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Immunization against Anthrax with Aromatic Compound-Dependent (Aro$^-$) Mutants of *Bacillus anthracis* and with Recombinant Strains of *Bacillus subtilis* That Produce Anthrax Protective Antigen

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The safety and efficacy of five prototype, live anthrax vaccines were studied in Hartley guinea pigs and CBA/J and A/J mice. Two of the strains, *Bacillus anthracis* FD111 and FD112, are Aro$^-$ mutants derived by Tn*916* mutagenesis of *B. anthracis* UM23-1. *Bacillus subtilis* PA1 and PA2 contain a recombinant plasmid, pPA101 or pPA102, respectively, that carries the gene from *B. anthracis* encoding synthesis of protective antigen (PA). The final strain, *B. subtilis* PA7, was isolated in this study from *B. subtilis* DB104 transformed with pPA101. All five strains were less virulent in guinea pigs and A/J and CBA/J mice than the toxinogenic, nonencapsulated *B. anthracis* veterinary vaccine Sterne strain. A/J and CBA/J inbred mice represent strains that are innately susceptible and resistant, respectively, to the Sterne strain. These differences in susceptibility are due to differences in ability to produce complement component 5. In guinea pigs, immunization with PA1 or PA2 vegetative cells or PA7 spores protected ≥95% from an intramuscular spore challenge with the virulent, "vaccine-resistant" *B. anthracis* Ames strain. Strain PA2 vegetative cells and strain PA7 spores were as effective as the Sterne strain in Sterne-resistant CBA/J mice, protecting 70% of the mice from Ames strain spore challenge. Immunization with FD111 or FD112 vegetative cells fully protected guinea pigs from challenge. Immunization with FD111 cells protected up to 100% of CBA/J mice and up to 70% of A/J mice.

Two anthrax vaccines are licensed for use in the United States. The veterinary vaccine is a suspension of viable spores of the toxigenic, nonencapsulated *Bacillus anthracis* Sterne strain (22). Its use occasionally results in necrosis at the inoculation site and, rarely, death. The human vaccine consists of aluminum hydroxide-adsorbed supernatant material from fermentor cultures of another toxigenic, nonencapsulated *B. anthracis* strain, V770-NP1-R (19). Undesirable characteristics of this vaccine include the need for numerous boosters and the reduced ability to protect laboratory animals against certain virulent strains of *B. anthracis* (15, 23), such as the Ames strain.

We are evaluating various living and chemical candidate vaccines for improved, safe, and efficacious prophylaxis against anthrax (10). Recently, we cloned the *B. anthracis* protective antigen (PA) gene into the asporogenic *Bacillus subtilis* strain 1S53 by using the vector pUB110 (9). Two of the clones, PA1 and PA2, carried recombinant plasmids pPA101 and pPA102, respectively, and produced PA in broth cultures at levels equal to or greater than those produced by the parent *B. anthracis* Sterne strain. In preliminary experiments, immunization with PA1 protected guinea pigs and mice from the lethality of a virulent spore challenge with *B. anthracis* Vollum 1B and partially protected rats from the lethality of an anthrax toxin challenge (9, 25). Although this preliminary evidence suggested that vegetative cells of PA-producing *B. subtilis* recombinants immunized against anthrax, there were no data on the efficacy of spores of PA-producing *B. subtilis*. In the present study, we transformed a spore-forming *B. subtilis* strain, DB104, with the plasmid from strain PA1. Spores from one of the clones isolated, PA7, were tested along with PA1 and PA2 vegetative cells as live vaccines in mice and guinea pigs against parenteral challenge by spores of *B. anthracis* Ames.

The mutagenesis of *B. anthracis* with Tn*916* was also previously described (11). Two of the insertion mutants were deficient in the synthesis of aromatic compounds (18). We tested these strains, designated FD111 and FD112 in the present study, as prototype live vaccines in guinea pigs and mice against *B. anthracis* Ames spore challenge. The Ames strain was used for challenge because it more readily overcomes vaccine-stimulated resistance to lethal infection with *B. anthracis* than do strains such as Vollum 1B (15, 23).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used are listed in Table 1.

