Whole-Genome Sequencing in Microbial Forensic Analysis of Gamma-Irradiated Microbial Materials

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Effective microbial forensic analysis of materials used in a potential biological attack requires robust methods of morphological and genetic characterization of the attack materials in order to enable the attribution of the materials to potential sources and to exclude other potential sources. The genetic homogeneity and potential intersample variability of many of the category A to C bioterrorism agents offer a particular challenge to the generation of attributive signatures, potentially requiring whole-genome or proteomic approaches to be utilized. Currently, irradiation of mail is standard practice at several government facilities judged to be at particularly high risk. Thus, initial forensic signatures would need to be recovered from inactivated (nonviable) material. In the study described in this report, we determined the effects of high-dose gamma irradiation on forensic markers of bacterial biothreat agent surrogate organisms with a particular emphasis on the suitability of genomic DNA (gDNA) recovered from such sources as a template for whole-genome analysis. While irradiation of spores and vegetative cells affected the retention of Gram and spore stains and sheared gDNA into small fragments, we found that irritated material could be utilized to generate accurate whole-genome sequence data on the Illumina and Roche 454 sequencing platforms.

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Biocrime, bioterrorism, and biological warfare constitute the three major categories of deliberate misuse of etiologic agents of disease, depending on the perpetrator, sponsor, target, and motive(s) (1). Depending on the agent employed, all three could result in dramatic harm to people, animals, economic activity, and social institutions (1). Instances of any deliberate release of a pathogen with intent to harm would require a civil defense and a law enforcement response, beginning with the characterization of the materials used in the attacks and concluding with the apprehension, trial, and conviction of a suspect (2), which, in the event of overt biological warfare, may extend to a national retaliatory response (3). Broadly defined, the discipline of forensics applies scientific principles to the acquisition of evidence, its analysis, and interpretation, to facilitate the attribution of evidentiary materials to potential sources (1, 4, 5). In the emerging subfield of microbial forensics, these principles are now being applied to the analysis of microbial materials, including potential and actual biocrime agents.

At least three significant instances of biocrimes using pathogens have been documented over the last 30 years. The 1984 attacks on salad bars in The Dalles, OR, by the Rajneesh cult inadvertently employed the common vaccine strain of Salmonella enterica serovar Typhi- murium (6). Likewise, attempts by the Aum Shinrikyo cult inadvertently employed the common vaccine strain of Bacillus anthracis in its attempted but ineffectual attack on the city of Tokyo, Japan (7). Finally, the successful anthrax biocrime of 2001 resulted in the most extensive and expensive criminal investigations in the history of law enforcement in the United States and led to the emergence of microbial forensics as one of the central investigative tools that facilitated the attribution of the attack materials to a set of potential sources (8, 9).

The employment of common laboratory strains in all three attacks reflects the relatively ready availability of such strains but complicates the forensic matching of the materials to potential source facilities. In the investigation of the 2001 anthrax attacks on the United States Postal Service (USPS; named Amerithrax by the FBI), the majority of spores were found to be indistinguishable from those of the widely utilized Ames strain of B. anthracis when lower-resolution typing methods were used. Even when whole-genome analysis of an organism was applied during the forensic investigation, the major morphotype in the letters was also found to match exactly the Ames genotype over more than 5 million base pairs. Attribution to a source required whole-genome analysis of several rare morphological variants in bacterial colonies cultured from the spore preparations. That analysis revealed a panel of mutations that was subsequently utilized to narrow down the list of potential sources to a select few laboratories. The origin of each of these samples could be traced to a single flask that was maintained at a U.S. Army laboratory (8). The ability to perform this analysis required the generation of whole-genome sequences of each of the variant morphotypes, a process that required growth, isolation, and a laborious sequencing and bioinformatics effort (8, 9).

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In any forensic investigation of a biocrime, protection of the responders, law enforcement officials, and forensic technicians from the biocrime agent is paramount. Thus, much of the evidence needs to be decontaminated (i.e., rendered nonviable) prior to processing. Gamma irradiation is a widely accepted method used in food processing (<10 kGy) (10) and for sterilization of medical and pharmaceutical products to kill bacteria (particularly spores) (11, 12), making these products safe for human consumption and use without destroying the flavor or utility of the product. In addition, gamma irradiation is also preemptively used by the U.S. Government (USG) to ensure safe handling at critical government agencies and facilities (13). Current practice for protecting workers in several major government buildings (e.g., those used by the U.S. Congress) is to irradiate all incoming mail with high-dose gamma radiation to inactivate biological threats. The USPS treats the select mail with at least 56 kGy of radiation (14), a lethal dose for all known biothreat agents. Gamma irradiation causes indirect damage to intracellular DNA by acting on water and other cellular components to produce free radicals that ultimately cause double-stranded breaks in the DNA, leaving cells incapable of reproducing (15). While irradiation protects workers in those buildings from mail-borne threats, it also potentially destroys forensic signatures and could confound the ability to rapidly identify, characterize, and develop assays for a bioterror directed at a USG facility. Because these might be some of the first (and, for the first few days, potentially the only) samples available in a bioterror incident, inactivated samples are likely to be sent forward for whole-genome sequencing (WGS) to identify specific markers that may suggest a source, as was performed by the FBI during the Amerithrax investigations of 2001 (9).

