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Rapid Detection of Viable *Bacillus anthracis* Spores in Environmental Samples by Using Engineered Reporter Phages

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***Bacillus anthracis*, the causative agent of anthrax, was utilized as a bioterrorism agent in 2001 when spores were distributed via the U.S. postal system. In responding to this event, the Federal Bureau of Investigation used traditional bacterial culture viability assays to ascertain the extent of contamination of the postal facilities within 24 to 48 h of environmental sample acquisition. Here, we describe a low-complexity, second-generation reporter phage assay for the rapid detection of viable *B. anthracis* spores in environmental samples. The assay uses an engineered *B. anthracis* reporter phage (Wβ::*luxAB*-2) which transduces bioluminescence to infected cells. To facilitate low-level environmental detection and maximize the signal response, expression of *luxAB* in an earlier version of the reporter phage (Wβ::*luxAB*-1) was optimized. These alterations prolonged signal kinetics, increased light output, and improved assay sensitivity. Using Wβ::*luxAB*-2, detection of *B. anthracis* spores was 1 CFU in 8 h from pure cultures and as low as 10 CFU/g in sterile soil but increased to 10^5 CFU/g in unprocessed soil due to an unstable signal and the presence of competing bacteria. Inclusion of semiselective medium, mediated by a phage-expressed antibiotic resistance gene, maintained signal stability and enabled the detection of 10^4 CFU/g in 6 h. The assay does not require spore extraction and relies on the phage infecting germinating cells directly in the soil sample. This reporter phage displays promise for the rapid detection of low levels of spores on clean surfaces and also in grossly contaminated environmental samples from complex matrices such as soils.**

Anthrax can be a fatal bacterial infection that occurs when *Bacillus anthracis* endospores enter the body through inhalation, ingestion, injection, or cutaneous exposure due to abrasions in the skin (1–3). Although anthrax infections occur infrequently in the natural environment, *B. anthracis* constitutes a biological threat as a military and/or terrorist weapon due to the longevity of its spores and the relative ease with which large quantities can be produced and stockpiled (2). The World Health Organization (WHO) estimated that 50 kg of dried spore powder dispersed over an urban population would result in ~100,000 deaths and a concomitant breakdown of medical resources and civilian infrastructure (2, 4).

The first use of *B. anthracis* during a bioterrorism event in the United States was in 2001, when envelopes containing *B. anthracis* spores were distributed via the U.S. postal system to political and media targets. In addition to causing five fatalities and extensive social disruption, the event incurred a substantial emergency response and remediation costs; testing and remediation for the 42 contaminated buildings cost ~$320 million (5). Given that these costs were the result of the distribution of only seven letters, the cost and scope of remediation and associated viability testing in the wake of a wide-area bioterrorism scenario would be extensive. The issues associated with remediation and the longevity of spores in soil are exemplified in the case of the Scottish island of Gruinard, which was deliberately contaminated with spores during biological weapon trials in World War II. Soil sampling 30 years after initial release indicated that although the numbers of spores were gradually declining, significant contamination remained and was likely to persist well into the next century (6). Cost-effective methodologies that expedite the large-scale and high-throughput sampling requirements for environmental detection and post-clearance testing for viable *B. anthracis* bacteria would be of value.

The use of traditional culturing onto blood agar to isolate and identify *B. anthracis* from environmental samples is limited due to several complicating factors. For samples from relatively clean environments, such as solid indoor or outdoor surfaces, processing a single sample through elution, dilution, plating, and incubation can take days. The sampling and viability analysis steps are further complicated with a more complex environment, such as soil. These additional complications include the findings that up to 2×10^9 background bacteria/g is found in the top 1 m of soil (7) and that closely related species within the *Bacillus cereus* group (e.g., *B. cereus*, *B. anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, and *Bacillus weihenstephanensis*) are present as natural inhabitants of soil. For example, environmental isolates of *B. anthracis* have been shown to be beta-hemolytic, and *B. cereus* isolates displaying *B. anthracis*-like characteristics (such as lack of hemolysis, nonmotility, penicillin sensitivity, and/or phage susceptibility) have been identified (8, 9). A further complication is that a small number of *B. anthracis* spores may be present among a large number of naturally occurring spores of other *Bacillus* spp. (8). A semiselective agar consisting of polymyxin, lysozyme, disodium EDTA, and thallium acetate (PLET) was previously developed for differential selection of *B. anthracis* (10–12). However, the use of PLET with

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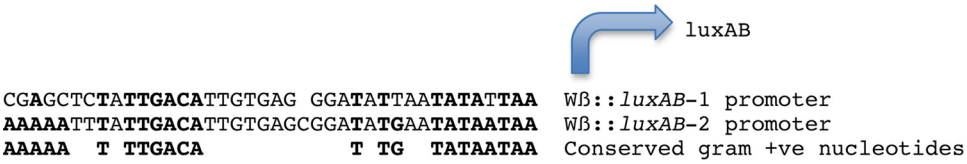

FIG 1 Promoter design of the first-generation (Wβ::*luxAB*-1) and second-generation (Wβ::*luxAB*-2) reporter phages. Conserved nucleotides of promoters from Gram-positive (Gram +ve) species (shown in bold) were identified in previous studies (35–37).

soil samples is limited for the following reasons: (i) multiple species within the *B. cereus* group are capable of growing on PLET, (ii) high numbers of *B. cereus* and *B. thuringiensis* bacteria naturally present in soil samples may outcompete growth of low numbers of *B. anthracis*, and (iii) polymyxin B is an ineffective inhibitor of Gram-negative bacteria (13).