Isolation and characterization of sporogenic *B. subtilis* PA-producing clones. Competent cells of the sporogenic *B. subtilis* strain DB104 were transformed with plasmid pPA101 DNA isolated from *B. subtilis* PA1 (4, 5, 9), as described previously (9). After isolating kanamycin-resistant (Km^r) colonies and identifying clones producing PA on immunoassay agar (17), we isolated and examined plasmid DNA from randomly selected recombinants (3, 9, 16). One of the clones, PA7, was selected and further characterized. The serological and biological activities of the PA produced by *B. subtilis* PA7 were assayed by the methods described for *B. subtilis* PA1 and PA2 (2, 6, 9, 13, 20, 24). PA production was assayed in cultures grown in defined *B. anthracis* R medium (20) with 0.8% yeast extract (RYE) or in RYE with 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid).

Preparation of vegetative cells for immunization. Vegetative *B. subtilis* cells were prepared for immunization by a modification of a previously described method (9). For the *B.*

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TABLE 1. Bacterial strains used

Strain	Plasmid(s)	Relevant characteristics[a]	Source or derivation
B. anthracis			
Ames	pXO1, pXO2	PA+ LF+ EF+ Cap+	USDA[b]
Sterne	pXO1	PA+ LF+ EF+	USAMRIID[c]
ΔSterne-1[d]	None		USAMRIID; 17
UM23-1[e]	pXO1	PA+ LF+ EF+ Str[r] Ura−	C. B. Thorne
FD111[f]	pXO1	PA+ LF+ EF+ Tc[r] (Tn*916*) Aro− Str[r] Ura−	USAMRIID; 11
FD112[f]	pXO1	PA+ LF+ EF+ Tc[r] (Tn*916*) Aro− Str[r] Ura−	USAMRIID; 11
B. subtilis			
BST1[g]	pUB110	Km[r], asporogenic	USAMRIID; 9
PA1	pPA101[h]	Km[r], asporogenic, PA+	USAMRIID; 9
PA2	pPA102[h]	Km[r], asporogenic, PA+	USAMRIID; 9
DB104	None	Sporogenic	Roy Doi; 12
PA7[i]	pPA101	Km[r], sporogenic, PA+	This study
PA8[i]	pPA101	Km[r], sporogenic, PA+	This study

[a] Abbreviations: Aro, aromatic compound biosynthetic pathway; PA, protective antigen; LF, lethal factor; EF, edema factor; Cap, capsule; Km, kanamycin; Sm, streptomycin; Tc, tetracycline; Ura, uracil.
[b] U.S. Department of Agriculture, Ames, Iowa.
[c] U.S. Army Medical Research Institute of Infectious Diseases.
[d] Derived by serial subculture of *B. anthracis* Sterne at 42.5°C to cure the cells of pXO1. ΔSterne-1 is nonencapsulated and nontoxinogenic.
[e] Derived from the veterinary vaccine Sterne (Weybridge) strain.
[f] Derived by conjugation of *B. anthracis* VNR-1-tet-1 (containing Tn*916*) with *B. anthracis* UM23-1 and selection for Aro− Tc[r] transposition mutants.
[g] *B. subtilis* BGSC 1S53 transformed with pUB110, a 4.5-kilobase plasmid vector encoding kanamycin resistance.
[h] Recombinant plasmid containing the *B. anthracis* PA gene ligated into pUB110; transformed into competent *B. subtilis* BGSC 1S53.
[i] *B. subtilis* DB104 transformed with pPA101 by methods described here and elsewhere (4, 5, 9).