In spite of the known effects of high-dose irradiation on spore viability (16–20), information about its impact on WGS methods or data quality is limited. Several works have demonstrated the utility of irradiated material for use in immunosay and PCR-based detection (15–17), and other investigators have reported the phylogenetic classification of Yersinia pestis strains from highly degraded samples originating from the period of the Black Death (21), which suffer DNA lesions and fragmentation similar to those suffered by irradiated samples. However, little literature describing the effect of irradiation on DNA within vegetative cells, spores, or naked DNA was available prior to the commencement of this study. A notable recent study by Hoile and coworkers examined the effects of gamma irradiation on spore viability, fingerprint recovery, and recovery of DNA from blood-soaked paper (22) but did not examine the recovery of microbial forensic signatures from the spores themselves.

Previous evaluations of the effect of gamma irradiation on bacterial DNA integrity concluded that there was little effect on the ability to amplify DNA extracted from irradiated bacterial samples, and that finding was verified using real-time PCR of the 16S rRNA gene extracted from cells postirradiation at a dose of 12 to 25 kGy (15, 16). However, unlike real-time PCR, which requires small DNA fragments (50 to 150 bases) for amplification, some WGS approaches (e.g., those that use paired-end/mate-pair sequencing and long-read sequencing platforms, such as the Roche 454, IonTorrent, or Pacific Biosciences SMRT platform) require larger fragments and potentially several enzymatic processing steps to construct the libraries used in sequencing instruments. With the decrease in cost and increase in speed, WGS is being incorporated in the biodetection and biosurveillance communities as a means of identification (23–25), characterization, and outbreak tracing (26–30) and is likely to be one of the first methods to be attempted when a sufficient quantity of a potential biocrime agent is available.

To evaluate whether irradiation would affect several microbial forensic signatures, we designed a study to evaluate the effect of gamma irradiation on the outcome of standard microbiological staining regimens and on the chromosomal DNA of bacterial samples. We selected two endospore-forming bacteria differentiated by the absence and presence of an exosporium, namely, Bacillus atrophaeus and B. thuringiensis serovar Kurstaki, respectively. The presence of exosporium and its safety record as a biopesticide make B. thuringiensis serovar Kurstaki a favored surrogate for anthrax (31). We also selected a Gram-negative strain (Yersinia pestis EV76) and purified DNA. Spores, vegetative cells, and purified DNA were exposed to 54.3 kGy of gamma radiation from a 60Co radiation source and processed for potential forensic signatures. In this report, we describe the effect of high-dose gamma irradiation on the ability to generate forensic signatures using conventional staining techniques and whole-genome sequence data from samples that mimic evidentiary materials.

MATERIALS AND METHODS

Bacterial strains and growth conditions. (i) Bacillus spore preparation. Bacillus atrophaeus variant globigii Dugway spores were received from the Dugway Proving Ground in May 1991 as a dry spore preparation (large-scale fermenter strain; lot 10-88; Bioferm) (32). A pure culture seed stock was prepared from the crude spores after colony isolation and removal of extracellular proteins and DNA. Briefly, the dry preparation was diluted with water and isolated on LB agar plates, from which a colony was chosen, cultured in LB broth overnight, and sporulated by lawn plating on nutrient sporulation medium agar (33). The formation of spores by Scheaffer-Fulton spore stain procedures (34,35) was monitored until a majority of the spores stained blue-green. Spores were carefully scraped off the agar plates, shaken in a shaker to break up clumped spores for 1 h, and incubated at 55°C with proteinase K (50 μg/ml) for 12 h. After pelleting and resuspension in 1× TE (10 mM Tris, pH 8.0, 1 mM EDTA), the spores were separated from the cellular debris via centrifugation through a 60/40 Percoll gradient. Measures were taken to remove any free DNA attached externally to the spore coats by treating the preparation with DNase I (1 unit/μl of spores) for 15 min, and the activity of DNase I was quenched with 0.5 M EDTA. The spores were again purified through a 60/40 Percoll gradient, reconstituted in 1× TE, and stored at 4°C until use.

PCR assays were performed using a primer assay targeting the recF gene to confirm the identity of the organism as previously described (32). B. thuringiensis serovar Kurstaki spores were prepared as dry powders as previously described (36).

(ii) Gram-positive bacterium vegetative cell preparation. The B. atrophaeus variant globigii spore preparation was plated for single colonies on LB agar and incubated overnight at 37°C. One colony of the plated B. atrophaeus variant globigii spore preparation was used to inoculate 10 ml tryptic soy broth (TSB), and the spore preparation was cultured overnight (14 h) at 37°C with shaking at 250 rpm.

(iii) Gram-negative bacterium vegetative cell preparation. A variant of the vaccine strain of Yersinia pestis EV76 (pgm negative, pCD1 positive, pMT1 positive, pPCP1 negative) previously utilized in our laboratory (37) was isolated on tryptic soy agar containing 5% sheep’s blood (TSA/SB) after incubation for 72 h at 30°C. One colony from the TSA/SB plate was added to 10 ml TSB and cultured overnight (22 h) at 30°C with shaking at 250 rpm.

Overnight cultures were centrifuged at 5,000 × g for 5 min to pellet the cells. The cells were reconstituted in 10 ml 1× TE (10 mM Tris, pH 8.0, 1 mM EDTA), and two 3-ml aliquots of all three samples were prepared in

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50-ml sterile conical tubes. One tube per sample was reserved for irradiation, and the remaining tube per sample functioned as the nonirradiated control. Samples were delivered to the irradiator on the morning after initial broth inoculation.

The purified DNA sample had previously been extracted from the B. atrophaeus variant globigii cell preparation using a Qiagen blood and cell culture DNA midikit and had previously been used for genomic analysis (32). Forty-eight micrograms in 40 μl was set aside for irradiation, with a second 48-μg aliquot acting as the control.