Due to the limitations of traditional culture methods, the gold standard for quantification and detection of *B. anthracis* in environmental samples is nucleic acid-based detection (endpoint and real-time PCR) (8, 14). However, due to genetic similarity within the *B. cereus* group, cross-reactivity has often been observed (8, 15). With the advent of more rapid and less expensive whole-genome sequencing approaches, discrimination between closely related species and strains is readily attainable (16, 17). Nevertheless, chemical constituents of soil (such as organics, humic acids, and/or heavy metals) often interfere with nucleic acid-based chemistry and make direct detection of *B. anthracis* extremely difficult (18–21). Consequently, the majority of detection assays incorporate sample processing methods to separate, concentrate, and purify *B. anthracis* spores from soil prior to DNA extraction (21). Further complications arise during clearance monitoring as small numbers of viable spores must be recognized amid a landscape of nonviable spores. Currently, rapid viability PCR (RV-PCR) is the only diagnostic validated by the Environmental Protection Agency (EPA) for the detection of live *B. anthracis* spores in environmental samples (22, 23). This diagnostic expands the current capabilities of real-time PCR by measuring changes in cycle threshold (C_T) values pre- and postincubation of sample. As only viable *B. anthracis* spores/cells can germinate and/or grow during the incubation period, reduced C_T values postincubation are indicative of the presence of viable cells in the original environmental sample. RV-PCR can detect 10 live spores in the presence of 10^6 autoclaved spores and can process ~96 samples in 24 h with a single robot and with personnel working in successive 8-h shifts. Although this technique has been validated for use with air filters and water and surface samples (24), it has yet to be validated for use in soil samples.

Bacteriophages (phages) are viruses which exclusively infect bacteria. This host tropism led to the development of phage typing schemes that have been used for decades to identify pathogens and that are now utilized in a number of different applications for bacterial detection (25, 26). A temperate *B. anthracis* phage (Wβ) identified in the 1950s was shown to display species specificity for *B. anthracis* by its ability to infect all *B. anthracis* strains tested ($n = 171$) (27) and an inability to lyse 242 out of the 244 strains (99% specificity) analyzed from 17 different non-*anthracis Bacillus* species (28). However, a few unusual *B. cereus* strains that manifest phenotypes of both *B. cereus* and *B. anthracis* have been identified which are phage susceptible (29). Using Wβ, we previously generated a light-tagged reporter phage by integrating the genes encoding bacterial luciferase (*luxA* and *luxB*) into the Wβ genome (30). Wβ::*luxAB*-1 transduces bioluminescence to cultured cells within 20 min. Although this reporter phage was able to detect 10^4 spores/ml in pure cultures within 3 h, improvements were required in order to facilitate detection from complex samples. Inclusivity experiments against wild-type *B. anthracis* isolates indicated that the reporter was able to confer bioluminescence to all *B. anthracis* strains tested ($n = 38$) (31). Specificity experiments with members of the closely related *B. cereus* group (*B. cereus*, *B. thuringiensis*, *B. weihenstephanensis*, and *B. mycoides*) indicated that 6 strains out of 119 analyzed displayed bioluminescence signals above those of background controls (95% specificity). Of these, five of the six positive strains elicited signals that were 10- to 100-fold lower than the *B. anthracis* signal. Fifteen other species of *Bacillus* and non-*Bacillus* members (comprising 47 strains) did not elicit a response with the reporter phage (31). We reconstructed Wβ::*luxAB* (Wβ::*luxAB*-2) to generate a brighter reporter phage with improved sensitivity, and here we demonstrate its utility in detecting viable *B. anthracis* spores from soils.

MATERIALS AND METHODS

Construction and propagation of Wβ::*luxAB*-2. The Wβ::*luxAB*-2 reporter phage was constructed by targeted homologous recombination as previously described (30), with the exception that a designed promoter, harboring consensus transcriptional signals, was placed immediately upstream of *luxAB* (Fig. 1A). As before, the gene encoding spectinomycin resistance was included within the reporter cassette to enable the selection of recombinant lysogens. Plate lysates of Wβ::*luxAB*-1 and Wβ::*luxAB*-2 stocks were eluted in brain heart infusion (BHI) broth saturated with chloroform and centrifuged twice at room temperature (RT) for 10 min at 4,000 × *g* before the supernatant was incubated with DNase 1 (1.7 units/ml) (Thermo Scientific) for 20 min at 37°C. Lysates were then vacuum filtered through 0.2-μm-pore-size polyethersulfone (PES) membranes.

Phage were concentrated by adding NaCl and polyethylene glycol (PEG) 8000 to the lysate to final concentrations of 0.75 M and 8% (wt/vol), respectively, and rotating the sample at 4°C for 3 h, followed by centrifugation at 4°C for 30 min at 11,000 × *g*. Pellets were gently resuspended in SMC buffer (50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.1 M NaCl, 8 mM $MgSO_4$, 0.01% gelatin), and titers were determined using soft-agar overlays (32). Phage stocks were stored in the dark at 4°C until needed.

