Tobery S, Bassett A, Sanz P, Darnell SC, Alem F, Welkos SL, O’Brien PC (ed). 2011. Anthrax spores make an essential contribution to vaccine efficacy. Infect. Immun. 77:1197–1207.

34. Shlyakhov EN, Rubinstein E. 1994. Human live anthrax vaccine in the former USSR. Vaccine 12:727–730.

35. Turnbull PC (ed). 2008. Anthrax in humans and animals, 4th ed. World Health Organization Press, Geneva, Switzerland.

36. Vergis JM, Cote CK, Bozue J, Alem F, Ventura CL, Welkos SL, O’Brien AD. 2008. Recombinant Bacillus anthracis spore proteins enhance protection of mice primed with suboptimal amounts of protective antigen. Vaccine 26:4927–4939.

37. Gauthier YP, Rubinstein E. 1994. Human live anthrax vaccine in the former USSR. Vaccine 12:727–730.

38. Turnbull PC (ed). 2008. Anthrax in humans and animals, 4th ed. World Health Organization Press, Geneva, Switzerland.

39. Cote CK, Rossi CA, Kang AS, Morrow PR, Lee JS, Welkos SL. 2002. Roles of the immunodominant protein (PA) associated with spores of Bacillus anthracis and the effects of anti-PA antibodies on spore germination and macrophage interactions. Microb. Pathog. 38:209–225.

40. Welkos SL, Cote CK, Rea KM, Gibbs PH. 2004. A microtiter fluorometric assay to detect the germination of Bacillus anthracis spores and the germination inhibitory effects of antibodies. J. Microbiol. Methods 56:253–265.

41. LePage GA, Morgan JF, Campbell ME. 1946. Production and purification of penicillin. J. Biol. Chem. 166:465–472.

42. Basu S, Kang TJ, Chen WH, Fenton MJ, Baillie L, Hibbs S, Cross AS. 2007. Role of Bacillus anthracis spore structures in macrophage cytokine responses. Infect. Immun. 75:2351–2358.

43. Severson KM, Mallozzi M, Bozue J, Welkos SL, Cote CK, Knight KL, Driks A. 2009. Roles of the Bacillus anthracis spore protein ExsA in exosporium maturation and germination. J. Bacteriol. 191:7587–7596.

44. Sylvestre P, Couture-Tosi E, Mock M. 2002. A collagen-like surface glycoprotein is a structural component of the Bacillus anthracis exosporium. Mol. Microbiol. 45:169–178.

45. Barnard JP, Friedlander AM. 1999. Vaccination against anthrax with attenuated recombinant strains of Bacillus anthracis that produce protective antigen. Infect. Immun. 67:562–567.

46. Cohen S, Mendelson J, Altboun M, Kobler D, Elhanany E, Bino T, Leitner M, Inbar I, Rosenberg H, Goezes Y, Barak R, Fisher M, Kroman C, Velan B, Shafferman A. 2000. Attenuated nontoxicogenic and nonencapsulated recombinant Bacillus anthracis spore vaccines protect against anthrax. Infect. Immun. 68:4549–4558.

47. Welkos SL, Keener TJ, Gibbs PH. 1986. Differences in susceptibility of inbred mice to Bacillus anthracis. Infect. Immun. 51:795–809.

48. Steward J, Lever MS, Simpson AJH, Sefton AM, Brooks TJG. 2004. Post-exposure prophylaxis of systemic anthrax in mice and treatment with fluoroquinolones. J. Antimicrob. Chemother. 54:95–99.

49. Gehring M, Chignard M, Mock M, GoosSENS PL. 2007. Murine splenocytes produce inflammatory cytokines in a MyD88-dependent response to Bacillus anthracis spores. Cell. Microbiol. 9:502–513.

50. Steichen CT, Kearney JF, Turnbough CL. 2005. Characterization of the exosporium basal layer protein BXP of Bacillus anthracis. J. Bacteriol. 187:5868–5876.

51. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389–3402.

52. Altschul SF, Wootton JC, Gertz EM, Agarwala R, Morgulis A, Schäffer AA, Yu Y-K. 2005. Protein database searches using compositionally adjusted substitution matrices. FEBS J. 272:5101–5109.

53. Pearson WR. 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. Methods Enzymol. 183:63–98.

54. Pearson WR, Lipman DJ. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. U. S. A. 85:2444–2448.

55. Gat O, Grossfeld H, Ariel N, Inbar I, Zaide G, Broder Y, Zvi A, Chitlaru T, Altboun Z, Stein D, Cohen S, Shafferman A. 2006. Search for Bacillus anthracis potential vaccine candidates by a functional genomic-serological screen. Infect. Immun. 74:3987–4001.

56. Ouyewumi MO, Kumar A, Cui Z. 2010. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev. Vaccines 9:1095–1107.