World J Microbiol Biotechnol (2014) 30:1453–1461
DOI 10.1007/s11274-013-1576-x

REVIEW

Bacillus thuringiensis as a surrogate for *Bacillus anthracis* in aerosol research

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Received: 16 September 2013 / Accepted: 3 December 2013 / Published online: 12 December 2013

Abstract Characterization of candidate surrogate spores prior to experimental use is critical to confirm that the surrogate characteristics are as closely similar as possible to those of the pathogenic agent of interest. This review compares the physical properties inherent to spores of *Bacillus anthracis* (*Ba*) and *Bacillus thuringiensis* (*Bt*) that impact their movement in air and interaction with surfaces, including size, shape, density, surface morphology, structure and hydrophobicity. Also evaluated is the impact of irradiation on the physical properties of both *Bacillus* species. Many physical features of *Bt* and *Ba* have been found to be similar and, while *Bt* is considered typically non-pathogenic, it is in the *B. cereus* group, as is *Ba*. When cultured and sporulated under similar conditions, both microorganisms share a similar cylindrical pellet shape, an aerodynamic diameter of approximately 1 μm (in the respirable size range), have an exosporium with a hairy nap, and have higher relative hydrophobicities than other *Bacillus* species. While spore size, morphology, and other physical properties can vary among strains of the same species, the variations can be due to growth/sporulation conditions and may, therefore, be controlled. Growth and sporulation conditions are likely among the most important factors that influence the representativeness of one species, or preparation, to another. All *Bt* spores may, therefore, not be representative of all *Ba* spores. Irradiated spores do not appear to be a good surrogate to predict the behavior of non-irradiated spores due to structural damage caused by the irradiation. While the use of *Bt* as a surrogate for *Ba* in aerosol testing appears to be well supported, this review does not attempt to narrow selection between *Bt* strains. Comparative studies should be performed to test the hypothesis that viable *Ba* and *Bt* spores will behave similarly when suspended in the air (as an aerosol) and to compare the known microscale characteristics versus the macroscale response.

Keywords Anthrax · Biological outdoor decontamination · Detection

Disclaimer 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 manuscript 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. Mention of trade names or commercial products does not constitute endorsement or recommendation for use of a specific product. This review was generated using some references (secondary data) that could not be evaluated for accuracy, precision, representativeness, completeness, or comparability and, therefore, no assurance can be made that the data extracted from these publications meet EPA's stringent Quality Assurance requirements.

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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 (Inglesby et al. 1999). The environmental and public health impacts of reaerosolization of *Ba* following an outdoor release in an urban environment are not well characterized, and 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.

Based on 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 (Greenberg et al. 2010). Although there may be some regulatory hurdles to overcome in the off-label use of a registered pesticide, *Bt* is currently used in wide area outdoor spraying as an insecticide (Van Cuyk et al. 2011) and, therefore, may have increased acceptance by the public for wide area releases. Additionally, the recent development of genetically tagged *Bt* kurstaki (Emanuel et al. 2012; Buckley et al. 2012) may facilitate its use in the field as it may allow the *Bt* under test to be differentiated from any *Bt* in the background. Another surrogate option is the use of inactivated *Ba* spores. Irradiation is one of several methods used to inactivate virulent *Ba* strains to prevent infection resulting from occupational exposure (Dauphin et al. 2008) and to sterilize contaminated samples and equipment from release sites (Fiester et al. 2012). 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.

This literature review, which builds on other published reviews, compares *Bt* and *Ba* spores in the context of physical attributes that may affect reaerosolization, discusses the suitability of *Bt* as a surrogate for *Ba* and factors that may impact use of *Bt* in field testing, and examines the impacts of irradiation on *Bacillus* spores and the resulting suitability of irradiated spores as a *Ba* surrogate. The key physical parameters compared are size, exosporium and spore coat 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.

Previous discussions in the literature regarding potential surrogates for *Ba* focused on narrowing the available choices by safety and genetic relatedness. This review provides more detailed information on the physical similarities between the two species specific to the use of *Bt* as a surrogate for *Ba* in aerosol testing, discusses the potential impact of sporulation and preparation conditions, and closely examines the surface morphology and characteristics of *B. cereus* (*Bc*) family spores as they apply to adhesion and resuspension.