***Bacillus* spore preparations.** *B. anthracis* ΔSterne (exempt select agent strain) spores were prepared and kindly provided by Tony Buhr (Navy Surface Warfare Center Dahlgren Division) (33). The final preparation consisted of >98% spores that were stored in 0.1% Tween 80 and maintained at −80°C until use. *B. anthracis* Sterne 34F2 and *B. thuringiensis* 4AG1 spores were generated as described in Schofield and Westwater (30). Final preparations consisted of >98% spores that were stored in sterile water. As required, vials of *Bacillus* sp. spores were diluted in 0.05% Tween 80 and enumerated via colony counting (after 24 h of growth on BHI plates at 35°C). Unless otherwise stated, all experiments were conducted using *B. anthracis* ΔSterne.

Signal kinetics and sensitivity of Wβ::*luxAB*-2. Signal kinetics between the Wβ::*luxAB*-1 and Wβ::*luxAB*-2 were compared by enriching *B. anthracis* spores (final concentration, 1×10^6 CFU/ml) in tryptic soy broth (TSB) (containing 0.1 M L-alanine) for 3 h with shaking (250 rpm) at 35°C ($n = 3$). The culture was divided and infected with either Wβ::*luxAB*-1 or Wβ::*luxAB*-2 (final concentration, 1×10^8 PFU/ml), incubated at designated time points (see Fig. 2A), and measured for bioluminescence. Detection limits of the Wβ::*luxAB*-2 reporter were determined by mixing *B. anthracis* spores (final concentration, 1 to 100 CFU/ml) with phage (final concentration, 3.4×10^8 PFU/ml) in TSB containing 0.1 M L-alanine. Samples were incubated for 8 h with shaking (250 rpm) at 35°C before being assayed for bioluminescence.

Soil sample preparation and spore inoculation. Mollisol HCB, a silty clay loam (USDA textural class), which is the predominant (21.5%) soil type in the United States (34), was purchased from Agvise Laboratories. A top layer (0 to 6 in.) of soil was passed through a 2-mm-pore-size sieve; samples (pH 7.8) contained 2.3% moisture and 7.5% organic matter. Where indicated (see Fig. 3 and 4), sterile soil was prepared by autoclaving (at 121°C for 60 min) soil samples three times in 50-ml Falcon tubes at 50% capacity. Sterility was assessed by the absence of growth following incubation on blood agar plates. For each experiment, the desired volume of soil (either 1.0 g or 0.1 g) was aliquoted into a 50-ml Falcon tube before 10 μl of an appropriate concentration of *B. anthracis* spores was inoculated into the center of each soil sample. Samples were maintained overnight (~16 h) at 4°C before further use.

To assess the ability of Wβ::*luxAB*-2 to produce a bioluminescent signal response in the presence of other spore-forming bacteria, sterile soil was inoculated with *B. anthracis* spores at 1.1×10^1 to 1.1×10^4 CFU/g and incubated in the presence of a fixed spore concentration (1.1×10^4 CFU/g) of a nonpermissive *B. thuringiensis* strain (4AG1).

Phage-mediated detection of *B. anthracis* in soil. TSB or beef infusion broth (containing 0.1 M L-alanine) was added to soil-spore mixtures at a 1:10 (for 1.0-g soil samples) or a 1:100 (for 0.1-g samples) ratio and vortexed vigorously before being mixed with Wβ::*luxAB*-2 at a final concentration of 4×10^7 PFU/ml. Where indicated (see Fig. 6), spectinomycin (final concentration, 100 μg/ml) was added to the culture-soil mixes 4 h after phage infection. Samples were incubated at 35°C with shaking (250 rpm) before bioluminescence analyses at various times. Soil samples containing Wβ::*luxAB*-2 served as negative controls.

Bioluminescence assays and statistical analysis. Samples (195 μl) were measured for bioluminescence at various times after phage infection using a Veritas Microplate Luminometer (Turner BioSystems). Samples were injected with *n*-decanal (67 μl of a 2% solution) and read for 10 s. Controls consisted of cells or phage alone. Bioluminescent signal intensities were reported as mean relative light units (RLU) from three experiments ± standard deviations (SD). Statistical analysis and graphing were performed with GraphPad Prism, version 5, software. Two-way analysis of variance (ANOVA) followed by *post hoc* Bonferroni multiple-comparisons tests were used to determine statistical significance ($P < 0.05$) between treatments.

RESULTS

Redesign of Wβ::*luxAB*-2 reporter phage and signal response. Detection of *B. anthracis* spores from environmental samples poses significant challenges due to the complexity of the matrices and the requirement for low-level detection. To improve reporter phage sensitivity, we optimized the *luxAB* promoter to include all important conserved regions of Gram-positive bacterial promoters, in addition to the −35 and −10 hexamers (Fig. 1) (35–37). These include an A tract and T 5′ of the −35 hexamer, a TG dinucleotide 5′ of the −10 hexamer (extended −10), two A nucleotides 3′ of the −10 hexamer, and the optimal 17-bp spacer between the hexamers. These alterations generated significant changes in the characteristics of the reporter phage. *B. anthracis* lysogens harboring Wβ::*luxAB*-2 were brighter than lysogens harboring the Wβ::*luxAB*-1 reporter (data not shown). A more quantitative comparison of signal kinetics between Wβ::*luxAB*-1 and Wβ::*luxAB*-2 reporters indicated that the former elicits a peak signal 60 min after infection, followed by a general decline over time. In contrast, Wβ::*luxAB*-2 elicits a signal response that increases in strength over time (Fig. 2A). Wβ::*luxAB* is a temperate phage, and if the multiplicity of infection is high, the lysogenic pathway is typically favored over lytic growth (38). The reduction in luminescence after infection by Wβ::*luxAB*-1 suggests that *luxAB* expression decreases in the prophage state. The consensus *luxAB* promoter in Wβ::*luxAB*-2 therefore seems to be independent of other transcriptional regulatory processes acting on the phage genome. Nevertheless, the short-lived signal from Wβ::*luxAB*-1 necessitated that phage addition had to follow a period of incubation. However, Wβ::*luxAB*-2 can be added at the onset of germination. This allows a longer time frame for the reporter phage to interact with the cells and allows the spores to germinate and amplify during the course of the incubation time, a prerequi-