**Irradiation.** Three-milliliter aliquots of B. atrophaeus variant globigii spores (3.3 × 10^6 CFU/ml), Gram-positive B. atrophaeus variant globigii cells (1.4 × 10^6 CFU/ml), Gram-negative Y. pestis cells (3.5 × 10^6 CFU/ml), and purified DNA (48 μg in 40 μl) were placed in sterile 50-ml Falcon centrifugation tubes and irradiated with rotation on ice for a total dose of approximately 54.3 kGy, which is the Edgewood Chemical Biological Center’s (ECBC) standard kill dose for anthrax spores (S. Heinlein, personal communication). The minimum dose for ~10^9 anthrax spores has been suggested to be 41.5 kGy (38, 39), although discoveries of surviving spores in spore preparations irradiated with 41.5 to 44 kGy have called this figure into question (38, 40). We utilized a 26,000-Ci 60Co gamma irradiator (Shepherd and Associates), which produces gamma photons with an average energy of 1.0 MeV. The B. atrophaeus variant globigii spores were treated for 9.18 h at a dose of 5.9 kGy/h, and Y. pestis samples were irradiated at a gamma photon flux of 5.4 kGy/h for 10.2 h.

B. thuringiensis spores were added to four 2-ml Eppendorf tubes (0.3 g each at 1.1 × 10^11 spores/g). Two of the Eppendorf tubes received 1 ml 1× TE and were designated aqueous samples, while the remaining two Eppendorf tubes were analyzed as desiccated samples. B. atrophaeus variant globigii spores from the original spore preparation described above were aliquoted into four 2-ml Eppendorf tubes (1 ml each), two of which were placed at 55°C for 3 days until the spores became desiccated. The remaining two tubes served as the aqueous samples. The samples were irradiated at a dose rate of 4 kGy/h in turntable position number 3 for 13.5 h for a total inactivating dose of 54.3 kGy. In all cases, control samples were placed on ice and set in the room outside the irradiator. The irradiation and control samples were otherwise treated identically.

**Microscopic analysis of irradiated spores.** Prior to delivery to the irradiator and upon return from the irradiator, all bacterial samples were stained to observe their physical integrity, and the bacteria were enumerated using LB agar plating (B. atrophaeus variant globigii) and TSA/SB plating (Y. pestis) to assess viability and concentration. B. thuringiensis serovar Kurstaki and B. atrophaeus variant globigii spores were stained according to the Schaeffer-Fulton method (34, 35). Using this method, bacterial spores stain green, while vegetative cells and cellular debris stain red. Briefly, about 10 μl of culture was heated fixed on glass slides, covered with a paper towel, and steamed for 5 min with 5% malachite green, applied dropwise. After rinsing, the slides were treated with safranin counterstain for 30 s, rinsed again, and visualized via oil immersion microscopy (magnification, ×1,000). The B. atrophaeus variant globigii vegetative cell samples were stained using both the Schaeffer-Fulton technique and Gram staining technique.

To verify inactivation by irradiation, the number of viable CFU in irradiated and control samples was determined as soon as possible on the day following irradiation. Tenfold serial dilutions were prepared in 1× TE, and 100 μl of each dilution was spread in triplicate on LB agar plates. Plates were incubated at 37°C overnight for B. atrophaeus variant globigii and 30°C for 3 days for Y. pestis, and the cells were counted on the following day.

**Nucleic acid extraction and quality control.** Nucleic acid was extracted from bacterial control and irradiated samples using a Qiagen DNeasy blood and tissue kit. The standard protocol was modified to accommodate spore/cell disruption using a FastPrep FP120 instrument (Thermo Corp.) in combination with Lysing Matrix E tubes (MP Biomedicals). One milliliter from each sample was added to a Lysing Matrix E tube and homogenized in the FastPrep instrument for 45 s (speed, 6.5).

After the samples were incubated on ice for 5 min, the homogenization step was repeated, followed by a 2-min incubation on ice. The tubes were centrifuged at 14,000 rpm for 5 min at room temperature, and 400 μl was transferred from the lysing tube into a sterile 2.0-ml Eppendorf tube.

The protocol for the purification of total DNA from animal tissues (spin column) from the Qiagen DNeasy blood and tissue handbook was followed by adding equal portions of buffer AL and 100% ethanol and mixing prior to loading on the spin column. Samples were eluted with 100 μl buffer AE and quantified using a Qubit (v1.0) fluorometer (Invitrogen). The DNA integrity for all samples was initially assessed by gel visualization (100 ng/sample) prior to sequencing using a 2% E-Gel with SYBR Safe (Invitrogen) and a 100-bp DNA ladder (New England Biolabs) following the manufacturer’s instructions. As an orthogonal assessment to gel electrophoresis, 1 μl of the cellular control and irradiated samples was run on an Agilent Technologies Bioanalyzer 2100 instrument in conjunction with the use of an Agilent DNA 12000 kit following the manufacturer’s protocols to visualize DNA sizing and integrity.

**Optical mapping.** Five hundred microliters of each sample was removed from each control cell and irradiated cell sample and washed twice in 500 μl of cell wash buffer contained in OpGen’s sample preparation kit (catalog number 14300-010). A spheroplasting step was necessary to weaken the walls of the Gram-positive B. atrophaeus variant globigii cells prior to chemical lysis. Two hundred microliters of OpGen spheroplasting buffer, 5 μl of mutanolysin (25,000 U/ml; catalog number M9901; Sigma-Aldrich), and 0.1 μl of Ready-Lyse lysozyme (36,000 U/μl; catalog number R1804M; Epicentre Biotechnologies) were added to the cells, and the cells were incubated at 37°C for 1 h. The Y. pestis cells did not require the spheroplasting step, and chemical lysis was continued with the Y. pestis cells.