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 (Van Cuyk et al. 2011). Like *Ba*, *Bt* is a gram-positive spore-forming *Bacillus* that is in the *B. cereus* group (Rasko et al. 2005). *Bt* is genetically similar to *Ba*, with *Bt* 9727, a strain that has shown wound infectivity and virulence in immunocompromised mice (Hernandez et al. 1998), demonstrating the highest homology (Radnedge et al. 2003). There have also been some isolated reports of infection caused by strains of *Bt* (Hernandez et al. 1998; Radnedge et al. 2003; Samples and Buettner 1983), and a recent mouse study indicating that repeated low-dose aerosol exposures can cause sub-chronic lung inflammation (Barfod et al. 2010). However, while exposure to *Bt* may cause skin and eye irritation, based on its use in the field and laboratory studies, *Bt* is typically considered a non-pathogenic Biosafety Level-1 (BSL-1) organism (Siegel 2001).

Bt and *Ba* spore physical properties

The key physical parameters considered critical in influencing the behavior of an aerosol, and how a particle interacts with a surface, are size, density, surface morphology, and hydrophobicity. 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 (Hinds 1999). Particle density is a key parameter because it impacts aerodynamic diameter, particle settling velocity and inertial properties (Hinds 1999). 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 (Hinds 1999). For example, surface roughness determines how many points of contact a particle makes with a surface, and particle elasticity plays a part in particle deformation onto a surface and an increase in the adhesive force (Hinds 1999). As with morphology, hydrophobicity influences particle–surface interactions, as has been demonstrated experimentally with hydrophobic *Bc* spores which adhere more strongly to hydrophobic than to hydrophilic surfaces (Rönner et al. 2008; Husmark and Rönner 1992).

Surface features such as morphology and hydrophobicity may help predict how strongly a spore will adhere to a particular surface type. However, after the first layer of spores has settled, the spore-on-spore adhesive properties may be stronger than the adhesive forces between the spores and the surface they initially landed on, causing spores to detach more easily as larger agglomerates than as

single particles from surfaces (Hinds 1999). Additionally, because of the increasing adhesive forces as particle size decreases, larger particles are more easily resuspended than smaller particles (Hinds 1999). For example, Hinds estimates the adhesive force on a 1 μm particle to be 10^{-7} N, and the adhesive force on a 10 μm particle to be a much larger 10^{-6} N. Resuspended single particles may also collide and stick together, causing reaerosolized particles to be larger than those initially released. This could have a significant impact on spore transport and residence time in the air since larger particles settle faster due to gravity but diffuse more slowly than smaller particles.

Size

Since size is the most important parameter predicting aerosol behavior, having similarly sized surrogates is of primary importance. Both *Bt* and *Ba* single spores are in the same respirable size range of about 1 μm and have similar aspect ratios and diameters (Plomp et al. 2005a; Zolock et al. 2006; Zandomeni et al. 2003; Carrera et al. 2007). In a recent study comparing the physical dimensions of seven hydrated strains of *Ba* spores to seven other strains of hydrated *Bacillus* spores (Carrera et al. 2007), *B. subtilis* (*Bs*) 1031 (source laboratory not given) and *B. atrophaeus* (formerly *B. globigii*, *Bg*) ATCC B-385 spores were found have smaller dimensions than all *Ba* strains compared, while *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 1.61 × 0.80 μm. The average length and width of the seven *Ba* strains measured were 1.42 × 0.83 μm, although there was variation between strains. There were more between-strain variations in the length of *Ba* spores (range of 1.23–1.67 μm) than in the diameters (range 0.81–0.86 μm).

In another study (Plomp et al. 2005a) 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 (Plomp et al. 2005a). The authors also noted differences of up to a factor of 2 in length and 1.5 in width between the smallest and largest spores measured in each population.

Both *Ba* and *Bt* have been described as oval (Giorno et al. 2007), cylindrical (Logan and Berkeley 1984) and ellipsoid (Carrera et al. 2007; Logan and Berkeley 1984) in shape, depending on the viewer. Carrera et al. (2007) 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 similar to a cylindrical pellet. 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 Eq. 1 (Branan 2005):

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

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

Using the physical dimensional measurements in Carrera et al. (2007) and the calculated average equivalent diameter of each spore species, the average aerodynamic size for the hydrated *Ba* species was calculated by Eq. 2 (Hinds 1999):

$$d_{ae} = d_e\sqrt{\frac{\rho_p C_{cde}}{\rho_0 \chi}} \quad (2)$$

where d_e is the equivalent diameter calculated above; ρ_p is the density of the particle as reported in Carrera et al. (2008); C_c is the Cunningham slip correction factor as calculated by equation 3.22 in Hinds (1999); ρ_0 is standard particle density (1 g/cm^3); and χ is the dynamic shape factor, as calculated by Sturm (2012) for the estimation of non-spherical particle transport in the human respiratory tract.

