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ORIGINAL ARTICLE

Correlations between available primary amines, endospore coat thickness, and alkaline glutaraldehyde sensitivity for spores of select *Bacillus* species

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Abstract

Alkaline glutaraldehyde (GTA) is a high-level chemical disinfectant/sterilant and has a broad microbial kill spectrum. The precise antimicrobial mechanism of GTA remains debated. GTA kill times are extremely variable across different organisms, illustrating the need for a better understanding of GTA kill mechanisms related to different organisms. A commonly proposed GTA kill mechanism suggests that it works by cross-linking accessible primary amines on important surface proteins. If true, the antimicrobial activity of GTA may directly correlate to the number of these available functional groups. *Bacillus* species form highly resistant bacterial endospores that are commonly used as one of the most stringent test organisms for disinfection and sterilization. In this study, we compared the log reduction times of alkaline GTA on spores from 4 *Bacillus* species to fluorescent profiles generated using Alexa Fluor™ amine-reactive dyes. GTA kill times were also compared to mean spore coat thicknesses as measured with scanning electron microscopy (SEM). Fluorescence values generated from bound amine-reactive dye showed a strong, positive correlation to GTA susceptibility, as measured by GTA 6-$\log_{10}$ reduction times. Spore coat thickness also showed a strong, positive correlation to reduction time values. Results support the hypothesis that GTA kill times are directly related to the number of available primary amines on bacterial endospores. Results also indicated that the killing efficacy of GTA may be influenced by its ability to penetrate the spore coat to reach additional targets, suggesting that damaging important biomolecules beyond surface proteins may be involved in GTA killing mechanisms.

KEYWORDS
Alexa Fluor, disinfection, endospore, glutaraldehyde, spore coat, thickness

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1 | INTRODUCTION

1.1 | Background

Disinfection of surfaces and materials is a subject that has been studied extensively to provide safe and effective inactivation of infectious organisms on everything from children's toys to precision surgical instruments. Proper understanding, use, and development of disinfectants are an essential aspect of decreasing the costs and complications associated with modern health care and curbing the spread and development of resistant microbes that pose an increasingly problematic threat to public health. Bacterial endospores have received particular attention due to their notorious stability, resistance to chemical germicides, and their clinical significance (Christensen et al., 1989; March et al., 2012). Because of this, sporicidal activity is an important aspect of liquid sterilization claims, and disinfection research generally. An enhanced understanding of the modus operandi of various disinfectants is essential to improving our ability to safely and consistently evaluate their efficacy and range, and to developing safer, more effective, and more economical disinfectants and disinfection protocols.

Endospores are structures that can be formed by some species of bacteria (most notably those in the genera *Bacillus* and *Clostridium*), typically in response to nutrient deprivation and environmental insult. The spore forms within the bacterial cell wall and is a highly resistant protective measure that is capable of germinating, upon the return of favorable conditions, to an active vegetative form. Endospores are comprised of layers known as (in order of increasing depth) the exosporium, spore coat, spore cortex, and germ cell wall. The core underlies the germ cell wall and contains protected DNA molecules and metabolically inactive proteins which are protected by the other layers of the spore. The spore coat has been most notably implicated in the protection of the spore and has been compared with a molecular sieve that excludes hazardous compounds (Driks & Eichenberger, 2016; Gerhardt, Scherrer, & Black, 1961; Ozin, Henriques, Yi, & Moran, 2000).

Glutaraldehyde (GTA) is a 5-carbon dialdehyde (1,5-pentanedial) commonly used as a cross-linking agent and is one of the most widely used liquid sterilants for reusable medical instruments such as endoscopes and bronchoscopes (Babb & Bradley, 1995). GTA's reactivity with the side chains of amino acids and proteins under various conditions has been studied in depth (Bowes & Cater, 1968; Quiocho & Richards, 1966).

Its principal target is primary amines, and it has been shown to interact only minimally with other functional groups on proteins (Habeeb & Hiramoto, 1968; Migneault, Dartiguenave, Bertrand, & Waldron, 2004; Russell & Hopwood, 1976). Of all these groups, previous studies suggest that the ε-amine group of lysine is most likely the main functional group involved in protein cross-linking (Migneault et al., 2004; Weetall, 1974). The interaction of GTA with other vital biomolecules, such as carbohydrates, lipids, and nucleic acids, has not received much attention, though the available data does not suggest these biomolecules to be significant targets for GTA disinfection (Adami & Rice, 1999; Hopwood, 1975).