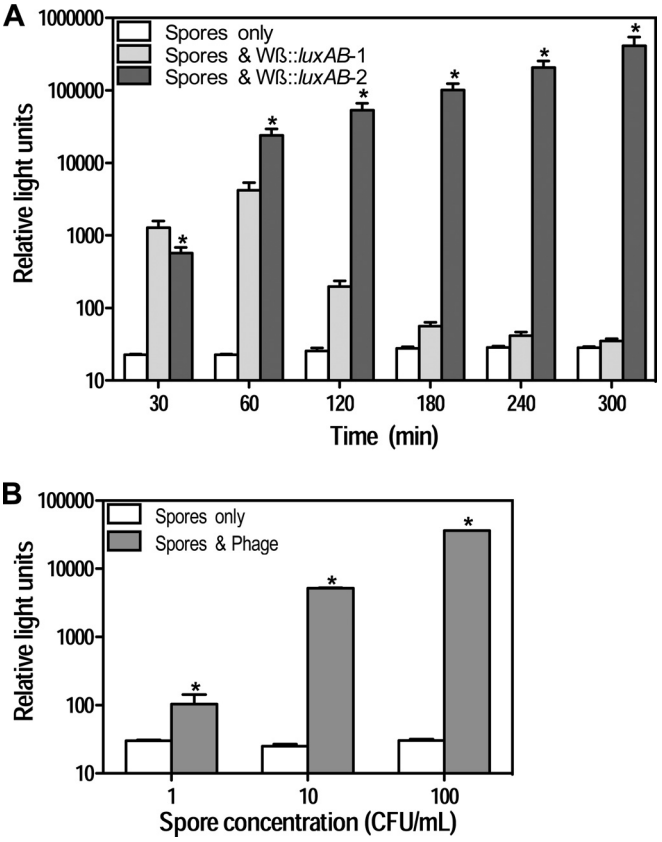

FIG 2 Reporter phage detection of *B. anthracis* Sterne 34F2. (A) Kinetics of light production by Wβ::*luxAB*-1 and Wβ::*luxAB*-2. Spores (1×10^6 CFU/ml) were incubated in TSB (containing 0.1 M L-alanine) for 3 h at 35°C. Phage (final concentration, 1×10^8 PFU/ml) were added, and bioluminescence was measured over time following the addition of *n*-decanal. (B) Detection sensitivity of Wβ::*luxAB*-2. Spores (1 to 100 CFU/ml) were mixed with reporter phage (final concentration, 3.4×10^8 PFU/ml) and incubated in TSB (containing 0.1 M L-alanine) for 8 h at 35°C. Bioluminescence was measured following the addition of *n*-decanal. Values represent the means ± SD ($n = 3$). *, $P < 0.05$ (two-way ANOVA) for the signal response from Wβ::*luxAB*-1 compared to that of Wβ::*luxAB*-2 (A) or compared to that of spore-only controls (B).

site for detection. As a result, the assay can readily detect a starting concentration of 1 CFU/ml of pure *B. anthracis* spores within 8 h (Fig. 2B). A similar level of sensitivity may be obtained from spores inoculated onto glass coupons, dried, and then immersed directly into outgrowth medium harboring the phage (data not shown).

Detection of *B. anthracis* in sterile soil. Soil is a complex matrix containing a myriad of factors that interfere with biological assays (20, 39). Soil microbes, humic acids, heavy metals, and other factors can reduce spore germination and growth, prevent phage infection, and/or quench bioluminescence. To reduce sample complexity, we initially used sterile (autoclaved) soil and examined the effect of varying the multiplicity of infection on the ability to detect *B. anthracis* (Fig. 3A). Soil was inoculated with a range of spore concentrations (10 to 10,000 CFU/g) and incubated with reporter phage at concentrations varying from 10^6 to 10^8 PFU/ml. At each spore concentration, the highest signal response was obtained using 10^7 PFU/ml ($P < 0.05$), irrespective of the multiplicity of infection. To examine the relationship between the number of PFU/ml, *B. anthracis* growth, and the number of infected cells (lysogens), soil samples were inoculated with 10^4 CFU/g and incubated with a phage concentration ranging from 10^6 to 10^8 PFU/ml (Fig. 3B). After 12 h of incubation, the samples were measured for bioluminescence, the number of CFU/ml, and the number of light-positive CFU/ml (lysogens). In the absence of phage, *B. anthracis* amplified to 10^7 CFU/ml during the course of the experiment. At the highest phage concentrations analyzed (10^8 PFU/ml), *B. anthracis* growth was reduced 100-fold compared to growth of phage-free controls. However, a very high proportion of these colonies (96%) were light positive (lysogens). Samples incubated with the lowest concentration of phage (10^6 PFU/ml) contained similar counts of CFU/ml as phage-free controls, but <10% of these colonies were lysogens. *B. anthracis* samples incubated with 10^7 PFU/ml grew to similar CFU/ml levels as phage-free controls, harbored 37% light-positive colonies, and displayed the highest total bioluminescence ($P < 0.05$). Thus, a reporter phage concentration of 10^7 PFU/ml was used in the subsequent experiments.