Long DNA fragments (200 kb and greater) were extracted from the cells using an Agencourt Genfind (v2) DNA purification system (catalog number A41499; Beckman Coulter). Briefly, the cells received 400 μl Agencourt lysis buffer premixed with Agencour proteinase K (9 μl) and were incubated for 30 min at 56°C. After incubation, 200 μl of washed Agencourt binding buffer was added to the cells, and the mixture was incubated at ambient room temperature for 15 min, followed by magnetic separation. The nucleic acid bound to the magnetic beads in the binding buffer was washed with Agencourt wash buffer 1 (800 μl), Agencourt wash buffer 2 (500 μl), and 70% ethanol (500 μl). Nucleic acid was eluted from the magnetic beads using OpGen’s elution buffer (90 μl), to which 10 μl of OpGen’s 10× loading buffer was added to facilitate the attachment of the nucleic acid to the glass surfaces for mapping.

The long DNA was visualized by affixing it linearly on glass slides and staining with Hojo dye (catalog number 50004; OpGen) following the protocols provided in the Argus optical mapping system user manual. Once the DNA was deemed suitable for mapping (see Fig. 5), it was treated with the appropriate restriction enzyme (Ncol for B. atrophaeus variant globigii and Nhel for Y. pestis) and imaged using an OpGen Argus optical mapper.

**Whole-genome sequencing.** (i) Roche 454 GS-FXL Titanium pyrosequencing. DNA samples were prepared for Roche 454 GS-FXL Tita- nium pyrosequencing (41) using the Roche GS FLX Titanium rapid library preparation method (January 2010), the Roche GS FLX Titanium series emulsion PCR method manual (Lib-L LV; January 2010), and the Roche GS FLX Titanium series sequencing method (November 2010) following the manufacturer’s protocols, except as noted. The DNA was sheared into fragments with an average size of 500 to 800 bp using a Covaris S2 ultrasonicator. Libraries were quantitated prior to emulsion PCR using an Agilent Bioanalyzer 2100 high-sensitivity DNA chip in conjunction with a 96-well Thermoskan Ascent fluorometer. Samples with DNA too damaged to be successfully prepared for Roche 454 pyrosequencing were treated by use of a Nextera DNA sample preparation kit as described, below as it could support a smaller fragment size.

(ii) Sequencing with the Illumina HiSeq 2000 platform. DNA was prepared using the Nextera DNA sample preparation kit, which requires less DNA (50 ng versus 500 ng for Roche 454 pyrosequencing) and shorter
Quantitative real-time PCR (qPCRs) were carried out in a 7900HT Fast real-time PCR system (Applied Biosystems, Foster City, CA) using equal quantities of DNA from each sample. All primers used for this study were obtained from Integrated DNA Technologies (Coralville, IA). The forward and reverse primers targeting the *B. atrophaeus* variant globigii *recF* gene were 5'-ACCAGACAATGCTCGACGTT-3' and 5'-CCCTGTTGAAATTCCCGAAT-3', respectively. The primers used for *Y. pestis* detection targeted the chromosomal locus from positions 560674 to 561038 in the *Y. pestis* CO92 genome (primers 3a-F [GGACGGCATCAGATTTCT] and 3a-R [CCGAAAACATTGGGACGATT]) (42, 43).

One-hundred picograms of genomic DNA was combined with 2 pmol each of the appropriate primers and 10 µl Fast SYBR green master mix (Applied Biosystems). The final volume was brought to 20 µl with water. After the DNA polymerase was activated with a 5-min incubation at 50°C, the DNA was amplified by an initial denaturation at 95°C for 20 s, followed by 40 cycles of 95°C for 1 s and 60°C for 20 s. The specificity of amplification was confirmed by performing a thermal dissociation step consisting of 60°C for 20 s followed by a slow ramp to 95°C (at approximately 0.02°C/s).

**Microarray data accession numbers.** Raw sequence data for all datasets have been deposited in the Sequence Read Archive under accession numbers SRR1514287, SRR1514286, SRR1513901, SRR1513647, SRR1513646, and SRR1513645.

**RESULTS**

Irradiation affects standard microscopic staining outcomes. We first examined whether irradiation might affect the observable properties of spores and vegetative cells under conventional microbiological staining regimens. We subjected irradiated material and appropriate control samples to Schaeffer-Fulton (35) and Gram staining procedures. As expected, the nonirradiated *B. atrophaeus* variant globigii spore sample and its control sample stained as expected, with vegetative cells retaining the malachite green and debris showing only the counterstain. In contrast, irradiated spores did not bind the green stain and appeared red.

**FIG 1** Effect of gamma irradiation on retention of standard microbiological stains by *B. atrophaeus*. Spores and vegetative cells were subjected as an aqueous suspension to irradiation with 54.3 kGy of gamma rays from a 24,000-Ci²⁶⁶Co source. Samples were subjected to Schaeffer-Fulton malachite green spore staining (35) or standard Gram staining protocols prior to irradiation (untreated samples) or after irradiation (control and irradiated samples). Spores stain green; vegetative cells and cellular debris stain red in the Schaeffer-Fulton procedure, whereas vegetative cells stain purple in the Gram staining procedure. The preirradiated sample and the control sample stained as expected, with the spore coat absorbing the malachite green and any vegetative cells remaining red. The irradiated spore lost its ability to bind the green stain and appears red. The numbers of remaining viable CFU were determined by dilution plating.
We also Gram stained irradiated and control vegetative B. atrophaeus variant globigii cells. In contrast to the controls, which retained the crystal violet stain in the standard Gram stain method, irradiated cells failed to stain with crystal violet, displaying instead the safranin counterstain and appearing as Gram-negative cells (Fig. 1). In contrast to the relatively robust Bacillus strains, which retained their shape following irradiation, the Gram-negative Y. pestis cells appeared to be less protected from the irradiation, suggested by the indistinctly defined cell margins postirradiation (Fig. 2).