N-hydroxysuccinimide(NHS)estersand2,4,5,6-Tetrafluorophenyl (TFP) esters are used commonly in the bioconjugation of molecular probes to proteins via acylation of ε-amino groups on lysine residues. Alexa Fluor™ dyes are a series of fluorophores developed by Molecular Probes, Inc., and a wide variety are currently sold as bioconjugates. They are commonly employed in immunology research to stain cells using antibody-dye conjugates, but they can be attached to other biomolecules as well (Disney, Zheng, Swager, & Seeberger, 2004; Friedman & Perrimon, 2006; Lakadamyali, Babcock, Bates, Zhuang, & Lichtman, 2012). Alexa Fluor dyes have also been used to non-specifically label surface protein on various organisms (Stojkovic et al., 2008; Zhang, Tan, Hanson, & Ooi, 2010; Zhang, Tan, & Ooi, 2011).

For bacterial endospores, it is commonly believed that GTA kills by cross-linking proteins important in spore germination. However, the exact location where GTA reacts and its exact antimicrobial mechanisms are still not well characterized (Tennen, Setlow, Davis, Loshon, & Setlow, 2000). Additionally, although GTA is a commonly used primary amine cross-linking agent and a high-level disinfectant, it has yet to be broadly demonstrated that its disinfection mechanism/antimicrobial kill efficacy is directly related to the number of primary amine functional groups readily accessible on a spore.

1.2 | Purpose of this study

The major goal of this study was to investigate the relationship between the resistance of endospores to GTA and the prevalence of accessible primary amines on or near their surfaces. We also sought to better understand where these targets might be located. Because GTA has been shown to preferentially react with lysine residues on exposed proteins, it should be possible to quantify the approximate number of amines available to GTA using amine-reactive dyes and labels. The number of these sites could be quantified on bacterial endospores using the mean fluorescence generated by the amine-reactive Alexa Fluor 488 TFP ester dye. Because the spore coat is an important outer protective layer, we also sought to determine whether the thickness of spore coats in different species correlated with their respective kill kinetics.

To accomplish these goals, we first determined the fluorescent profiles of various spore species from the genus *Bacillus* stained with AlexaFluor™ amine-reactive dyes and then compared them to the 6-$\log_{10}$ kill times using GTA as a disinfectant. Quenching assays were also used to verify their shared binding sites. Spore coat thickness of various spores was measured using TEM and also compared with their 6-$\log_{10}$ kill times.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and spore production

Bacillus anthracis Sterne 1043, *Bacillus subtilis* ATCC 19659, and *Bacillus atrophaeus* ATCC 51189 were maintained on Columbia agar plates (BD Diagnostic Systems) and grown at 37°C. Spore suspensions were prepared using the method of Leighton and Doi (1971). Spore stock solutions were quantified by serial dilutions and viable plating. Stocks typically yielded between 1 × 10^8–1 × 109 CFU/ml. Suspensions were stored at 4°C until use. *Geobacillus stearothermophilus* ATCC 12980 was maintained on Columbia agar. Spore suspensions were created by growing lawns of *G. stearothermophilus* on nutrient agar supplemented with Mg^{+2}, Mn^{+2}, Ca^{+2}, K^{+}, and Fe^{+2} ions at 55°C for 8–10 days until sporulation was greater than 90%. Spores were harvested by adding cold PSS +0.01% Tween 80 (PSST) to plates, scraping off the spores, and centrifugation at 4000 × *g* for 15 min to pellet the spores. Washing by centrifugation and resuspension in PSST was repeated 5 times, and suspensions were checked for purity via phase-contrast microscopy before storage at 4°C until use.