subtilis isogenic strains PA1, PA2, and BST1, approximately 10^3 CFU was inoculated into a 250-ml Erlenmyer flask containing 100 ml of brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) with 10 μg of kanamycin per ml and the culture was incubated overnight with rapid shaking at 37°C. Twenty milliliters of culture was then inoculated into a 2-liter Erlenmyer flask containing 1,000 ml of BHI with kanamycin, and the culture was shaken for 4 h at 37°C. The cells were pelleted by centrifugation at 10,000 × *g*, washed once with phosphate-buffered saline (PBS), suspended in PBS with 12% glycerol, and frozen at −70°C. Survival during storage at −70°C was monitored by periodic assay of viability on plates of sheep blood agar to ensure correct dosages of viable cells, or spores, during immunization. *B. anthracis* Sterne and ΔSterne-1 vegetative cells were prepared similarly, except that they were grown in BHI without kanamycin.

B. anthracis FD111 and FD112 cells were also prepared for immunization studies. Plates of sheep blood agar consisting of 5% sheep blood in blood agar base (Difco) with tetracycline (10 μg/ml) were inoculated with FD111 and FD112 and incubated overnight at 37°C. Isolated colonies were then inoculated into 250-ml flasks containing 50 ml of Aro growth medium (AGM) consisting of the following: BHI, 37 g; yeast extract, 10 g; L-tyrosine, 144 mg; L-phenylalanine, 85 mg; L-tryptophan, 265 mg; *p*-aminobenzoic acid, 20 mg; 2,3-dihydroxybenzoic acid, 10 mg; *p*-hydroxybenzoic acid, 10 mg; and deionized water, 1 liter. Tetracycline hydrochloride was then added to a concentration of 10 μg/ml. The cultures were incubated for 12 h at 37°C with shaking at 100 rpm. One milliliter of each culture was inoculated into a 250-ml Nephelo sidearm culture flask (Bellco Glass, Inc., Vineland, N.J.) containing 50 ml of AGM with tetracycline; 10 ml of each culture was inoculated into a 2-liter flask containing 500 ml of AGM with tetracycline. All cultures were incubated at 37°C with shaking until the A_{540} (measured on a Spectronic 21 spectrophotometer; Bausch & Lomb, Inc., Rochester, N.Y.) of the cultures in the Nephelo flasks had increased 30-fold. The bacteria in the 500-ml cultures were then collected by centrifugation at 9,000 × *g* for 15 min. The cell pellets were suspended in PBS, pelleted again, suspended to 1% of the original culture volume in PBS with 12% glycerol, dispensed (2 ml) into polypropylene tubes (12 by 75 mm), and frozen at −70°C. *B. anthracis* UM23-1 cells for immunization were prepared similarly from cultures not containing tetracycline.

Preparation of spores for immunization. For preparation of *B. subtilis* PA7 spores, approximately 10^3 vegetative CFU was inoculated into 100 ml of Leighton-Doi broth (14) with 10 μg of kanamycin per ml; the culture was incubated for 18 h with vigorous shaking at 37°C. Two-liter Erlenmyer flasks, each containing 200 ml of Leighton-Doi broth, were inoculated with 5-ml amounts of the culture. After the cultures were shaken for 24 h at 37°C, 800 ml of sterile distilled water was added to each flask, and incubation with shaking was continued for 40 h. The cells and spores were pelleted at 10,000 × *g* and then suspended in PBS containing 0.1% gelatin (PBSG). The cell suspension (10 ml) was added to 35-ml centrifuge tubes containing 8 ml of 60% Renografin (E. R. Squibb & Sons, Princeton, N.J.) overlaid with 14 ml of 50% Renografin in PBSG. These discontinuous gradients were centrifuged in a Sorvall HB-4 hanging bucket rotor (Du Pont Co., Wilmington, Del.) for 3 h at 12,000 × *g*. The spores, which formed a band at the interface of the 50 and 60% Renografin layers, were removed with a pipette and diluted with an equal volume of PBSG. The spores were pelleted at 10,000 × *g*, suspended in PBSG containing 12% glycerol, and frozen at −70°C. Spores from *B. anthracis* Sterne and Ames and *B. subtilis* DB104 were prepared as described above from cultures in Leighton-Doi medium not containing kanamycin.

