EPA/600/R-12/596 | September 2012 | www.epa.gov/ord

United States
Environmental Protection
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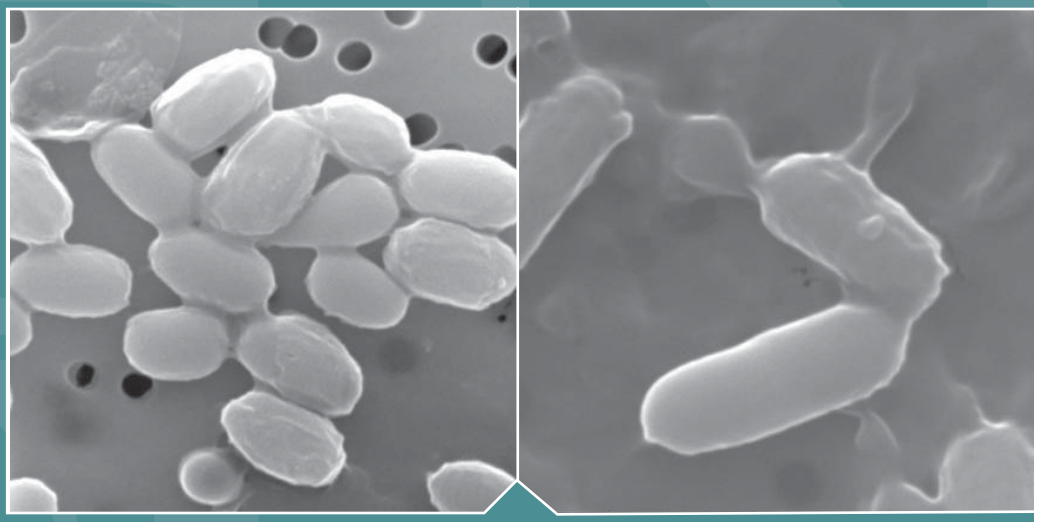
On the Use of *Bacillus thuringiensis* as a Surrogate for *Bacillus anthracis* in Aerosol Research

TECHNICAL REPORT



Office of Research and Development
National Homeland Security Research Center

Technical Report

On the Use of *Bacillus thuringiensis* as a Surrogate for *Bacillus anthracis* in Aerosol Research

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

RESEARCH TRIANGLE PARK, NORTH CAROLINA 27711

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The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development's National Homeland Security Research Center, funded, directed and managed this work. This report has been peer and administratively reviewed and has been approved for publication as an EPA document. Note that approval does not signify that the contents necessarily reflect the views of the agency.

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Questions concerning this document or its application should be addressed to:

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Acronyms and Abbreviations

AFM	Atomic Force Microscope
Ba	*Bacillus anthracis*
Ba S	*Bacillus anthracis* var Sterne
Bg	*Bacillus atrophaeus*
Bs	*Bacillus subtilis*
Bt	*Bacillus thuringiensis*
Bt k	*Bacillus thuringiensis* var kurstaki
BSL	Biological Safety Level
CDC	Centers for Disease Control and Prevention
DNA	Deoxyribonucleic acid
EBI	Electron Beam Irradiation
ECBC	Edgewood Chemical Biological Center
EPA	Environmental Protection Agency
kGy	kiloGray
LLNL	Lawrence Livermore National Laboratory
LANL	Los Alamos National Laboratory
MGB	Microbial Genomic and Bioprocessing Research Unit
NCAUR	National Center for Agricultural Utilization Research
NHSRC	National Homeland Security Research Center
PCR	Polymerase Chain Reaction
QA	Quality Assurance
QC	Quality Control
SEM	Scanning Electron Microscope
USAMRIID	U.S. Army Research Institute of Infectious Diseases

Acknowledgments

Contributions of the following individuals and organizations to the development of this document are gratefully acknowledged:

Post-Graduate Research Contractor

Jenia A. Tufts, M.S, ESE, for performing the literature review and authoring this document.

United States Environmental Protection Agency (EPA)

M. Worth Calfee, Ph.D. (US EPA/ORD/NHSRC, Decontamination and Consequence Management Division) for technical reviews and mentorship.

Dugway Proving Grounds

Angelo J. Madonna, Ph.D. (Chief, Microbiology Branch, West Desert Test Center, U.S. Army Test & Evaluation Command) for providing Scanning Electron Microscope (SEM) images of crystalliferous *Bt*.

Alion Science and Technology Corporation

SEM images and the Polymerase Chain Reaction (PCR) analysis of irradiated and non-irradiated spore samples were completed under U.S. EPA contract #EPD10070 with Alion. This support provided by Alion and their subcontractor, RTI International, is gratefully acknowledged.

Additionally, the authors would like to thank the peer reviewers for their significant contributions. Specifically, the efforts of Gene Rice, Vipin Rastogi and Timothy Dean are recognized.

Executive Summary

This project supports the U.S. Environmental Protection Agency (EPA), through its National Homeland Security Research Center (NHSRC), by providing relevant information pertinent to the selection of a *Bacillus anthracis* spore surrogate for use in aerosol and reaerosolization testing.

The primary focus of this effort is to investigate the physical properties of spores of *B. anthracis* (*Ba*) and *B. thuringiensis* (*Bt*) that impact their movement in air and interaction with surfaces, including size, shape, density, surface morphology, structure and hydrophobicity. Also compared are pathogenicity, genetic relatedness, and the impact of irradiation on the physical properties of both spore species.