2.2 | Spore labeling and flow cytometry

Spores were labeled using a modified protocol from Boyana et al. Suspensions were adjusted to 1 × 107 CFU/ml, then diluted 1:10 in PSST containing 0.1 M sodium bicarbonate, pH 8.3 (wash buffer) to give approximately 106 CFU/ml. Desiccated Alexa Fluor™ 488 TFP-ester (AF488) dye was re-suspended in sterile DMSO to give a final concentration of 1 µg/µl. Fifteen microliters of dye was added to 1 ml of each spore suspension, followed by incubation in the dark for at least 2 hr at room temperature. Unbound dye was removed by centrifugation at 12,000 × *g* for 30 min and washing the spores 3 times in 1 ml of wash buffer. Prepared samples were kept at room temperature in the dark until assayed in a Blue/Red Applied Biosystems Attune Flow Cytometer (Hopwood, 1975). As spore preparations were all greater than 90% purity, spore gates were made around populations that showed relatively high side scatter and low forward scatter, specific to spore complexity and size, respectively, to reduce noise from other particulates and contaminants. Unstained spores were used as standards for gating stained spore populations. Runs were done in triplicate, and each experiment was performed 6 times. Raw data were processed using FlowJo™ software where fluorescent means were obtained by gating on spores, then sub-gating intensities to exclude extreme outliers and unstained spores.

2.3 | Sporicidal suspension tests

All spore suspensions were tested against GTA using the ASTM Standard Guide for Assessment of Antimicrobial Activity using a Time-Kill Procedure, E2315-03 (ASTM E2315). One hundred microliters of a 109 CFU/ml spore suspension was added to 9.9 ml of GTA and incubated at 20°C for predetermined contact times. At a given contact time, 1 ml of this GTA-spore suspension was added to 9 ml of 1% glycine for at least 5 min, before serially diluting the suspension further to predetermined dilutions. One ml from these dilutions was then plated in triplicate onto Columbia agar using membrane filtration, and plates were incubated at 37°C (55°C for *Geobacillus* spores). Colonies on each membrane were counted at 24 and 48 hr. The average number of CFU per plate was used to determine the log_{10} reduction in spores for each contact time. Tests were replicated 3 times, using 4 contact times per test. Time-Kill regression curves and 6-log_{10} reduction estimates were generated using Minitab™ software and Microsoft Excel from contact times and their associated log_{10} reductions.

2.4 | Glutaraldehyde

CIDEX™ (2.4% alkaline glutaraldehyde) was purchased from Advanced Sterilization Products™ and was activated just before use in disinfection tests.

2.5 | Fluorescence-quenching assay

Fluorescence-quenching assays were performed to verify the shared binding sites of GTA and AF488 dye. To test GTA's ability to inhibit AF488 binding to bacterial spores, stock spore suspensions were diluted to approximately 107 CFU/ml and 100 µl of this stock was added to 900 µl of GTA. Spores of *B. anthracis* were incubated for 5, 10, 15, and 30 min before 1 ml of 6% glycine was added to neutralize the reaction. *B. subtilis* spores were incubated for 45, 90, 130, and 150-min contact times. The spores were then washed 3 times by centrifugation at 12,000 × *g* for 30 min, and re-suspending in wash buffer to remove the glycine-GTA solution. Ten microgram of AF488 was added to each tube and these suspensions were incubated at room temperature in the dark for at least 2 hr. Once staining was complete, the suspensions were washed 3 times as described above and analyzed by flow cytometry. Generated intensities were analyzed, and mean fluorescence values were measured in triplicate. Each spore species was tested 3 times.

2.6 | Spore coat measurement

Endospores were prepared for electron microscopy by fixing with 2% glutaraldehyde overnight. Spores were washed and then stained with OsO_4 for 3 hr. After staining, spores were embedded in 2% LMP agar and dehydrated using an acetone series. Spore-agar pellets were then soaked in 50% acetone /50% resin of Spurr

for 4 hr, twice, and then 100% resin overnight. Spore pellet-resins were then cured overnight at 70°C. Thin sections were cut to a gold color and stained with 2% uranyl acetate and lead citrate. Thin sections were then attached to copper grids with formvar-carbon supports and imaged in an FEI Helios Nanolab 600 by SEM. Measured spores were selected at random from 3 different slides per sample, using 2 samples. Spores were excluded from measurement based on deformity, lack of morphology consistent with the species being measured, and having been cut obliquely or near the end of a spore. Measurements included both the inner and outer spore coats, and excluded exosporia or spore crusts where applicable. Each spore was measured in 6 different locations along axes approximately 60° apart, and measurements were averaged to create an average coat thickness value. Measurements were started perpendicular from the outer edge of the spore, indicated by the dark, electron deflecting region, and measured inwards until the cortex was reached, indicated by a large electron penetrating region before the core.