Determination of generation time for *B. anthracis*. Nephelo sidearm culture flasks containing 25 ml of AGM (without tetracycline) were inoculated in triplicate with 10^2 to 10^3 CFU of *B. anthracis* UM23-1, FD111, or FD112. The cultures were incubated with shaking at 37°C. After the cultures reached an A_{540} of 0.01 (approximately 2×10^6 CFU/ml), turbidity measurements were performed as described above at 0.5-h intervals. The generation time was the time required for the A_{540} to double during logarithmic growth.

Reversion of *B. anthracis* FD111 and FD112 to the parental phenotype. One-liter Erlenmyer flasks containing 100 ml of AGM or AGM with tetracycline (10 μg/ml) were inoculated with approximately 10^2 CFU of *B. anthracis* FD111 or FD112. The cultures were incubated with shaking at 37°C for 48 h. Samples of the cultures were periodically tested for viability and growth characteristics on the following media with or without tetracycline: BHI agar (Difco), unsupplemented Brewer agar (1, 11), and Brewer agar supplemented with phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic

acid, 2,3-dihydroxybenzoic acid, and *p*-hydroxybenzoic acid added at the same concentrations as those in AGM.

Experimental animals. Female Hartley guinea pigs (8) weighing 350 to 375 g at the beginning of the vaccination regimens and CBA/J and A/J mice (25, 26) weighing 25 g were used in the immunization experiments. Female Hartley guinea pigs were also used in determinations of anthrax edema-producing toxin activity (21). Male Fischer 344 rats weighing 200 to 300 g were used to determine anthrax lethal toxin activity (6).

Immunization and challenge of guinea pigs. Groups of Hartley guinea pigs were immunized intramuscularly (i.m.) with 0.1- to 0.5-ml doses of either spores or log-phase vegetative cells or with 0.5-ml doses of the human anthrax vaccine, MDPH-PA (19), prepared by the Michigan Department of Public Health. Eight weeks after the first immunizations, guinea pigs were challenged i.m. with spores of the virulent *B. anthracis* Ames strain (100 spores = 1 50% lethal dose [LD_{50}]). The numbers of animals that died within 2 weeks after challenge were recorded.

Lethality of *B. anthracis* FD111, FD112, and UM23-1 for A/J mice. A/J mice were injected subcutaneously (s.c.) in groups of 20 with 10^4, 10^5, 10^6, 10^7, or 10^8 CFU of *B. anthracis* FD111 or FD112 or the Tc^s Aro^+ parent strain, UM23-1. Mortalities were noted for 14 days after injection.

Immunization and challenge of mice. Groups of CBA/J or A/J mice were immunized s.c. with 0.2-ml doses of spores, log-phase vegetative cells, or MDPH-PA. Eight weeks after the first immunizations, mice were challenged s.c. with 0.2 ml of *B. anthracis* Ames spores. The LD_{50} of Ames spores are <1 spore (calculated LD_{50} = 0.5 spore) for A/J mice and 35 spores for CBA/J mice. Deaths within 2 weeks after challenge were noted.

Serological studies. Prechallenge sera from guinea pigs (15) and mice (25) were examined for antibody to PA by using an enzyme-linked immunosorbent assay (ELISA) (15). Anti-PA titers were determined (8).