Many physical features of *Bt* and *Ba* have been found to be similar and, while *Bt* is considered non-pathogenic, *Bt* is in the same family as *Ba*. When prepared similarly, both microorganisms share a similar cylindrical pellet shape, an aerodynamic diameter of approximately 1 μm (in the respirable size range), and have higher relative hydrophobicities than other species. While spore size, morphology, and other physical properties can vary among strains of the same species, the variations can be due to sporulation conditions and may therefore be controllable. All *Bt* spores may therefore not be representative of all *Ba* spores. Characterization of candidate surrogate spores prior to experimental use is therefore critical to confirm that the characteristics of the surrogate are as closely similar as possible to the characteristics of the pathogenic agent.

Both anecdotal and direct evidence of spore structural damage caused by electron beam and gamma irradiation contraindicates the use of irradiated spores in aerosol testing as the structural damage has unknown effects on the aerosol properties of these particles. Based on the results presented here, irradiated spores do not appear to be a good surrogate to predict the behavior of non-irradiated spores, and irradiated spores should not be used for studies to investigate the reaerosolization potential of spores.

Based on this review and comparisons of the physical properties of *Bt* and *Ba*, the use of *Bt* as a surrogate for *Ba* in aerosol testing appears to be well supported. Comparative studies should be performed to test the hypothesis that the two species will behave similarly when suspended in a gas (as an aerosol).

1.0 Introduction

The Category A biological agent *Bacillus anthracis* (*Ba*) has the potential to produce mass casualties and its spores are highly persistent in the environment.[1] The environmental and public health impacts of reaerosolization of *Ba* following an outdoor release in an urban environment are not well characterized. There are currently insufficient data in the literature to adequately quantify, predict, or model the risks associated with an outdoor release. Research to address these gaps is an immediate need. Before research can begin, however, a surrogate must be identified that is an appropriate model for *Ba* in aerosol and reaerosolization testing that could be released in the environment without concerns of pathogenicity. *Bacillus thuringiensis* (*Bt*) has been discussed in the literature as an appropriate surrogate for *Ba* in aerosol testing[2] and is currently used in wide area outdoor spraying as an insecticide.[3] The purpose of this document is to summarize the similarities and differences between *Bt* and *Ba* in the context of reaerosolization, discuss the suitability of *Bt* as a surrogate for *Ba* and factors that may impact use of *Bt* in field testing.

Based on a review of the available literature and comparisons of the physical properties of *Bt* and *Ba*, the use of *Bt* as a surrogate for *Ba* appears to be well supported.[2] A recent paper by Greenberg *et al.*[2] examined several *Bacillus* species as potential surrogates for *Ba* in aerosol and water testing. The authors comprehensively reviewed the literature for historical *Ba* surrogates and compared the properties of those surrogates to select the one that would most closely mimic *Ba* during aerosol and water testing.

The properties examined by the authors included the physical properties that impact the movement of spores in air and water, including size, shape, density, surface morphology, structure and hydrophobicity. Also compared were pathogenicity and genetic relatedness to *Ba,* a potential indicator of similar characteristics,[2] and survivability of spores exposed to extreme conditions.

Of the potential surrogates considered (which included *B. atrophaeus*, *B. cereus*, *B. subtilis*, *B. thuringiensis*, *B. anthracis* Sterne (*Ba* S), *B. megaterium*, *B. mycoides*, and *Geobacillus spp.)*, *Bt* was identified as the most appropriate for aerosol and water testing. The authors based this selection on the lack of pathogenicity, the high degree of genetic relatedness, and, most importantly, on the many physical similarities between *Bt* and *Ba*.

Drawing on the literature, the following sections outline the similarities between *Ba* and *Bt*, justifying the selection of *Bt* as a surrogate for *Ba* in aerosol and reaerosolization testing. The key physical parameters compared are size, surface morphology, hydrophobicity and density. These parameters are considered critical in influencing the behavior of an aerosol, and of a particle interacting with a surface.

2.0 Materials and Methods

2.1 Sources of Secondary Data

Sources of data for this literature review were unclassified peer reviewed journal articles, conference proceedings and textbooks found by searching citation databases including the EPA Desktop Library, PubMed, Google Scholar, and books. Articles included research, reviews and epidemiological studies. Due to the dearth of published data on the physical properties of these spore species, the search was not limited by the age of the data. However, books cited were published within the last 7 years or are the most recent edition of an established source.

2.1.1 Source Selection Rationale

Peer-reviewed sources are generally accepted as reliable in the scientific community because the study methods and results have been verified by independent reviewers. Important sources were defined as those crucial to answering research questions pertaining to the physical properties of the target spore species because they pertain to aerosol testing as well as pathogenicity and genetics. Five general assessment factors were considered in the evaluation of scientific and technical information, as outlined in the EPA General Assessment Factors for Evaluating the Quality of Scientific and Technical Information (EPA/100/B-03/001):

- ***Soundness:*** The extent to which the scientific and technical procedures, measures, methods, or models employed to generate the information was reasonable for, and consistent with, the intended application.
- ***Applicability and Utility:*** The extent to which the information was relevant for the intended use.
- ***Clarity and Completeness:*** The degree of clarity and completeness with which the data, assumptions, methods, QA, and analyses employed to generate the information are documented.
- ***Uncertainty and Variability:*** The extent to which variability and uncertainty (quantitative and qualitative) related to results, procedures, measures, methods, or models are evaluated and characterized.
- ***Evaluation and Review:*** The extent of independent verification, validation, and peer review of the information or of the procedures, measures, methods, or models.