The sensitivity limits of detection and signal response times were examined using sterile soil. Soil samples inoculated with spores were directly mixed with medium harboring reporter phage (Fig. 4A). Thus, spores were not extracted from the soil samples, and the reporter phage infected germinating cells directly in the soil-medium mix. A detection sensitivity of 100 CFU/g was achieved in 7 h but could be lowered to 10 CFU/g after an incubation time of 12 h (Fig. 4B). Signal responses were phage dose dependent at early times but saturated at 24 h after infection. These results indicate (i) that spore extraction from the (originally sterile) soil is unnecessary and (ii) that phage infection is not inhibited by the soil matrix.

Sterile soil lacks organisms that could compete with *B. anthracis* germination and growth. We therefore assayed for *B. anthracis* in the presence of *B. thuringiensis* 4AG1, which is nonpermissive to Wβ::*luxAB*. Sterile soil was inoculated with 10 to 10,000 *B. anthracis* spores in the presence of a fixed concentration (10,000 CFU/g) of *B. thuringiensis* spores and analyzed for bioluminescence after 12 h of incubation (Fig. 4C). The presence of *B. thuringiensis* reduced the signal elicited by phage-infected *B. anthracis*. Nevertheless, 100 CFU/g of *B. anthracis* bacteria is readily detected in the presence of 10,000 CFU/g of *B. thuringiensis* bacteria; similar data were recorded when a non-phage-susceptible *B. cereus* strain (ATCC 14579) was used (data not shown). Presumably, these closely related bacteria affected the luciferase assay by reducing *B. anthracis* germination or growth and/or by sequestering the phage.

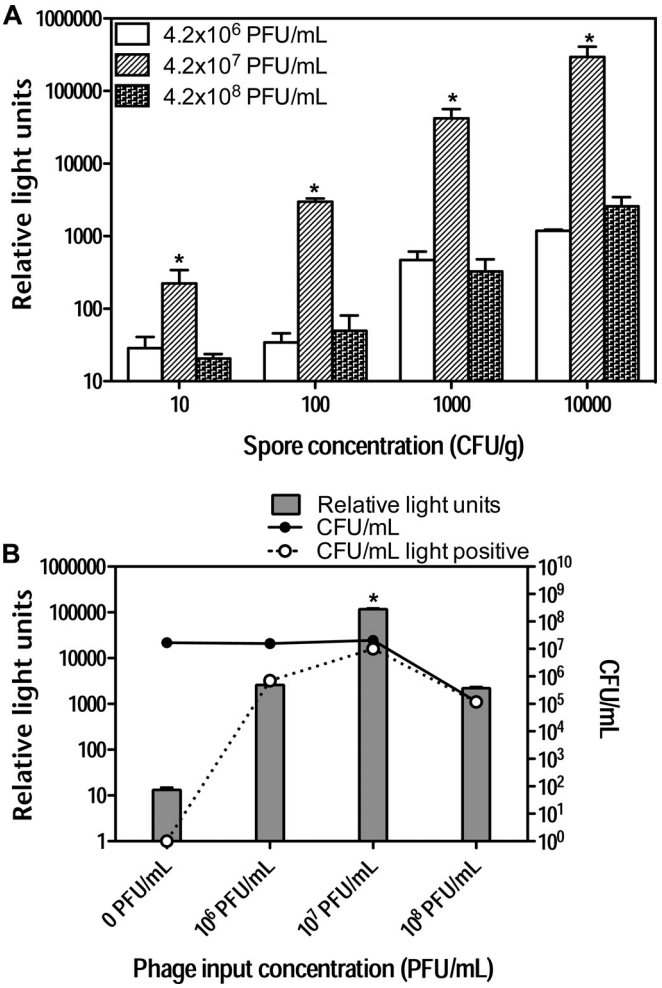

FIG 3 Effect of varying the reporter phage concentration on *B. anthracis* detection. (A) Effect of varying the multiplicity of infection. Spores (10 to 10,000 CFU/g) were inoculated into sterile soil (1.0 g) and mixed with Wβ::*luxAB*-2 (final concentration, 4×10^6 to 4×10^8 PFU/ml) and TSB (containing 0.1 M L-alanine) at a 1:10 soil/medium ratio. Samples were then incubated for 12 h at 35°C and measured for bioluminescence following the addition of *n*-decanal. (B) Effect of the number of PFU/ml on bioluminescence and the number of CFU/ml. Spores (1×10^4 CFU/g) were inoculated into soil (1.0 g) and mixed with a range of concentrations of Wβ::*luxAB*-2 (4×10^6 to 4×10^8 PFU/ml final) in TSB (containing 0.1 M L-alanine). After 12 h at 35°C, samples were measured for bioluminescence and plated for counts of CFU/ml. To determine the number of phage-infected colonies (light-positive lysogens) plates were exposed for 10-min to *n*-decanal and examined under dark-field illumination. Values represent the means ± SD ($n = 3$). *, $P < 0.05$ (two-way ANOVA) for comparisons of the results at the various phage concentrations used.