TABLE 2. Immunization of guinea pigs with *B. subtilis* PA1, PA2, and PA7

Immunization[a]	Doses	No. survived/no. challenged (%)[b]	Serologic response[c]
PBS	3	0/16 (0)	<10
MDPH-PA	3	12/16 (75)	20,535
B. anthracis Sterne			
10^6 CFU	1	11/15 (73)	392
	2	14/16 (88)	10,700
10^7 CFU	1	10/12[d] (83)	4,641
	2	10/10[e] (100)	3,981
B. subtilis PA1			
10^8 CFU	2	10/14 (71)	13,895
10^9 CFU	1	0/16 (0)	1,225
	2	19/20 (95)	5,623
B. subtilis PA2			
10^8 CFU	2	5/14 (36)	1,912
10^9 CFU	1	1/16 (6)	277
	2	18/19 (95)	3,450
B. subtilis PA7			
10^8 CFU	2	1/17 (6)	667
10^9 CFU	1	0/17 (0)	115
10^{10} CFU	2	18/18[f] (100)	9,380

[a] Guinea pigs were immunized i.m. at 0 weeks (one dose), 0 and 4 weeks (two doses), or 0, 2, and 4 weeks (three doses). They were immunized with vegetative preparations of PA1 or PA2 or with spore preparations of Sterne or PA7.

[b] Animals were challanged 4 weeks after the last vaccine dose with 43 LD_{50}s of Ames spores.

[c] Reciprocal geometric mean anti-PA ELISA titer of prechallenge sera.

[d] Five guinea pigs died from the immunization.

[e] Six guinea pigs died from the immunization.

[f] Two guinea pigs died from the immunization.

RESULTS AND DISCUSSION

PA-producing transformants of *B. subtilis* DB104. Approximately 3,000 Km^r colonies (600/μg of DNA) were isolated after transformation of *B. subtilis* DB104 with pPA101 DNA. Over 400 colonies were then transferred to immunoassay agar (17) to identify putative PA-producing clones. All of the tested clones were surrounded by immunoprecipitin halos. Eighteen of the clones were randomly selected and serially subcultured five times. They remained halo positive. Plasmid DNAs from these 18 clones were isolated (3) and analyzed by agarose gel electrophoresis (9, 16). The plasmid DNAs from all 18 strains appeared identical in size to the 7.8-kilobase pPA101 plasmid (data not shown).

Plasmids from 2 of the 18 clones described above, PA7 and PA8, were compared with the pPA101 plasmid in agarose gel electrophoresis. Both PA7 and PA8 were identical in electrophoretic profile to pPA101, either undigested or after digestion with *Bam*HI, *Hin*dIII, *Eco*RI, or *Pvu*II (data not shown). Thus, it was apparent that *B. subtilis* PA7 and PA8 possessed plasmid pPA101. *B. subtilis* PA7 was examined further; it produced PA (9) that was biologically (6, 21) and serologically (9, 24) identical to the PA produced by *B. anthracis* Sterne and *B. subtilis* PA1.

Immunization of guinea pigs with *B. subtilis* PA-producing recombinant strains. The protective efficacy of the asporogenic, PA-producing *B. subtilis* recombinant strains, PA1 and PA2 (9), was tested. Two immunizations at 0 and 4 weeks with 10^8 or 10^9 CFU of the nonrecombinant, pUB110-containing strain, *B. subtilis* BST1, gave no protection from an i.m. challenge of 20 LD_{50}s of *B. anthracis* Ames spores at 8 weeks. In contrast, with PA1 and PA2, guinea pigs were completely protected from lethality by two doses of 10^8 or 10^9 CFU. *B. anthracis* Sterne cells completely protected the guinea pigs with two doses of 10^6 or 10^7 CFU; however, one animal injected with 10^8 CFU died.

The safety and efficacy of *B. subtilis* PA1 and PA2 vegetative cells, *B. subtilis* PA7 spores, *B. anthracis* Sterne spores, and MDPH-PA were then compared in guinea pigs (Table 2). Two doses of 10^7 CFU of Sterne spores completely protected against the Ames spore challenge. However, 11 of 33 guinea pigs were killed by the immunization. In contrast, two doses of 10^9 CFU of PA1 or PA2 vegetative cells protected 95% of the animals and caused no deaths. Two injections of 10^{10} CFU of PA7 spores killed two guinea pigs but completely protected the remaining 18 challenged animals. Although immunization with MDPH-PA elicited the highest anti-PA titers, only 75% of the immunized animals were protected against challenge (Table 2). These data confirm previous reports (15, 23, 25) that there is no strict correlation between anti-PA titers and protection against spore challenge. The data also demonstrate that the PA-producing *B. subtilis* recombinant strains protect guinea pigs from virulent spore challenge and that they are safer than *B. anthracis* Sterne spores.