2.2 Sources of Primary Data

To determine what effects gamma irradiation may have on exposed spores, EPA compared samples of irradiated *Bt 9727* and *Ba* S with viable preparations of *Bt kurstaki* (*Bt* k) and *Ba* S using polymerase chain reaction (PCR) and scanning electron microscopy (SEM). The irradiated samples were inactivated using an exposure dose of 44 ± 4 kiloGray (kGy).

PCR is a standard microbiological technique to identify microorganisms through the isolation, amplification and analysis of DNA in a sample.

SEM is a widely used microscopy technique that allows viewing and imaging of a sample on a submicron scale.

3.0 Quality Assurance/Quality Control

Quality assurance (QA)/quality control (QC) procedures were performed in accordance with the EPA QA program. QA/QC procedures are summarized below.

3.1 Quality Requirements

A criteria based approach was followed to select the data and information to develop this report. Although the quality of the data used cannot be quantified or qualified based on the source selection and assessment process, we have some certainty that the information is credible.

Data collected were from peer-reviewed journals, conference proceedings and textbooks, all of which were critically assessed through peer critique, typically by experts in the field, prior to publication. This peer review process usually ensures a high level of quality in the reported data.

3.2 Procedures for Determining Quality

Peer-reviewed publications were identified by reviewing the editorial statements of the journal. Research articles presenting data were considered to be of sufficient quality if the data were produced using accepted analytical methods and techniques. Accepted analytical methods are previously documented, commonly utilized, and recognized laboratory techniques, procedures, measures, methods, and models employed to generate information. Information on quality parameters considered for the literature review includes the following:

- ***Source****:* The characteristics of the source were considered as an indicator of quality. Publications that were peer-reviewed and published in a reputable journal were generally regarded as reliable, although, because the quality of peer-reviewed articles can vary greatly, care was taken to assess each article carefully as outlined in Section 2.2.
- ***Experimental Design****:* Articles that contained details of experimental design (quality control samples, statistically valid conclusions) indicating that appropriate scientific methodology was employed were regarded as high quality.
- ***Data Quality:*** The quality of data was assessed by examining the types and numbers of QC samples used, the consistency of results and other statistical analyses of data. Since most peer-reviewed articles do not include all QA/QC information and data generated during a study, a review of such information and data was not possible. Instead, sources were assessed for evidence of the use of controls, replicates and other QC samples as applicable to the study and the evaluation of variability and uncertainty in the data.
- ***Data Presentation:*** Concise, well-substantiated articles were sought and regarded as high quality. Poorly presented information (disorganized, rambling, and poorly supported by factual information) was viewed with extreme caution.
- ***Data Interpretation:*** The authors' interpretation and conclusions were reviewed for scientific soundness. Results were reviewed to ensure they supported the conclusions presented, and other published sources substantiating the conclusions were sought out.

3.3 Primary Data Sources

The SEM images shown in Figure 1 were obtained from Dugway Proving Ground rather than the literature. The PCR data and SEM images discussed in Section 4.2.1 and shown in Figures 2-4 were collected by EPA during efforts to characterize spore preparations that had the potential for use in aerosol testing in the planning process.

All of the data were collected using established methods. QC samples for PCR analysis include positive, negative and internal controls. Positive controls ensure that the test is sufficiently sensitive and have an expected positive result for the target DNA sequence. Negative controls ensure aseptic techniques and are expected to contain no target DNA. Internal controls are present in all samples, including control samples, to ensure that the method has the expected sensitivity and that PCR inhibitors did not negatively bias the results.

QA/QC procedures for SEM include detector calibration, detector beam alignment and the periodic measurement of a known standard.

4.0 Results and Discussion

4.1 Background and Taxonomy

Bt is widely distributed in nature and is commonly used as an insecticide in the management of mosquitoes, moths and black flies.[3] Like *Ba*, *Bt* is a gram-positive spore-forming *Bacillus* that is in the *B. cereus* group.[4] *Bt* is genetically similar to *Ba*, with *Bt* 9727, a strain that has shown wound infectivity and virulence in immunocompromised mice,[5] demonstrating the highest homology.[6] There have also been some isolated reports of infection caused by strains of *Bt,*[5-7] and a recent mouse study indicated that repeated low-dose aerosol exposures can cause sub-chronic lung inflammation in mice.[8] However, while exposure to *Bt* may cause skin and eye irritation,[9] based on its use in the field and laboratory studies,[9] *Bt* is considered a non-pathogenic Biosafety Level-1 (BSL-1) organism.