Detection of *B. anthracis* in soil. *B. anthracis* spores were tested in natural (nonsterile) soil samples. Soil was inoculated with 1.3×10^5 to 1.3×10^7 CFU/g and assayed for bioluminescence. Although 10^5 CFU/g could be detected within 3 h (Fig. 5), the signal strength was attenuated compared to that in sterile soil and then declined even further. The reduced signal may be due to

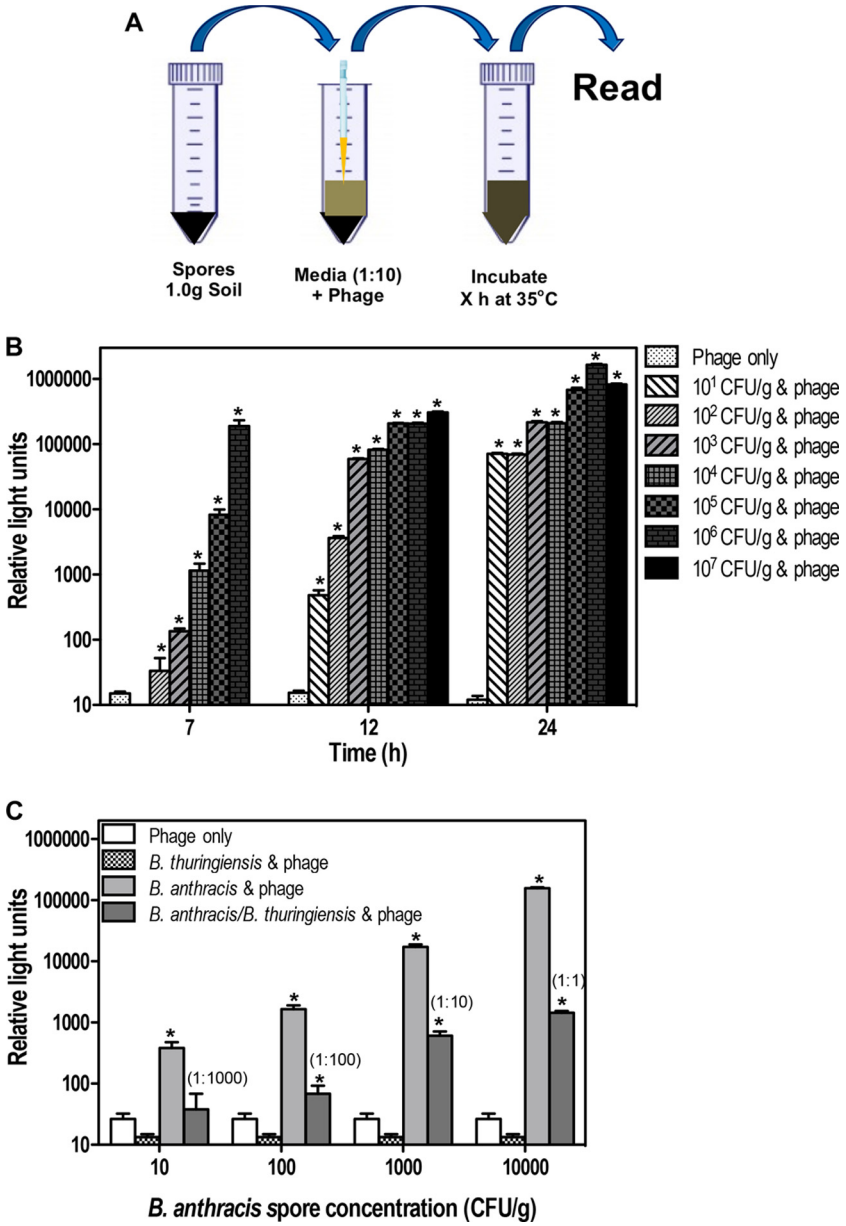

FIG 4 Detection of *B. anthracis* spores from defined soil. (A) Assay procedure. Unless otherwise stated, spores at the desired concentration were inoculated into sterile soil (1.0 g) and maintained overnight (~16 h) at 4°C before the addition of Wβ::*luxAB*-2 (final concentration, 4×10^7 PFU/ml) and medium (containing 0.1 M L-alanine) at a 1:10 soil/medium ratio. Samples were then incubated for *X* h (where *X* is 7, 12, or 24 h) at 35°C and measured for bioluminescence following the addition of *n*-decanal. (B) Sensitivity limit of detection of *B. anthracis* spores in sterile soil. Spores (1.3×10^1 to 1.3×10^7 CFU/g) were inoculated into soil and measured for bioluminescence after 7, 12, and 24 h of incubation. (C) Effect of mixed bacterial populations on detection in sterile soil. *B. anthracis* spores (10 to 1,000 CFU/g) were inoculated into soil in the presence or absence of an excess of spores (10,000 CFU/g) from the nonpermissive *B. thuringiensis* strain 4AG1. Bioluminescence was measured after 12 h of incubation. Values represent the means ± SD ($n = 3$). *, $P < 0.05$ (two-way ANOVA) for results compared to those with the phage-only controls. In panel C, values in parentheses indicate the initial ratio of *B. anthracis* spores to *B. thuringiensis* spores.