Using Eq. 1, d_e was calculated to be 1.01 and 1.03 μm for *Ba* and *Bt*, respectively. To calculate d_{ae} using Eq. 2, ρ_p values in the range of 1.16–1.19 g/cm^3 were used for *Ba*, and a ρ_p value of 1.03 g/cm^3 was used for *Bt*, resulting in an average aerodynamic size for the hydrated *Ba* spores of 0.95 μm (ranging between 0.90 and 1.01 μm) and 0.91 μm for *Bt* 4055. The estimated aerodynamic diameters for hydrated *Bg* B-385 and *Bs* 1031 are estimated to be 0.68 and 0.50 μm, respectively.

Dried *Bt* spores expand by 4 % under high humidity conditions and hydrated spores contract at low humidity (Plomp et al. 2005a), so the size range will vary depending upon environmental conditions (Westphal et al. 2003). Similar size variations occur in *Bg*, which was shown to shrink by as much as 12 % from a hydrated state when air-dried (Plomp et al. 2005a), and may also be true for *Ba*, because it is a close relative. However, there are no confirmatory studies currently in the literature addressing this property for *Ba*.

Surface morphology

Numerous imaging studies are available in the literature (Plomp et al. 2005a, b, c; Plomp and Malkin 2008; Plomp et al. 2007; Malkin et al. 2005), 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. In the *Bc* group, differences in adhesion have also been correlated to the presence (Faille et al. 2007) and number of appendages (Tauveron et al. 2006; Faille et al. 2010; Mercier-Bonin et al. 2011), exosporium length, hydrophobicity and zeta potential (Faille et al. 2010). Other published imaging studies investigated *Bc* group spore adhesion onto different surfaces and the relationship between surface morphology and adhesion. Faille et al. (2007) reported that damaged and missing *Bc* exosporium resulted in reduced adhesion to stainless steel; Lequette et al. (2011) found adhesion to stainless steel decreased with the removal of the hairy nap via *bc1A* deletion in *Bc* spores (reduced resistance to detachment); Chen et al. (2010) found that adhesion to silica sand increased in mutant spores of *Ba* (both for *bc1A* and *cotO* deletions); and Zolock et al. (2006) found adhesion to graphite to be stronger for *Ba* than *Bt* lacking exosporium due to filtration and the authors correlated this to differences in hydrophobicity in the spore coat.

However, because spore surface properties are impacted by their preparation, and the culturing and sporulation conditions are not always reported or the same between imaging studies, previously published atomic force microscopy (AFM) studies may not be useful in planning large-scale field reaerosolization studies. New microscopy images (AFM or scanning electron microscope (SEM)) should be obtained as part of surrogate spore characterization prior to field testing.

Hairy nap and surface proteins

An important glycoprotein unique to the *Bc* group is Bc1A, and is responsible for the production of the hairy nap (Sylvestre et al. 2002; Boydston et al. 2005; Lequette et al. 2011) located on the exosporium of spores from this *Bacillus* group. The glycoprotein hairy nap is attached to the crystal lattice basal layer (Kailas et al. 2011) of the exosporium. The nap length varies among *Bacillus* strains and between species (Sylvestre et al. 2002; Kailas et al. 2011; Faille et al. 2010). The hairy nap has been positively linked to adhesion (Lequette et al. 2011) and it has been shown that a lack of hairy nap reduces adhesion to stainless steel (Lequette et al. 2011).

Surface proteins have a significant impact on morphology (Mallozzi et al. 2008). The exosporium of *Bc* group spores have more than 20 proteins and glycoproteins (Fazzini et al. 2010). Many of these proteins are linked to exosporium and spore coat morphology (Mallozzi et al. 2008; Driks book chapt 2011). For example, Cotβ is found in the spore coat of *Ba* and deletion has been shown to smooth the surface and add a ridge along the spore axis (Mallozzi et al. 2008). Similarly, deletion of the exosporium protein ExsM from *Bc* spores yielded smaller, rounder spores, some with a double layer of exosporium, both having a hairy nap (Fazzini et al. 2010). It was suggested that this double layer may increase resistance to decontamination methods (Fazzini et al. 2010).

Spore coat

In the *Bc* family there are differences in spore coat morphology, however it is not currently known if these differences will result in different adhesive properties. For example, the spore coat of *Ba* has been shown to have bumps and ridges along the axis (Chada et al. 2003; Giorno et al. 2009), while *Bt* has a honeycomb structure (Sun et al. 2011). In another study of *Ba* and *Bt* where the authors found the surfaces to be very similar in appearance, the average surface grain diameter for *Ba* and *Bt* were 8.24 and 9.79 nm, respectively (Zolock et al. 2006), although the differences were not found to be statistically significant. The authors also reported comparable aspect ratios of both species studied: 2.49 for *Ba* and 2.53 for *Bt* (Zolock et al. 2006). These are larger than the aspect ratios reported by Carrera et al. however the spore preparations and strains were not the same between studies which may have had an impact on the aspects of the resulting spores.