We next asked whether the aqueous buffer in which the initial spores were irradiated might have aided chemical processes that would render the spores incapable of binding the malachite green spore stain. We also asked whether the phenomenon was unique to B. atrophaeus variant globigii spores or whether spores that contain an exosporium layer would likewise be affected by irradiation. To test this hypothesis, desiccated and aqueous B. atrophaeus variant globigii and B. thuringiensis serovar Kurstaki spores were irradiated and stained. As before, B. atrophaeus variant globigii spores irradiated in an aqueous buffer proved incapable of preserving the malachite green stain in the coat (Fig. 3A). However, desiccated B. atrophaeus variant globigii spores retained the spore stain upon irradiation, indicating that the radicals generated during irradiation damage the spore coat and lead to the failure to retain the malachite green stain. In contrast, B. thuringiensis serovar Kurstaki spores appeared to be visually unaffected postirradiation in both dry and aqueous environments and maintained the ability to retain the spore stain, displaying the malachite green stain both before and after irradiation (Fig. 3B).

High-dose irradiation fragments genomic DNA into small DNA fragment species. Given the known reactivity of DNA with hydroxyl radicals generated by interaction of gamma rays with water molecules (11), we examined to what extent genomic DNA would be impacted by irradiation. DNA was extracted from irradiated and control samples and visualized using gel electrophoresis. As expected, irradiation had a profound effect upon the integrity of the genomic DNA isolated from all kinds of samples. In contrast to the control DNA samples extracted from unirradiated control samples, which migrated at a large molecular size, DNA extracted from irradiated samples appeared as an indistinct smear in the adjacent lanes, indicative of sheared remnants from the irradiation (Fig. 4A). We confirmed the result using the Agilent bioanalyzer (Fig. 4B). By these analyses, DNA in spores appeared to be more protected than that in vegetative cells, having a larger average molecular size (1,610 bp [average] versus 965 bp). The most dramatic effect of irradiation on DNA integrity was observed when unprotected DNA was subjected to irradiation, resulting in the almost complete fragmentation into fragments less than 100 bp in length (Fig. 4A).

Irradiation prevents generation of optical genome maps. To gain another visual readout of DNA integrity, we utilized the optical mapping technique from OpGen, which arrays long (>50-kb) fragments of DNA onto slides for direct optical observation. While spores are not susceptible to the gentle lysis techniques required for the generation of long DNA fragments, we were able to observe DNA from vegetative cells. Whereas long DNA fragments from both unirradiated Y. pestis and B. atrophaeus variant globigii samples could be visualized as long strands on the optical mapping cards, no long fragments were observed in DNA isolated from irradiated samples (Fig. 5), thus providing a direct visual readout of the fragmentation effects.

Effects of irradiation on whole-genome analysis. While damage to DNA was readily evident in all irradiated samples, we reasoned that as long as sufficiently long fragments (~400 to 1,000 bp) remained, it might be possible to generate libraries for WGS. We therefore attempted to generate WGS libraries from control and irradiated samples for both the Roche 454 pyrosequencing and Illumina sequencing platforms.

NGS library preparation for Roche 454 pyrosequencing and Illumina sequencing platforms. Table 1 shows the results of our attempts to generate sequencing libraries from control and irradiated materials. The irradiated B. atrophaeus variant globigii spore preparation yielded DNA that was sheared to the appropriate fragment size of 400 to 800 bp and that was therefore suitable for shotgun sequence analysis. Using the Roche 454 pyrosequencing method, libraries could be prepared from both irradiated and control B. atrophaeus variant globigii spores and vegetative cells. In contrast, libraries could be produced only from unirradiated Y. pestis samples and from naked DNA. While Roche 454 library preparation to obtain DNA from irradiated vegetative B. atrophaeus variant globigii cells was successful, subsequent steps (emulsion PCR) were not, suggesting additional uncharacterized degradation of the DNA. However, DNA from irradiated spores passed

![FIG 2 Irradiation affects the integrity of Gram-negative bacterial cells. Y. pestis EV76 cells were irradiated with 54.3 kGy of gamma radiation and subsequently stained as described in the legend to Fig. 1. Irradiated or control samples were Gram stained to visually assess irradiation damage. Plate counts enabled viability and concentration determination. Viable cells were determined by dilution plating.](http://aem.asm.org/attachment/1/123/123_1.png)
FIG 3 Aqueous sample buffer contributes to the loss of spore integrity upon irradiation. Spores were irradiated in an aqueous buffer or as desiccated powders as described in the legends to Fig. 1 and 2 and then resuspended and stained using the Schaeffer-Fulton endospore staining method (35). (A) Bacillus atrophaeus spores; (B) B. thuringiensis serovar Kurstaki spores.
all quality checks and was successfully sequenced on the Roche 454 platform.