Immunization of mice with *B. subtilis* PA-producing recombinant strains. Like guinea pigs, CBA/J mice immunized

TABLE 3. Immunization of CBA/J mice with *B. subtilis* PA2 and PA7

Immunization[a]	Spore challenge doses (LD_{50}s)[b]	No. survived/no. challenged (%)	Serological response[c]
PBS	6	0/5 (0)	<10
	17	0/5 (0)	<10
MDPH-PA	10	1/10 (10)	100,000
	20	0/20 (0)	67,608
B. anthracis			
ΔSterne-1	6	0/10 (0)	<10
Sterne[d]	6	8/12 (67)	3,162
Sterne[d]	17	6/9 (67)	12,589
Sterne spores	17	9/20 (45)	2,818
B. subtilis			
DB104	17	0/10 (0)	<10
BST1	6	0/10 (0)	<10
PA2	6	8/12 (67)	4,217
PA2	17	2/3 (67)	10,000
PA7	17	7/10 (70)	5,623

[a] Mice were immunized s.c. three times at 2-week intervals. They were immunized with vegetative preparations of PA2 or BST1 (10^8 CFU each) or ΔSterne-1 or Sterne (2×10^6 CFU each) or with spore preparations of DB104 or PA7 (10^8 CFU each) or Sterne (2×10^6 CFU).

[b] The LD_{50} for CBA/J mice is 35 *B. anthracis* Ames spores.

[c] Reciprocal geometric mean anti-PA ELISA titer of prechallenge sera.

[d] Log-phase vegetative cells. Eleven of 32 mice died after the first immunization.

with MDPH-PA develop high titers to PA (25). Unlike guinea pigs, however, these vaccinated mice are not protected against virulent anthrax spore challenge (25). Furthermore, doses of *B. anthracis* Sterne spores required to protect CBA/J mice against virulent challenge are only slightly less than those which kill some of the mice (25). Since mice differ from guinea pigs in their response to anthrax vaccines, we tested the PA-producing recombinant strains for safety and efficacy in CBA/J mice. Virtually no protection against Ames spore challenge was seen in mice immunized with three doses of MDPH-PA; *B. subtilis* DB104 or BST1, which do not possess the PA gene; or *B. anthracis* ΔSterne-1, which does not contain the genes for PA, lethal factor, or edema factor (Table 3). *B. subtilis* PA1, PA2, and PA7 were as efficacious as *B. anthracis* Sterne vegetative cells, protecting ≤70% of the mice against Ames challenge. Thirty-four percent of the mice vaccinated with Sterne vegetative cells were killed by the immunization, whereas no vaccine-related mortalities occurred in the other immunization groups (Table 3). Both the Sterne spore vaccine and strain PA2 protected 100% of CBA/J mice against challenge with *B. anthracis* Vollum 1B (25). Thus, the PA-producing *B. subtilis* recombinant strains were safe and effective prototype live anthrax vaccines in CBA/J mice, as they were in Hartley guinea pigs.

Characterization and reversion of *B. anthracis* Aro^- strains. Two mutants derived by Tn*916* mutagenesis and deficient in synthesis of aromatic compounds were described previously (11). The *B. anthracis* Aro^- strains, FD111 and FD112, grew substantially slower than the parent UM23-1 strain, even in the nutritionally rich AGM. Doubling times were 24, 80, and 64 min, respectively, for UM23-1, FD111, and FD112.