4.2 *Bt* and *Ba* Spore Physical Properties

The key physical parameters compared are size, density, surface morphology, and hydrophobicity. These parameters are considered critical in influencing the behavior of an aerosol, and how a particle interacts with a surface. Identifying a surrogate spore in the same size range as *Ba* is essential for aerosol and reaerosolization studies because particle size is the most important factor in the behavior of aerosols, and all aerosol properties are dependent upon this parameter.[10] Particle density is a key parameter because it impacts aerodynamic diameter, particle settling velocity and inertial properties.[10] Surface morphology plays an important role in particle adhesion because the primary adhesive forces (van der Waals, electrostatic, and capillary) are influenced by particle surface features.[10] For example, surface roughness will determine how many points of contact a particle makes with a surface, and particle elasticity will play a part in particle deformation onto a surface and an increase in the adhesive force.[10] As with morphology, hydrophobicity influences particle-surface interactions, as has been demonstrated experimentally with hydrophobic *B. cereus* spores which adhere more strongly to hydrophobic than to hydrophilic surfaces.[11,12]

On a micro level, surface features such as morphology and hydrophobicity may help predict the likelihood that a spore will adhere to a particular surface type. However, after the first layer of spores has settled, the spore-on-spore adhesive properties are stronger than the adhesive forces between the spores and the surface they initially landed on, and a layer of particles will be detached more easily as larger agglomerates than as single particles from surfaces.[10]

4.2.1 Size

Both *Bt* and *Ba* single spores are in the same size range and have similar aspect ratios and diameters.[13-16] In a recent study comparing the physical dimensions of seven hydrated strains of *Ba* spores to seven other hydrated spores of *Bacillus* species,[16] *B. subtilis* (*Bs*) 1031 (source laboratory not given) and *B.atrophaeus* (*Bg*) ATCC B-385 spores were found have smaller dimensions than all *Ba* strains compared, while *B. cereus* (*Bc*) ATCC 10702 and *Bt* 4055 (Microbial Genomic and Bioprocessing Research Unit, NCAUR, Peoria, Il, USA) spores were found to have the dimensions most similar to the *Ba* species compared. All spores were prepared under similar conditions using the same media to ensure comparability. The *Bs* and *Bg* strains tested were smaller in length and diameter than the *Ba* strains. However, the *Bt* strain tested was closer in both aspects with an average of 1.42 x 0.83 μm. The average length and width of the seven *Ba* strains measured were 1.42 x 0.83 μm, although there was variation between strains.

There were more between-strain variations in the length of *Ba* spores (range of 1.23 to 1.67 μm) than in the diameters (range 0.81 to 0.86 μm). Table 4-1 outlines the results obtained by Carrera *et al*.[16]

In another study[13] where more variation in length than width was also seen, the authors prepared *Bt israelensis* (ATCC 35646) and *Bg* ATCC 9372 spores using both a plate-wash method and a liquid sporulation approach. For *Bt*, the resulting average length for plate-grown dried spores was 2.17 μm with an absolute deviation (D) of 0.18 μm and an average width of 0.937 μm (D = 0.049 μm). For solution-grown dried *Bt*, the average length was 2.00 μm (D = 0.16 μm) and the average width was 0.872 μm (D = 0.047 μm). For *Bg*, plate-grown dried spore average length was 1.68 μm (D = 0.13 μm) and width was 0.647 μm (D = 0.028 μm); solution-grown dried *Bg* spores averaged 1.79 μm (D = 0.19 μm) and width 0.686 μm (D = 0.027 μm). These results indicate that there are differences in the size of spores as determined by the two methods.[13] The authors also noted differences of up to a factor of 2 X in length and 1.5 X in width between the smallest and largest spores measured in each population. These wide size distributions in unique spore populations will have significant impact on environmental fate and transport models.[13]

Both *Ba* and *Bt* have been described as oval,[17] cylindrical[18] and ellipsoid[16,18]in shape, depending on the viewer. Carrera *et al*. (2007)[16] used the shape of an ellipsoid to calculate the volume of a spore. However, this calculation may underestimate the volume as the actual shape of these spores is more like a cylindrical pellet. However, to calculate the volume of a cylindrical pellet one needs the radius at each end. In the absence of radius data, the equation for the equivalent diameter for a cylindrical pellet was used to estimate the volume of a spore, and this value was used to estimate the aerodynamic diameters. Equivalent diameter was calculated by

$$d_e = \sqrt[3]{\frac{6\left[0.7h\left(\frac{d^2\pi}{4}\right)\right]}{\pi}} \qquad (1)^{19}$$

where h is the hydrated length (μm), and d is the hydrated diameter (μm).

Using the physical dimensional measurements in Carrera *et al.* (2007)[16] and the calculated average equivalent diameter of each spore species, the average aerodynamic size for the hydrated *Ba* species was calculated by

$$d_{ae} = d_e\sqrt{\frac{\rho_p C_{c\,de}}{\rho_0 \chi}} \qquad (2)^{10}$$

where d_e is the equivalent diameter calculated above; ρ_p is the density of the particle as reported in Carrera *et al*. (2008);[20] C_c is the Cunningham slip correction factor as calculated by equation 3.22 in Hinds (1999);[10] ρ_0 is standard particle density (1 g/cm^3); and χ is the dynamic shape factor, as calculated by Sturm (2011)[21] for the estimation of non-spherical particle transport in the human respiratory tract.