(i) competitive exclusion of *B. anthracis* germination or growth, (ii) phage binding to nonspecific components in the soil, and/or (iii) vegetative cell death. In unsuccessful attempts to address the cause of the reduced signal, we changed the multiplicity of infection and incorporated a 1-h 70°C heat pretreatment prior to outgrowth in order to reduce the viability of vegetative cells and partially select for spores. Neither treatment increased signal intensity or prolonged signal kinetics (data not shown).

A spectinomycin resistance gene was used as a selection marker in the construction of Wβ::*luxAB*. Consequently, all phage-infected *B. anthracis* bacteria should have a competitive growth advantage in medium supplemented with spectinomycin. To test whether spectinomycin could improve signal responses, soil was inoculated with 1.3×10^7 CFU/g and mixed with medium and phage. After 4 h of incubation, samples were supplemented with spectinomycin (100 μg/ml). In the absence of spectinomycin, peak signal intensity occurred at 6 h and then decreased (Fig. 6A). In contrast, the presence of spectinomycin allowed a significantly

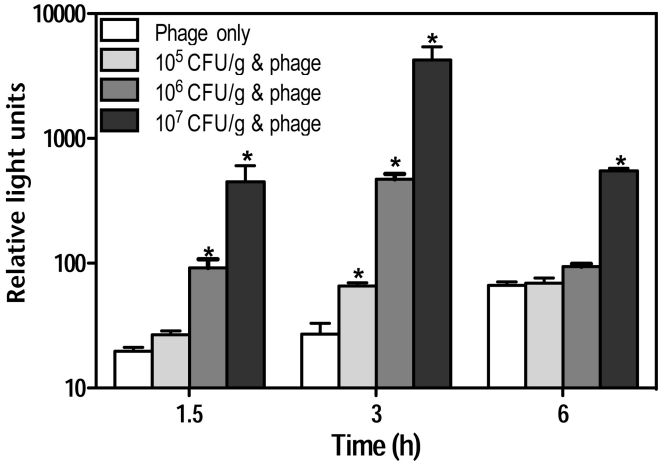

FIG 5 Detection of *B. anthracis* spores in soil. *B. anthracis* spores (1.3×10^5 to 1.3×10^7 CFU/g) were inoculated into soil (1.0 g) and maintained overnight (~16 h) at 4°C before being mixed with Wβ::*luxAB*-2 (final concentration, 4×10^7 PFU/ml) and incubated in TSB (containing 0.1 M L-alanine) at 35°C. Bioluminescence was measured 1.5, 3, and 6 h following the addition of *n*-decanal. Values represent the means ± SD ($n = 3$). *, $P < 0.05$ (two-way ANOVA) for results compared to those with phage-only controls.

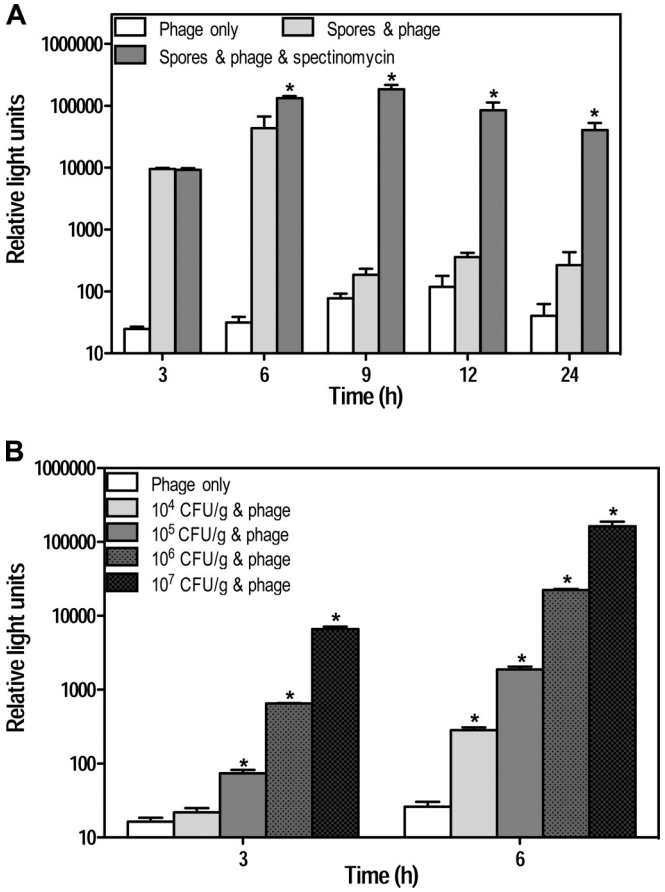

FIG 6 Effect of spectinomycin on signal kinetics and limits of detection. (A) Detection of *B. anthracis* spores in soil in the presence of spectinomycin. Spores (1.3×10^7 CFU/g) were inoculated into soil (0.1 g) and maintained overnight (~16 h) at 4°C before being mixed with Wβ::*luxAB*-2 (final concentration, 4×10^7 PFU/ml) and incubated in beef infusion broth (containing 0.1 M L-alanine) at 35°C. Spectinomycin (100 μg/ml) was added 4 h after phage infection, and bioluminescence was measured over time following the addition of *n*-decanal. (B) Limits of detection. *B. anthracis* spores (1.3×10^4 to 1.3×10^7 CFU/g) were inoculated into soil and incubated with the reporter phage and spectinomycin as described above. Samples were read for bioluminescence after 3 and 6 h. Numbers represent the means ± SD ($n = 3$). *, $P < 0.05$ (two-way ANOVA) for results with spectinomycin-treated samples compared to those with untreated samples (A) or for results compared with those of phage-only controls (B).