It is essential for live, attenuated vaccines to be stable and not revert to the more virulent, parental phenotype. Therefore, we tested *B. anthracis* FD111 and FD112 for reversion from Aro^- to Aro^+ and from Tc^r to Tc^s. No reversion was detected when cultures were grown in the presence of

TABLE 4. Reversion of *B. anthracis* FD111 and FD112 in the absence of tetracycline[a]

Strain	Culture age (h)	% Tc^s	% Aro^+
FD111	0	<0.01[b]	<0.01
	30[c]	<0.01	<0.01
	48[d]	0.49	0.49
FD112	0	<0.01	<0.01
	24[e]	<0.01	<0.01
	42[d]	1.06	1.06

[a] No reversion to the Tc^s or Aro^+ phenotype was detected when cells were grown with tetracycline.

[b] No revertants were detected among 10^4 colonies examined.

[c] Sample taken during log phase, after 19.1 generations.

[d] Sample taken during stationary/death phase of culture.

[e] Sample taken during log phase, after 18.3 generations.

tetracycline. In medium lacking tetracycline, no revertants were detected during logarithmic growth. However Aro^+ and Tc^s revertants appeared after the cultures entered the stationary phase (Table 4). The reason for reversion only after cessation of logarithmic growth was not investigated. Neither the Aro^- nor the Tc^r phenotype reverted separately, suggesting that excision of Tn*916* regenerated a functional gene in the *B. anthracis* aromatic compound biosynthetic pathway and resulted in the loss of Tn*916* from the cell. There was no evidence of either transposition of Tn*916* to another site on the genome or loss of Tn*916* without regeneration of functional DNA. All revertants were phenotypically indistinguishable from the parent, UM23-1, on several minimal and complex media, with or without tetracycline. The in vivo rates of reversion of *B. anthracis* FD111 and FD112 after injection into mice or guinea pigs were not examined in these experiments.

Immunization of guinea pigs with *B. anthracis* Aro^- strains. Immunization with either one dose of 10^9 or two doses of 10^8 CFU of *B. anthracis* FD111 or FD112 cells gave strong protection to guinea pigs against an Ames spore challenge, without killing any animals during vaccination (Table 5). Immunization with the same doses of *B. anthracis* UM23-1 also protected guinea pigs from challenge, but 10^9 CFU killed 8 of 17 animals.

TABLE 5. Immunization of guinea pigs with *B. anthracis* Aro^- strains

Immunization[a]	Doses	No. survived/no. challenged (%)[b]	Serological response[c]
PBS	2	2/15 (13)	<10
B. anthracis FD111			
10^8 CFU	2	17/17 (100)	9,345
10^9 CFU	1	16/16 (100)	1,540
B. anthracis FD112			
10^8 CFU	2	16/16 (100)	6,978
10^9 CFU	1	13/15 (87)	1,848
B. anthracis UM23-1			
10^8 CFU	2	17/17 (100)	6,225
10^9 CFU[d]	1	9/9 (100)	5,275

[a] Guinea pigs were immunized i.m. at 0 and 4 weeks with 10^8 CFU or at 4 weeks with 10^9 CFU of vegetative cells.

[b] Animals were challenged 4 weeks after the last vaccine dose with 16 LD_{50}s of Ames spores.

[c] Reciprocal geometric mean anti-PA ELISA titer of prechallenge sera.

[d] Eight guinea pigs died from the immunization.

TABLE 6. Immunization of A/J mice with *B. anthracis* FD112

Immunizing dose (CFU)[a]	No. survived/no. challenged (%)[b]
10^4	0/20 (0)
10^5	3/20 (15)
10^6	3/19 (16)
10^7	9/20 (45)
10^8	8/20 (40)

[a] Mice were immunized s.c. at 0 and 4 weeks.
[b] Mice were challenged at 8 weeks with approximately 100 LD_{50}s of Ames spores.