Using the above-cited equation, the average aerodynamic size for the hydrated *Ba* spores is 0.95 μm (ranging between 0.90 and 1.01 μm) and 0.91 μm for *Bt* 4055. The estimated aerodynamic diameters for *Bg* B-385 and *Bs* 1031 are estimated to be 0.68 μm and 0.50 μm, respectively.

Dried *Bt* spores expand by 4% under high humidity conditions and hydrated spores contract at low humidity,[13] so the size range will vary depending upon environmental conditions.[22] This type of size variation is also true for *Bg*, which was shown to shrink by as much as 12% from a hydrated state when air-dried,[13] and may also be true for *Ba*, because it is a close relative. However, there are no confirmation studies

currently in the literature addressing this property for *Ba*.

4.2.2 Surface Morphology

Numerous atomic force microscopy (AFM) image studies of several *Bacillus* species are available in the literature,[13,23-28] detailing three-dimensional views of the surface architecture of different *Bacillus* spore species, size distributions, changes in spore size due to different hydration states, and changes in ultrastructure due to different spore treatments or preparations. However, because spore surface properties are impacted by their preparation, previously published AFM studies may not be useful in planning large-scale field reaerosolization studies. New microscopy images (AFM or SEM) should be obtained as part of surrogate spore characterization prior to field testing.

4.2.2.1 Crystal-Producing *Bt* Strains

One factor not considered by Greenberg *et al.*[2] in their discussion of *Ba* surrogate selection is the production of crystal proteins in some *Bt* strains[33] that are not present in *Ba*. While it is possible these crystals adhere to *Bt* spores, impacting their aerodynamic and other properties, the effect of these crystals, shown in Figure 1, on reaerosolization is currently unknown because it this has not yet been studied. Accordingly, until more is known about the impact of the crystals on the movement or adhesion of *Bt* spores, acrystalliferous *Bt* strains should be favored as *Ba* surrogates.

4.2.3 Hydrophobicity

Hydrophobicity studies with hexadecane and other hydrophobic solvents, conducted to understand *Ba* and *Bt* spore behavior in the aqueous phase better, have shown that both *Bt* and *Ba* spores can bind to hydrophobic solvents.[29-31] However, the degree of hydrophobicity varies both between species and within strains.[29] While these results indicate that both *Ba* and *Bt* spores have potentially similar hydrophobic properties in aqueous media, whether this similarity is applicable to spore resuspension under ambient conditions is uncertain. Wshile these studies are not determinative for the degree of hydrophobicity of each species, they do indicate that both *Ba* and *Bt* have higher relative hydrophobicities than other *Bacillus* species. Hydrophobicity for *Bt* and *Ba* spores may be linked to the presence and makeup of their exosporia.[30,32]

4.2.4 Density

When prepared in the same manner, the wet spore density and volume of some *Bt* species are in the same range as some strains of *Ba*.[16,20] The average wet and dry densities for seven strains of *Ba* were determined to be 1.17 g/cm^3 and 1.42 g/cm^3, respectively.[16] In the same study, the average wet density of *Bt* was measured as 1.17 g/m^3 and the dry density was not determined.

4.3 Effects of Irradiation

Irradiation is one of several methods used to inactivate virulent *Ba* strains to prevent anthrax infection resulting from occupational exposure[34] and to sterilize contaminated samples and equipment from release sites.[35] The use of irradiated *Ba* surrogate spores has been suggested to eliminate concerns of pathogenicity, to facilitate approval for outdoor surrogate releases, and to better compare the behavior of inactivated *Ba* to inactivated surrogates in laboratory studies. For these reasons, the effects of irradiation on spore properties should be understood. A recent study[35] on the effects of electron beam irradiation (EBI) on *Bg* spores in solution revealed that EBI of up to 20 kGy (2 megarads) resulted in structural damage, DNA fragmentation, reduction in spore size, and other effects. The changes seen in the EB irradiated spores was dose-dependent, with increasing damage seen at higher doses.[35] While there are currently no published studies or images of the physical or structural effects of gamma

irradiation, there are some anecdotal observations in the literature from researchers studying other aspects of *Bacillus* spore irradiation as well as some unpublished data suggesting gamma irradiation can cause structural damage to spores similar to that described for EB irradiated spores.

A study by CDC authors[34] explored the gamma radiation dose needed to inactivate 0.1 mL aliquots from suspensions of live virulent spores of eight *Ba* strains, including Ames. These authors report a dose of 25 kGy (2.5 megarads) to achieve a 6 log reduction (99.9999%) of spores of a concentration of 10^7 CFU/mL across all strains tested. At 15 kGy (1.5 megarads), the authors reported >99% reduction in the suspension aliquots tested. The authors reported that under microscopic examination, irradiated spores "appeared irregularly shaped" and concluded that gamma irradiation "induces changes in structural components." Since this was not the focus of their study, no further details were provided. However, the authors also noted an increase in chromosomal DNA detection by real-time PCR, and hypothesize that as a result of spore structural damage caused by irradiation, DNA is more readily accessible in the suspension from damaged irradiated spores than from non-irradiated spores. These observations are consistent with the unpublished EPA research, discussed below, indicating that irradiation causes internal structural damage and the evacuation of the spore core. In another earlier study[36] that examined the reaction of irradiated and non-irradiated *Ba* Ames spores to monoclonal antibodies, the researchers noted structural damage to spores following irradiation at a dose of 30 kGy (3 megarads). No further information was given by the researchers.