higher signal response by 6 h ($P < 0.05$), and the signal remained fairly stable up to 24 h (Fig. 6A). Spectinomycin also improved the assay sensitivity. Soil was inoculated with *B. anthracis* bacteria at 10^4 to 10^7 CFU/g and tested as described before; bacteria at 10^4 CFU/g were easily detected in 6 h, a 10-fold improvement over nonselective medium (Fig. 6B).

DISCUSSION

B. anthracis spores are the infectious agent of anthrax and may persist long-term in the environment if they are deliberately released (40–42). Viable *B. anthracis* spores were still detected 30 years after their release on Gruinard Island and have been revived from sediments ~1,000 years old (42). For over a decade, scientists have been striving to develop rapid, sensitive, and specific methods for the detection of *B. anthracis* spores in environmental samples (8). Reporter phages constitute an alternative means that may fit this need. In contrast to conventional PCR methodologies, which are independent of host viability, reporter phages generate a signal only if metabolically active cells are present (43). This is a critical element required for environmental detection especially following remediation to ensure that viable and potentially infectious cells have been effectively decontaminated. However, while detection of viable cells may be considered an advantage, it introduces assay variability as individual cells in a population may not be at the same stages of growth (asynchronous) or may be compromised, leading to differences in signal strength. Moreover, detection requires spore germination, which in the presence of other microorganisms in soil samples may be subject to competitive exclusion of nutrients necessary for *B. anthracis* germination and growth. As assay success requires both germination and growth, we used a nonselective rich medium to achieve this objective. Moreover, in order to maximize signal expression, we initially engineered a second-generation reporter phage, Wβ::*luxAB*-2, which was able to detect 1 CFU/ml in 8 h and displayed improved sensitivity and kinetics. The improvements, in comparison to the performance of Wβ::*luxAB*-1, are likely due to (i) the presence of a *Bacillus* consensus promoter driving constitutive expression of the *luxAB* reporter genes, rendering it independent of a lytic or lysogenic response by the phage, and (ii) the addition of the reporter phage to the spores at the onset of the assay. Thus, there is a longer window for the phage to find and interact with the germinating cells and elicit a response. The improvement in assay sensitivity of nonsterile soil when spectinomycin was employed further suggests that use of the Wβ::*luxAB* reporter phage will be effective for the detection of moderately to grossly contaminated soil samples.

Other phage-based methodologies using the closely related γ phage are being explored for the detection of *B. anthracis*. Cox et al. utilized the fact that phage will amplify in the presence of their host as a means of detecting whether *B. anthracis* cells are present (44). When γ phage amplification was used in combination with

an inexpensive lateral flow immunochromatographic device which harbors an antiphage antibody, 10^4 CFU/ml could be detected within 2 h. Alternatively, Schuch et al. used the phage lysin PlyG as a means of detecting *B. anthracis* (45). Germinating spores, when incubated in the presence of the *B. anthracis*-specific PlyG, rapidly lyse and release ATP. ATP is a cofactor for eukaryotic luciferase. Thus, when γ phage are mixed with luciferase/luciferin, light is emitted, indicating the presence of *B. anthracis*. Using this method, about 100 spores could be detected within 60 min. To the best of our knowledge, it is unknown if these methodologies have been assessed for the detection of *B. anthracis* from complex environmental samples. The transition from noncomplex to complex samples can be challenging. The differences in our results in terms of the efficiency of detection between pure cultures/cultures inoculated into sterile soil versus detection in "real" soil samples were significant. The limit of detection in pure cultures was 1 CFU/ml (in 8 h), but this decreased 1,000-fold to 10^4 CFU/g (in 6 h) in soil. The reasons for the drop in sensitivity in soil may be due to a multitude of factors, including a reduced ability of *B. anthracis* to grow during the course of incubation (C. Nguyen, N. J. Sharp, and D. A. Schofield, unpublished results). Future experiments may therefore require an initial spore extraction step to clean up and concentrate the spores and remove any soil-mitigating component prior to phage addition. It should be noted that for *B. anthracis* PCR detection methodologies, the use of commercial DNA extraction kits is dependent on the soil type, which can vary greatly and thus adds assay complexity. Moreover, input spore concentrations of $\geq 10^5$ CFU/g are required (39). The reporter phage technology in its current configuration is therefore at least as sensitive as, or perhaps more sensitive than, currently available commercial kits. Importantly, spore extraction and extensive processing of soil samples are not necessary for the reporter phage assay. Therefore, it offers the potential for high throughput.

During the 2001 anthrax event, the Federal Bureau of Investigation opted for traditional culture methods as their simplicity permitted inoculation of culture medium in hot zones by nonspecialists (46). This eliminated the laboratory bottlenecks previously experienced with molecular techniques that necessitate multiple sample preparation and assay steps and require highly trained technicians. Reporter phage technology may provide a direct and more sensitive approach that is potentially compatible with large-scale, high-throughput sampling that can be processed by technicians with no prior experience with this phage assay.

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