We generated Roche 454 sequence data from irradiated spores that were comparable in quality and coverage to those for the unirradiated control sample. Roche 454 pyrosequencing results from the initial spore study produced similar average read lengths (sequenced library fragments) between the control and irradiated spore samples, with only a 0.44-bp difference in average length being found. Table 2 presents the base caller metrics and the results of the de novo assembly of the raw reads, as well as the results of template-assisted assembly using the complete genome sequence of Bacillus atrophaeus 1942 (GenBank accession number NC_014639.1) as the reference (32). Despite the comparable quality scores, reads from the irradiated sample assembled into a higher percentage of smaller contigs with an overall larger number of contigs and a smaller N\textsubscript{50} contig size. The overall quality scores of the assembled contigs were unaffected. We considered the possibility that the assemblies were affected by the presence of PCR duplicates that affected assembly quality. Only 0.08% and 0.24% of the reads in the control and irradiated Roche 454 data set, respectively, were PCR duplicates; therefore it is unlikely that these account for the differences observed between these samples. Illumina data sets had larger and more variable numbers of duplicate reads, ranging from 8 to 20%, but a larger number of duplicate reads did not correlate with the de novo assembly metrics.

When the reference genome of B. atrophaeus 1942 (a direct ancestor of the Dugway variant) (32) was utilized as a template for assembly, the number of overall contigs was unaffected by irradiation. Reference-assisted assemblies were similar for the control and irradiated sample, with a ≤1% difference in the percentage of mapped reads being detected between irradiated and unirradiated material. Reads from both samples assembled equally well to the B. atrophaeus reference sequence with similar percentages, N\textsubscript{50} sizes, and percentages of bases with Phred quality scores of Q40+, with approximately 98% of reads mapping correctly to the reference sequence (Table 3). Roche 454 contigs from both samples that aligned to the B. atrophaeus 1942 reference sequence mapped similarly against the optical map prepared from the DNA (not shown). Single nucleotide polymorphisms (SNPs) and small insertion/deletion calls were identical between both types of samples (control and irradiated) for mutation calls for which ≥90% of individual reads differed from the reference sequence (see Table S1 in the supplemental material). All variants identified in B. atrophaeus in our previous study (32) were identified in both the irradiated and the control samples, and no additional high-confidence mutation calls were evident (see Table S1 in the supplemental material).

**Illumina sequencing of irradiated material.** The failure to generate sequence data from irradiated vegetative bacteria on the Roche 454 platform prompted us to ask whether the shorter-read Illumina platform could be used to generate sequence data. In contrast to the failure of Roche 454 library generation or the emulsion PCR method, we were able to generate libraries and clusters on the Illumina HiSeq platform and subsequently generate sequence data for irradiated samples of both vegetative B. atrophaeus variant globigii and Y. pestis (Table 1).
TABLE 1 Library generation and sequencing results from irradiated samples

<table>
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<th>Sample</th>
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<th>Roche 454 GS-FLX Titanium platform</th>
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<td>Library preparation</td>
<td>Emulsion PCR</td>
<td>Sequencing</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Library preparation</td>
<td>Sequeing</td>
</tr>
<tr>
<td>B. atrophaeus spores</td>
<td>-</td>
<td>Pass</td>
<td>Pass</td>
<td>NA*</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Pass</td>
<td>Pass</td>
<td>NA</td>
</tr>
<tr>
<td>B. atrophaeus vegetative</td>
<td>-</td>
<td>Pass</td>
<td>Pass</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Pass</td>
<td>Fail</td>
<td>Pass</td>
</tr>
<tr>
<td>B. atrophaeus DNA</td>
<td>-</td>
<td>Pass</td>
<td>Pass</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Fail</td>
<td>Fail</td>
<td>NA</td>
</tr>
<tr>
<td>Y. pestis EV76 vegetative</td>
<td>-</td>
<td>Pass</td>
<td>Pass</td>
<td>Pass</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>Fail</td>
<td>Fail</td>
<td>Pass</td>
</tr>
</tbody>
</table>

*NA, not attempted.

In spite of the loss of DNA integrity from irradiation, Illumina sequencing of short 100-bp fragments was still attainable. The results are captured in Tables 4 and 5, which show the results produced from the de novo and reference-assisted assemblies, respectively. We noted that sequence data from the irradiated sample exhibited indications of DNA degradation that affected assembly quality. As with the Roche 454 data, the reads generated from irradiated samples assembled into a higher number of shorter contigs in a de novo assembly and covered slightly less of the reference sequence. Most previously reported variants from the 1942 strain were also detected using Illumina sequence data, although some did not meet the ≳85% read congruence threshold (see Table S1 in the supplemental material). The numbers of variants from the Y. pestis CO92 reference sequence were comparable.

Detection of microbial contamination. Approximately 1 to 5% of the spores in one of the Amerithrax letters were derived from an uncharacterized B. subtilis strain, which presumably contaminated one of the cultures during the preparation of the attack material. To test the ability to detect lower-abundance contamination in irradiated materials, we spiked reads from a previously generated B. thuringiensis serovar Kurstaki HD-1 data set (44) into the irradiated B. atrophaeus data set to 5% and ran our in-house identification pipeline (to be published elsewhere), which, briefly, identifies closest neighbors by automated BLAST analysis of contigs from a de novo assembly to identify potential near neighbors. Reads mapping to the near neighbor are subtracted from the data set, and then the process is iteratively repeated until either no reference sequence can be found or the reads are exhausted. Mapping of a total of 14.5 million reads in the first iteration generated a 265× average coverage of B. atrophaeus 1942 (GenBank accession number CP002207.1), whereas the second iteration identified B. thuringiensis HD-1 (CP010005.1) with a 7.6× average coverage.