Exosporium

The size of exosporium in *Bc* family spores vary between strains (Hachisuka et al. 1984; Tauveron et al. 2006; Faille et al. 2007, 2010), and even within the same batch of spores (Plomp et al. 2005b, Faille et al. 2010). Size variation can be the result of sporulation differences (Faille et al. 2007, 2010). In general, the exosporium of *Ba* is smaller than that of *Bc* and *Bt* (Hart et al. 2006). This could

impact the aerodynamic diameter of spores with intact exosporium and result in larger differences than are reported in the literature.

Exosporium fragility also differs between *Bc* strains (Tauveron et al. 2006), and a damaged or missing exosporium may decrease adhesion and/or increase resistance to removal from surfaces (Faille et al. 2007). Sheer stress damages and sometimes removes exosporium (Faille et al. 2007), and filtration can also remove the exosporium (Zolock et al. 2006; Chada et al. 2003). Damage to the exosporium from shear stress differed between strains of *Bc* in one study (Faille et al. 2007), and the authors hypothesized that the variance in damage was the result of altered chemical composition of basal layer or exosporium proteins resulting from different sporulation conditions (Faille et al. 2007). Spores produced under optimal conditions exhibited the least damage.

Appendages

Unlike *Bc* and *Bt*, which both have appendages whose numbers vary between strains (Faille et al. 2008, 2010), *Ba* has no appendages (Hachisuka and Kozuka 1981, Hachisuka et al. 1984; Faille et al. 2010). In a study with seven strains of *Bc* with the same sporulation and preparation conditions, the authors reported high variation in the length and number of appendages between strains with the number varying between 1 and 23 and length between 0.6 and 2 μm (Tauveron et al. 2006). While some authors report a link between appendages and adhesion (Mercier-Bonin et al. 2011; Faille et al. 2007, 2010) on a microscale, macroscale differences may be difficult to distinguish.

Hydrophobicity

The BATH (bacterial adhesion to hydrocarbons) test developed by Rosenberg et al. (1980) is used to assess hydrophobicity, whereby spores are suspended in saline solution to which hexadecane or other hydrophobic solvents are added. Following a period of vortexing, the degree of hydrophobicity is quantified by measuring the turbidity in the aqueous phase, where a decrease in turbidity indicates that hydrophobic spores are bound to the surface of solvent droplets in the solvent phase. Hydrophobicity studies using this method have shown that both *Bt* and *Ba* spores can bind to hydrophobic solvents (Doyle et al. 1984; Koshikawa et al. 1989; Leishman et al. 2010). However, the degree of hydrophobicity varies both between species and within strains (Doyle et al. 1984, Tauveron et al. 2006). While these results indicate that both *Ba* and *Bt* spores have potentially similar hydrophobic properties in aqueous media, spore-surface interaction under ambient conditions for spore resuspension requires further investigation. While 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 (Koshikawa et al. 1989; Brahmbhatt et al. 2007). Removal of the exosporium from *Bc* via shear stress increased spore hydrophobicity in one study (Faille et al. 2007), and in another study the authors demonstrated increased hydrophobicity in Δbc1A *Ba* strains heat treated during sporulation (Brahmbhatt et al. 2007). The same study reported decreased hydrophobicity in Δbc1A *Ba* over wild type (Brahmbhatt et al. 2007).

Density

When prepared in the same manner, the wet spore density (mass per unit volume) and volume of some *Bt* species are in the same range as some strains of *Ba* (Carrera et al. 2007; Carrera et al. 2008). To measure density, wet spores were suspended in water and dry, crushed spores were suspended in solvent, and both suspensions were compared to marker beads of known density, similarly suspended (Carrera et al. 2008). The average wet and dry densities for seven strains of *Ba* were determined to be 1.17 and 1.42 g/cm^3, respectively (Carrera et al. 2008). In the same study, the average wet density of *Bt* was measured as 1.17 g/cm^3 and the dry density was not determined.

Crystal-production

One factor not previously considered is the production of crystal proteins in some *Bt* strains (Roh et al. 2007) that are not produced in *Ba*. While most crystals produced by the various strains of *Bt* separate from spores following sporulation, there are a few strains where the crystal remains within the exosporium (Lopez-Meza and Ibarra 1996; Ji et al. 2009). For *Bt* kurstaki, the crystals disassociate from the spore. It is possible these crystals adhere to *Bt* spores, impacting their aerodynamic and other properties. However, the effect of these crystals on reaerosolization is currently unknown because 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.