4.3.1 Primary Data on Irradiation Effects

Analysis of DNA purification wash fractions from irradiated *Ba* S preparations by gel electrophoresis showed substantial amounts of DNA present. The irradiated *Bt* 9727 spores appeared more intact than the irradiated *Ba* S, with little to no DNA visualized on the gels from the washes. No amplification was seen on the irradiated spores, although the *Bt* primers should amplify both the *Bt* k and the *Bt* 9727. This lack of amplification could indicate that the quality of the extracted DNA was too poor to readily perform PCR on due to damage from irradiation or that the PCR reactions require further optimization.

Figure 2, below, shows SEM images of viable (non-irradiated) intact spores of *Bt k* and *Ba* S in liquid. In contrast, Figure 3 shows SEM images of irradiated *Ba* S and *Bt* k spores in liquid. A thick particulate layer in all of the irradiated samples was likely comprised of cell debris and expended media. All images in Figures 2 and 3 were taken of liquids under vacuum at a scale of 2.0 μm. Figure 4 contains SEM images of the irradiated spores following centrifugation-based washes. This figure demonstrates that damaged spores are still present after the wash steps and are, therefore, likely to comprise a large fraction of the preparation.

Table 4-1. Size Comparison of Hydrated *Bacillus* Spores (Carrera *et al.*; 2007)[16]

Type	Species	Size Comparison of Hydrated *Bacillus* spores (Carrera *et al.*; 2007)[16]							
		Hydrated Length (µm)				Hydrated Diameter (µm)			
		Mean (n=100)	±	Range		Mean (n=100)	±	Range	
				Min	Max			Min	Max
Pathogenic anthracis	*B. anthracis* 1087 (USAMRIID)	1.67	0.20	1.66	2.17	0.85	0.09	0.53	1.11
	B. anthracis 1029 (USAMRIID)	1.26	0.13	0.92	1.65	0.81	0.06	0.69	0.95
	B. anthracis Ames[a]	1.52	0.19	1.14	2.27	0.81	0.06	0.70	1.00
	B. anthracis LA1 (1088) (USAMRIID)	1.23	0.08	1.09	1.35	0.81	0.07	0.67	1.03
Attenuated anthracis	*B. anthracis* Sterne (ECBC)	1.49	0.17	1.09	2.13	0.85	0.08	0.66	1.09
	B. anthracis Δ-Sterne (ECBC)	1.55	0.15	1.23	2.05	0.86	0.07	0.67	1.06
	B. anthracis Pasteur 3132 (USAMRIID)	1.23	0.11	0.96	1.47	0.81	0.07	0.65	0.96
Surrogates	*B. atrophaeus* ATCC B-385	1.22	0.12	1.05	1.63	0.65	0.05	0.58	0.86
	B. subtilis 1031 (Source unknown)	1.07	0.09	0.89	1.52	0.48	0.03	0.41	0.67
Neighbors	*B. cereus* ATCC 10702	1.55	0.16	1.20	1.99	0.90	0.07	0.76	1.14
	B. thuringiensis 4055 (MGB, NCAUR)	1.61	0.18	1.07	1.99	0.80	0.07	0.59	0.96
	B. megaterium CDC 684	1.73	0.16	1.35	2.18	0.88	0.06	0.69	1.01
	B. stearothermophilus ATCC 12980	1.74	0.14	1.45	2.04	0.98	0.07	0.76	1.16
	B. sphaericus ATCC 4245	1.07	0.10	0.82	1.47	0.85	0.06	0.74	1.00

[a]Biological Defense Research Division, US Navy, Washington, D.C.