Effects of irradiation on qPCR detection. To test whether the effects on our library preparation and sequencing results might be reflected in the ability to detect these agents using real-time PCR methods, we also investigated the effect of gamma irradiation on the sensitivity of real-time quantitative PCR assays directed at the pathogens (Table 6). While the generation of sequencing results proved challenging, identification of the presence of biological agents using real-time qPCR was easily achieved, as previously reported (16, 17). The cycle threshold (CT) values obtained from irradiated and control material were compared, albeit irradiation had detectable effects on the integrity of the DNA isolated from vegetative cells. The CT value for irradiated B. atrophaeus variant globigii spores was within the experimental error for the spore control. The irradiated B. atrophaeus variant globigii cell CT value was 2.7 cycles higher than that of its control sample, while the irradiated Y. pestis CT value was 3.3 cycles higher than that of its control counterpart, representing a decline of amplifiable DNA of approximately an order of magnitude (Table 6). These results are consistent with our visual observations of DNA quality as well as with the decrease in sequence quality obtained and illustrate that irradiated material can be detected via PCR, yet the sensitivity is not

TABLE 2 Roche 454 pyrosequencing read-level sequencing and de novo assembly results

<table>
<thead>
<tr>
<th>Sample</th>
<th>Read-level metrics</th>
<th>De novo assembly metrics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of reads</td>
<td>Read length (bp)</td>
</tr>
<tr>
<td>Control</td>
<td>544,215</td>
<td>280.04</td>
</tr>
<tr>
<td>Irradiated</td>
<td>523,766</td>
<td>279.60</td>
</tr>
</tbody>
</table>

*Average quality score of all the DNA bases in the filtered reads (after trimming).

* Large contigs were ≥1,000 bp; all contigs were ≥200 bp.

* Equivalent to the Phred quality score.

* Sequence statistics are derived from de novo and reference assemblies using Newbler GSAssembler (v2.3) software.
sacrificed (16, 17). In contrast to the requirements for longer DNA fragments during 454 pyrosequencing and Illumina library preparation, qPCR detection schemes typically amplify fragments of less than 140 bp (131 bp for \textit{B. atrophaeus} variant globigii and 67 bp for \textit{Y. pestis} in this study). The probability of occurrence of radiation damage in any given fragment increases with the strand length; therefore, it is not surprising that methods that are dependent on smaller fragments would be less affected by irradiation than those that require longer strands.

**DISCUSSION**

Our gamma irradiation procedures completely inactivated the spores and vegetative cells in each batch, with no viable colonies being recovered from any of our irradiated samples (Fig. 1 to 3). In contrast, recent events and earlier reports indicate that the current standard kill dose of 41.5 kGy may be insufficient for the complete inactivation of \textit{B. anthracis} cultures (38–40). Because our results with \textit{B. atrophaeus} variant globigii and \textit{B. thuringiensis} serovar Kurstaki strains will need to be verified with \textit{B. anthracis}, we are currently investigating the use of 54.3-kGy doses on \textit{B. anthracis} Sterne strains. However, the decimal reduction value for \textit{B. anthracis} has been estimated to be as high as 5.5 kGy (38), which would make a 54.3-kGy dose sufficient for almost a 10-log reduction in the count of viable organisms. In contrast, 41.5 kGy would be sufficient for a 7-log kill, leaving open the possibility that significant numbers of viable cells could be recovered from starting populations of as many as 10^9 cells (38).

To our surprise, \textit{B. atrophaeus} variant globigii spores irradiated in the presence of a liquid milieu presented false characteristics. Mining the Amerithrax investigation contained an environmental strain of \textit{B. subtilis} at a ratio of 1 to 5% (45). Given the phylogenetic, morphological, and physical similarity of \textit{B. subtilis} to the \textit{B. atrophaeus} test organism used here (31, 32, 46, 47), it is possible that a Gram stain result such as that presented in this report would have led to an incorrect conclusion regarding the presumptive contaminant organism. Therefore, our results with both Gram-positive and Gram-negative organisms indicate that caution should be exercised when choosing PCR assays for detection of pathogens in irradiated materials on the basis of morphological characteristics. We also note that other characteristics (e.g., protein and polysaccharide components) that were not investigated in this study may also be affected, although the target size for individual proteins is considerably smaller, and thus, they would be relatively unaffected by irradiation (48). In this study, aqueous irradiated Gram-positive bacterial spores appeared as both a non-spore substance and Gram-negative rods (Fig. 1 and 2). Irradiation of a dry powder did not affect spore staining, indicating that the sample matrix should be clearly recorded when microbial materials are processed for irradiation.

The DNA degradation induced by gamma irradiation did not interfere with Roche 454 shotgun sequencing of DNA prepared from spores, short-read Illumina WGS methods, or PCR-based detection. Although the Roche 454 technology is becoming obsolete, other technologies (e.g., IonTorrent) that require fragment sizes similar to those required for the Roche 454 technology for shotgun sequencing are in widespread use. On the basis of our results with the Roche 454 platform, it is likely that such methods could also successfully use a template from irradiated samples. Assuming that sufficient sample is available, particularly with amplification methods, it would be reasonable to expect to generate a shotgun WGS from some evidentiary samples. Shotgun sequencing and de novo assembly would thereby enable the discovery of artificially introduced or naturally acquired genetic material, such as chromosomal integrations or novel plasmids, information that would be critical in the early phases of a response. However, irradiation-induced degradation of the DNA into short fragments would handicap the use of emerging long-read sequencing (i.e., PacBio and nanopore-based sequencing) and would severely restrict the insert size for long-range paired-end and mate-pair sequencing libraries. Given these limitations, it is unlikely that a finished genomic sequence could be derived from a degraded sample, such as samples degraded by irradiation. Future studies should aim to determine the effects of various irradiation regimens that still safely inactivate the spores on DNA integrity and utility for sequencing. In addition, future studies should