Lethality of *B. anthracis* FD111, FD112, and UM23-1 for A/J mice. A/J mice are quite susceptible to lethal infection by various strains of *B. anthracis*, including the toxinogenic, nonencapsulated Sterne vaccine strain (26). Injection of as few as 10^4 CFU of *B. anthracis* UM23-1 vegetative cells killed 100% of A/J mice, whereas injections of up to 10^8 CFU of *B. anthracis* FD111 or FD112 cells killed none of the mice. Thus the Aro^- strains were substantially less virulent in mice than the parent UM23-1 strain.

Immunization of mice with *B. anthracis* Aro^- strains. A/J mice are unable to be immunized with *B. anthracis* Sterne since Sterne spores are lethal to them at immunogenic doses (26). In contrast, the A/J mice were partially protected from a challenge with Ames spores by immunization with $\geq 10^7$ CFU of *B. anthracis* FD112 cells (Table 6). Although avirulent for the mice, the Aro^- strains were probably able to replicate sufficiently to generate a protective immune response against a subsequent, virulent *B. anthracis* spore challenge.

Finally, both A/J and CBA/J mice were immunized with 10^7 or 10^8 *B. anthracis* FD111 cells. Two doses of FD111 cells protected ≥80% of CBA/J mice against challenge with 6 LD_{50}s of Ames spores (Table 7). One or two doses of FD111 cells protected ≤70% of A/J mice against challenge with approximately 60 LD_{50}s of Ames spores (Table 7). The Aro^- strains were the first vaccines tested which were able to protect A/J mice against challenge with a fully virulent strain of *B. anthracis*.

Hoiseth and Stocker (7) used Tn*10* mutagenesis to obtain Aro^- strains of *Salmonella typhimurium* as live vaccines. Similarly, the objectives of our initial research with *B. anthracis* and Tn*916* (11) were to demonstrate the utility of transposon mutagenesis in *B. anthracis* and to generate

TABLE 7. Immunization of A/J and CBA/J mice with *B. anthracis* FD111

Mouse strain	FD111 immunization[a]		Ames spore challenge[b]	
	CFU	No. of doses	Dose (LD_{50}s)	Survival (%)
A/J	0 (PBS)	1	60	0/10 (0)
	10^7	1	60	7/10 (70)
	10^7	2	60	5/9 (56)
	10^8	2	60	4/6 (67)
CBA/J	0 (PBS)	1	6	1/20 (5)
	10^7	1	6	6/10 (60)
	10^7	2	6	10/10 (100)
	10^8	1	6	4/10 (40)
	10^8	2	6	8/10 (80)

[a] Mice were immunized s.c. at 0 and 4 weeks or at 4 weeks alone.
[b] Mice were challenged at 8 weeks.

Aro^- mutants as prototype live vaccines. The immunization experiments described here with both mice and guinea pigs indicate that *B. anthracis* FD111 and FD112 were efficacious as live vaccines and were far safer and less virulent than the *B. anthracis* UM23-1 parent strain, which is a Str^r Ura^- variant of the veterinary vaccine Sterne (Weybridge) strain. Obvious drawbacks of these prototype Aro^- live vaccines include the possession of a self-transmitting tetracycline resistance factor and their ability to revert to the Aro^+ Tc^s parental phenotype. These drawbacks would be eliminated in *B. anthracis* Aro^- mutants developed by chemical mutagenesis or by mutagenesis with a non-self-transmitting transposon that does not excise precisely to restore the function of the insertionally inactivated segment of DNA.

These experiments demonstrated that new, live vaccines can be developed against anthrax which are efficacious in protecting against challenge with the highly virulent *B. anthracis* Ames and which are safer in experimental animals than the live *B. anthracis* Sterne veterinary vaccine strain. Future studies of immunization, as well as the elucidation of the mechanisms of immunity to anthrax, will facilitate our goal of a safer, more efficacious, and long-lasting vaccine against anthrax in humans.

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