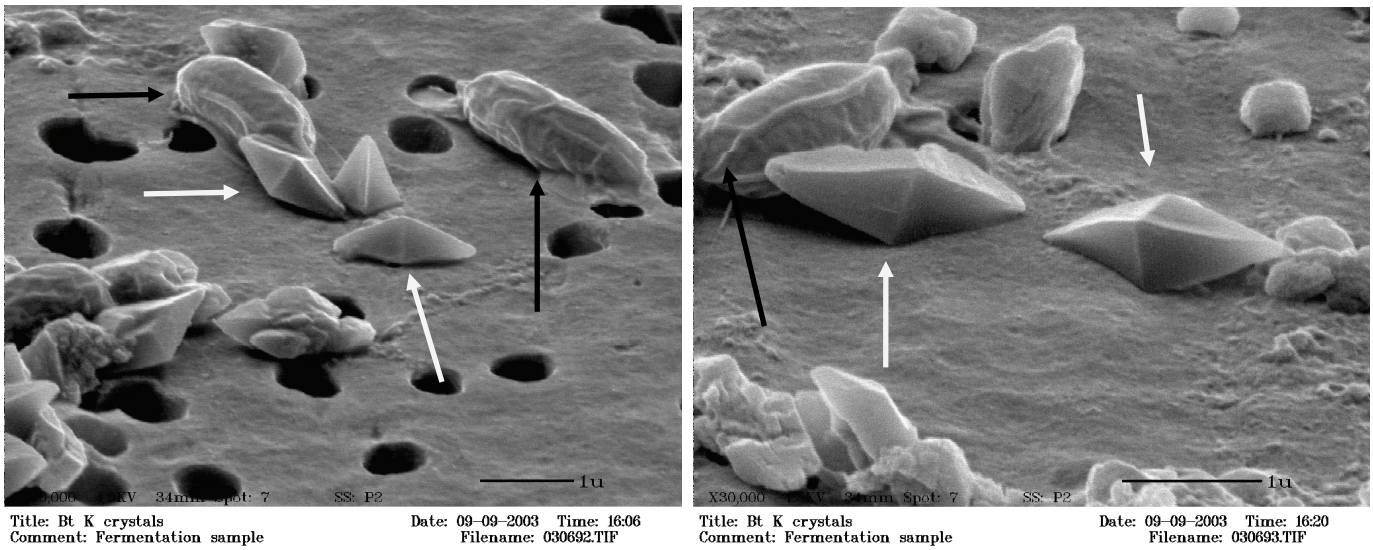

Figure 1. SEM images of *Bt* kurstaki strain spores and crystal proteins (spores indicated by black arrows, crystal proteins by white arrows)

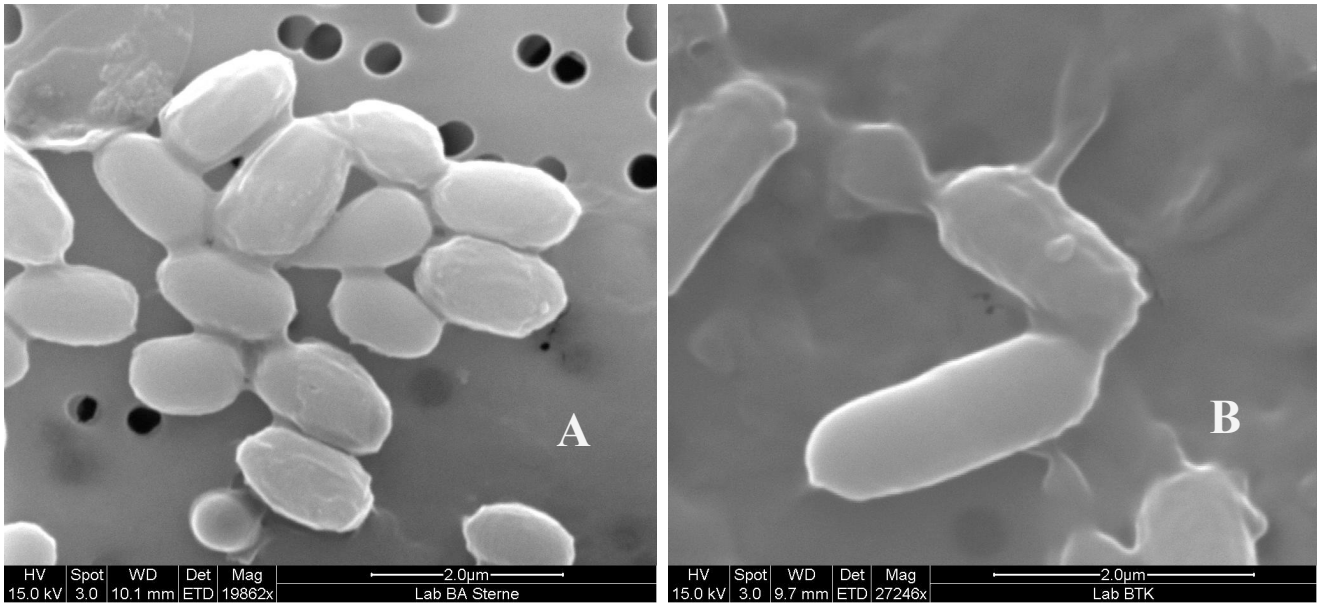

Figure 2. SEM images of viable (non-irradiated) *Ba* S (A) and *Bt* k (B) at 2 µm