---

**TABLE 3** 454 pyrosequencing reference mapping results

<table>
<thead>
<tr>
<th>\textit{B. atrophaeus} spore sample</th>
<th>% reads mapped</th>
<th>No. of contigs</th>
<th>No. of large contigs</th>
<th>Avg contig size (bp)</th>
<th>( N_{50} ) contig size (bp)</th>
<th>Q40+ bases in assembly (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98.35</td>
<td>17</td>
<td>13</td>
<td>275,739</td>
<td>1,048,322</td>
<td>99.93</td>
</tr>
<tr>
<td>Irradiated</td>
<td>97.35</td>
<td>16</td>
<td>16</td>
<td>257,477</td>
<td>1,048,279</td>
<td>99.94</td>
</tr>
</tbody>
</table>

\( a \) Sequence statistics are derived from \textit{de novo} and reference assemblies using Newbler GS Mapper (v2.3) software.

\( b \) Large contigs were \( \geq 500 \) bp; all contigs were \( \geq 100 \) bp.

\( c \) Equivalent to the Phred quality score.

---

**TABLE 4** de novo assembly of Illumina reads from control and irradiated material

<table>
<thead>
<tr>
<th>Sample</th>
<th>% reads assembled</th>
<th>Total no. of contigs</th>
<th>Avg contig size (bp)</th>
<th>( N_{50} ) contig size (bp)</th>
<th>Total assembly size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{B. atrophaeus} (54.3 kGy)</td>
<td>93.2</td>
<td>80</td>
<td>51,872</td>
<td>4,149,724</td>
<td></td>
</tr>
<tr>
<td>Dugway</td>
<td>98.9</td>
<td>92</td>
<td>44,789</td>
<td>4,120,630</td>
<td></td>
</tr>
<tr>
<td>\textit{Y. pestis}</td>
<td>93.5</td>
<td>405</td>
<td>11,067</td>
<td>4,483,431</td>
<td></td>
</tr>
<tr>
<td>EV76</td>
<td>85.0</td>
<td>502</td>
<td>9,185</td>
<td>26,970</td>
<td></td>
</tr>
</tbody>
</table>

\( c \) Irradiated material
evaluate different reagent chemistries for Illumina sequencing of irradiated material, as the NexTera kit used in this analysis, selected for its speed and simplicity of processing, requires longer fragment sizes than the TruSeq chemistry. Alternative reagents would likely provide even more sequence data than the reagents selected for this study. While the shotgun methods attempted here can assemble contigs that contain much of the relevant information and produce a panel of known SNPs that can themselves serve as forensic markers, assembly of larger-order scaffolds of microbial genomes, particularly of repeat-rich genomes, such as the genome of *Yersinia pestis* (49), requires the retention of fragments that physically bridge the repeats. Insert sizes of 3 to 5 kb are required to bridge most repetitive sequences (e.g., insertion sequences of 0.6 to 2.5 kb), but for RNA gene segments and large repetitive sequences, an ~9- to 10-kb average insert size is required to provide the structural information required to link contigs that are separated by such repetitive regions.

It is important to note that, in the context of forensic attribution, the ability to exclude potential suspects is as important as the ability to definitively match the evidentiary materials to a reference collection. While limiting the ability to provide a finished sequence that allows the latter, variant profiles generated even from a draft sequence can help exclude potential suspects (32, 50). When intact bacterial cells, whether they were spores or vegetative cells, offered some degree of protection from the effects of gamma irradiation, such that libraries could be constructed and sequence data could be generated, irradiation of aqueous solutions of purified DNA rendered the material incompatible with the sequencing methods selected for this study. Degradation of extracellular DNA would likely render any nonagent DNA (e.g., contaminating human or animal DNA) with probative value significantly compromised for forensic purposes. The sensitivity of unprotected DNA and the relative resistance of DNA in microbial cells (and particularly spores) to gamma radiation may prove useful in metagenomic analyses as a means to ablate unprotected DNA that may otherwise confound the analysis of microbial communities.

On the basis of the results presented in this report, irradiated spore material could be used for WGS in a forensic investigation with the following caveats: (i) that discovery of rare variants (such as the rare colony morphotypes in the Anthrax samples) within the DNA obtained from a population of nonviable cells would be difficult and (ii) that processes requiring long DNA fragments (e.g., optical mapping, long-insert paired-end libraries, finishing) are not likely to be possible using conventional techniques. However, recent developments may make it possible to reduce the baseline sequencing error by using error-correcting methods to access lower-abundance variants. Bioinformatics analysis of high-coverage data sets using quality-aware algorithms, such as LoFreq, allow sensitive detection of variants present in low abundances down to 0.2% (51), and directed amplicon sequencing using error-correcting barcodes can further increase both sensitivity and the amounts of such variants quantified in populations (52). Significantly, one of the five morphotypes that were critical for attributing the Amerithrax materials (morph A) was revealed only by the discovery of inconsistent long-insert read pairs and chimeric reads (9). In a shotgun sequence library without paired-end information, only the chimeric reads might have been evident, making the characterization of the nature of the duplication in that strain much more difficult. Despite the loss of information on the overall genome architecture, the discovery of informative SNPs and variants remains possible, at least for the aggregate population. Alternative sources of samples containing large populations of viable organisms (e.g., environmental swabs from nonirradiated areas) would be required for an accurate assessment of the phenotypic diversity of the populations in attack material.

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