Effects of irradiation

Several studies have reported *Bacillus* spore damage following irradiation (Dauphin et al. 2008; Fiester et al. 2012; Sun et al. 2011). Sun et al. (2011) reports an imaging study of dose-dependent damage and damage variation between several *Bt* strains studied. Sun et al (2013) reported that Bt insecticidal crystals appear unaffected by gamma irradiation at 20 and 60 kGY doses, and that spore and crystal damage appears to vary by strain and preparation. Another recent study (Fiester et al. 2012) 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 EBI spores was dose-dependent, with increasing damage seen at higher doses (Fiester et al. 2012). In addition to these studies, there are some anecdotal observations in the literature reported by researchers studying other aspects of *Bacillus* spore irradiation, suggesting gamma irradiation can cause structural damage to spores similar to that described by Sun et al. (2011) and Fiester et al. (2012).

Another recent study (Dauphin et al. 2008) 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 deoxyribonucleic acid (DNA) detected by real-time polymerase chain reaction (PCR), and hypothesize that as a result of spore structural damage caused by irradiation, DNA was more readily accessible in the suspension from damaged irradiated spores than from non-irradiated spores. In another earlier study (Phillips et al., 1988) 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).

Discussion

The properties considered in this literature review included the physical properties that impact the movement of spores in air and water, including size, shape, density, surface morphology, structure and hydrophobicity. Also examined was the impact of irradiation on the physical properties of both *Bacillus* species. Based on this review, previous reviews (Greenberg et al. 2010) 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. Both *Ba* and *Bt* spores are of similar aerodynamic size, aspect ratio, diameter, have an exosporium and a hairy nap, and have higher relative hydrophobicities and similar wet densities as other *Bacillus* species. All of these features may impact how the spores interact with the environment.

While it is possible that macroscale forces will dominate in large-scale reaerosolization and may overwhelm any microscale differences resulting from variation between spore species, comparative tests should be carried out to test the hypothesis that the two species will behave similarly when suspended in air (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 (Plomp et al. 2005a; Baweja et al. 2008; Setlow 2006). Differences in sporulation media can impact *Bc* family spore coat contents (Hirota et al. 2010), spectral signatures (Laflamme et al. 2011), spore size and exosporium fragility (Faille et al. 2007), altered sensitivity to denaturants and increased resistance to cleaning (Baweja et al. 2008). Temperature and RH differences during sporulation may alter resistance to wet heat and to HCl, NaOH and H_2O_2 (Baweja et al. 2008), and variations in sporulation temperature and pH may also impact spore size, although temperature effects may greater (Baweja et al. 2008).

Since size is the most important parameter predicting particle behavior, having similarly sized surrogates is of primary importance. *Ba* and *Bt* spores, when prepared in the same manner, appear to be very close in aerodynamic diameter. Much of the literature on sizing appears to not take into account the exosporium, which could impact the aerodynamic diameters. Since exosporium size can vary within species (being species or sporulation condition specific) and between species (*Ba* has a smaller exosporium than *Bt*), this may impact the movement of the spore as an aerosol.

Preparation following sporulation is another consideration. For example, filtration may damage the exosporium (Zolock et al. 2006; Faille et al. 2007) and alter spore adhesion characteristics (Faille et al. 2007; Lequette et al. 2011). Spores with an intact exosporium from the same lot as those with missing exosporium (from filtering or other shear stress) would have different aerodynamic diameters, because the aspect ratio of a spore with an intact exosporium would be greater than that of a spore with no

exosporium, resulting in differences in particle behavior and adhesion.

For these reasons, 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 EBI 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. While the best option for a *Ba* spore surrogate would be the use of inactivated *Ba* spores, irradiated spores do not appear to be a good surrogate for non-irradiated spores. Future research to test the hypothesis that *Bt* is a good surrogate for *Ba* in aerosol testing should include correlating differences in surface morphology to observable macro scale detachment behavior under different conditions; the impact of spore hydration state on hydrophobicity and the impact to d_{ae} on the presence or absence of exosporium.

Acknowledgments This project was supported in part by an appointment to the Research Participation Program at the National Homeland Security Research Center, U.S. Environmental Protection Agency, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and EPA. Additionally, the authors would like to thank Gene Rice (U.S. Environmental Protection Agency, Cincinnati, Ohio), Vipin Rastogi (US Army – ECBC, APG, MD) and Timothy Dean (U.S. Environmental Protection Agency, Research Triangle Park, NC) for their critical review of this manuscript.

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