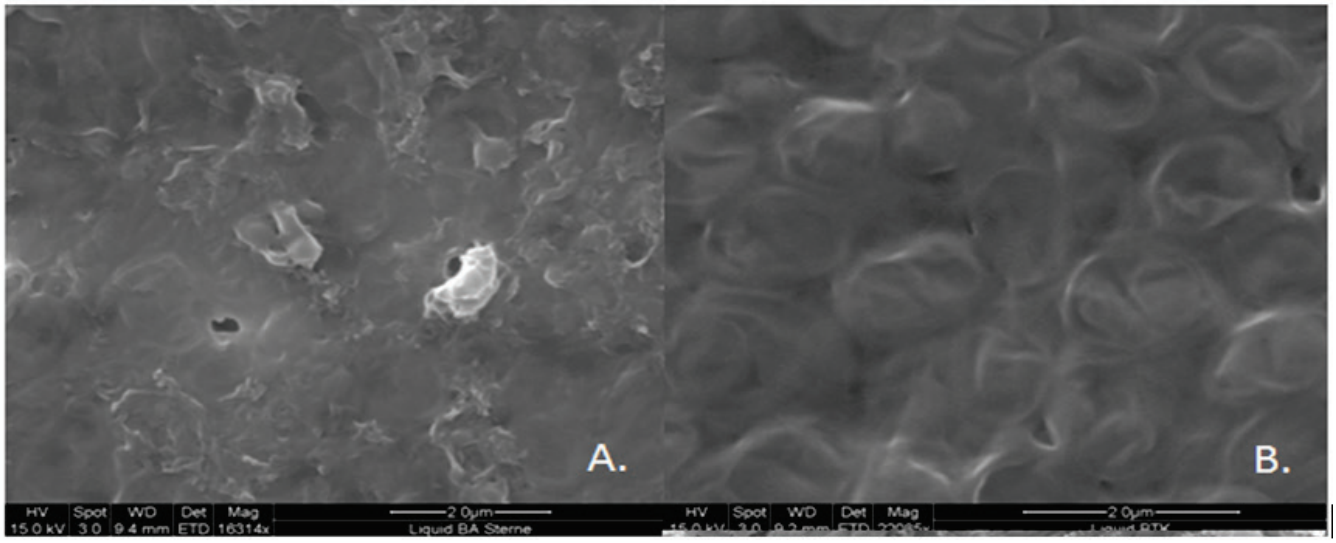

Figure 3. SEM images of irradiated *Ba* S (A) and *Bt* 9727 (B) (mislabeled as *Bt* k) at 2 µm

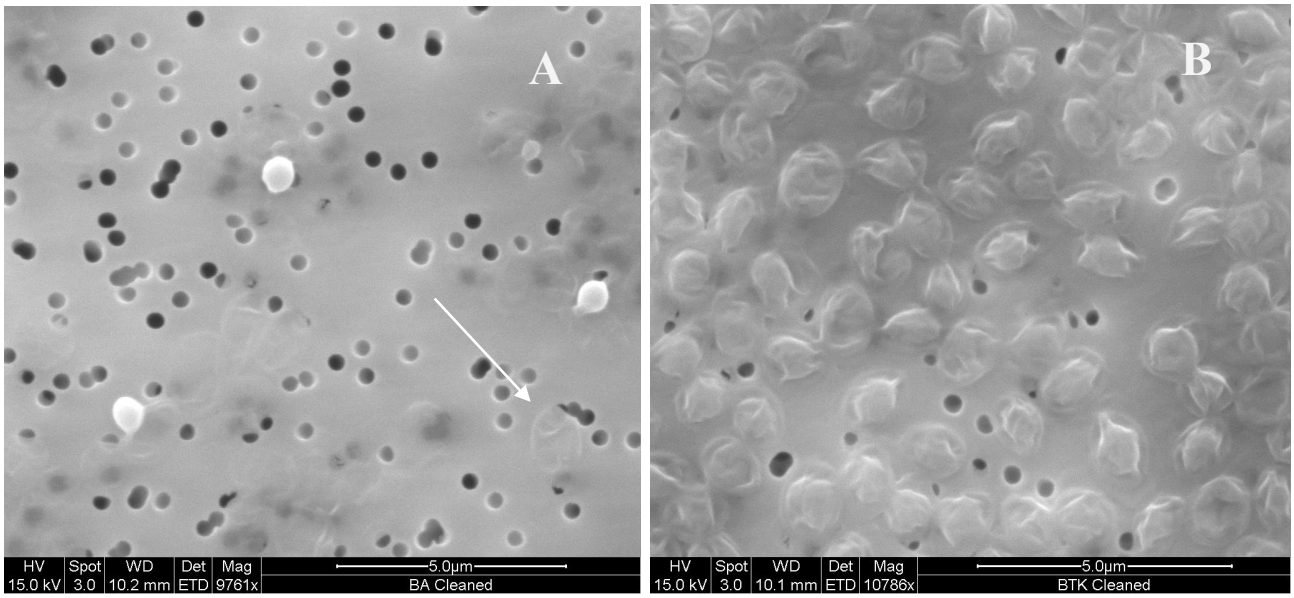

Figure 4. SEM images of washed irradiated liquid spore preparations for *Ba* S (A) and *Bt* 9727 (B) (mislabeled as *Bt* k). The white arrow in (A) points to a faintly evident collapsed spore.

5.0 Summary

The properties investigated in this effort included the physical properties that impact the movement of spores in air and water, including size, shape, density, surface morphology, structure and hydrophobicity. Also compared were pathogenicity, genetic relatedness, and the impact of irradiation on the physical properties of both spore species. Based on this review and comparisons of the physical properties of *Bt* and *Ba*, the use of *Bt* as a surrogate for *Ba* in aerosol testing appears to be well supported. Comparative tests should be carried out to test the hypothesis that the two species will behave similarly when suspended in a gas (as an aerosol).

While there are generally many features of *Bt* and *Ba* that are similar, spore size, morphology, and other physical properties are variable even between strains of the same species. The variations can be due to sporulation conditions, among other factors.[13,37,38] For this reason, one cannot conclude that all *Bt* spores are representative of all *Ba* spores. Prior to any experimentation, it is critical to characterize the surrogate to be used sufficiently and confirm that the characteristics of the surrogate are adequately similar to the characteristics of the live agent for the intended use.

Both anecdotal and direct evidence suggests that spore ultrastructure damage occurs as a result of irradiation. Evidence of spore structural damage caused by EB and gamma irradiation calls into question the use of irradiated spores for reaerosolization experiments as the noted structural changes have unknown effects on the aerosol properties of these particles. Based on the results of the EBI study[35] and the EPA comparison, irradiated spores do not appear to be a good surrogate for non-irradiated spores.

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