2.7 | Statistical analysis

Statistical differences between spores were first determined using single-factor ANOVA. Tukey multiple comparison procedures were then performed to compare spore coat thicknesses and fluorescence values. Two-sample *t* tests, assuming equal or unequal variances, were then used to determine if the differences between individual spore species were statistically significant. Regression lines were calculated to compare log reduction times with fluorescence and spore coat thickness values. Standard errors were calculated throughout and ±1 standard error was used uniformly as a measure of uncertainty. Analyses were conducted using Minitab™ software and Microsoft Excel.

3 | RESULTS

3.1 | Maximum fluorescence of spores with AF488

To determine the concentration of AF488 necessary to achieve maximum fluorescence (by occupying a maximal number of binding sites available on the spores), 106 CFU of *B. anthracis* and *B. subtilis* spores (selected as representative of both extremes of the data set as determined by preliminary data) were incubated with 0.001, 0.01, 0.1, 1, 10, 20, and 50 μl of AF488 (1 μg/μl) for at least 2 hr. ANOVA with Tukey HSD procedures was used to determine statistical differences between mean fluorescence values. Incubation for longer than 2 hr made no significant difference in mean fluorescence. Figure 1 shows the fluorescence intensities generated from this experiment. Increasing the amount of dye from 20 to 50 μg did not result in a significant increase in mean fluorescence for either spore type. However, increasing dye concentration from 10 to 20 μg in *B. anthracis* and *B. subtilis* did increase the mean fluorescence significantly. Overall, it was determined that greater than 10 μg of AF488 incubated for at least 2 hr was required to achieve maximum fluorescence on *B. anthracis* and *B. subtilis* spores.

3.2 | Fluorescent profiles of spores generated by bound dye

Staining *Bacillus* spores with AF488 yielded higher fluorescent intensity values relative to controls. The mean fluorescent intensity for each *Bacillus* spore type was calculated and is shown in Figure 2. *B. anthracis* displayed the greatest fluorescent intensity while *B. subtilis* displayed the least. Statistical analyses showed that the mean fluorescence of *B. anthracis* spores was significantly higher than that of the other spore species. Mean

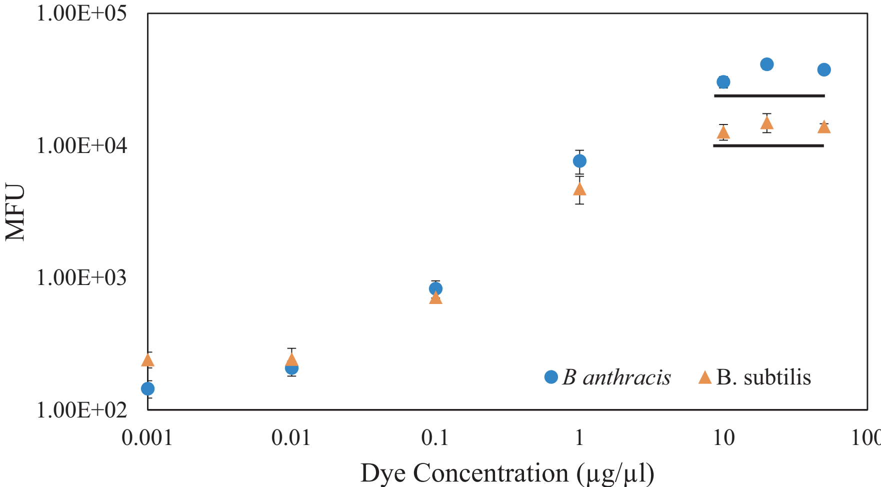

FIGURE 1 Mean fluorescence units (MFU) of *Bacillus anthracis* and *B. subtilis* spores after staining with different concentrations of Alexa Fluor™ 488 TFP dye. Blue circles indicate MFU for spores from *B. anthracis*, while orange triangles indicate MFU for *B. subtilis*. Each symbol represents the mean of 3 replications. Error bars represent ±1 standard error. Horizontal lines connect means from the same species with no statistical difference at a 95% confidence level

fluorescence values for *G. stearothermophilus* and *B. atrophaeus* were not statistically different from each other and mean fluorescence for *B. atrophaeus* and *B. subtilis* were not significantly different from each other; however, mean fluorescence values for *G. stearothermophilus* and *B. subtilis* were significantly different from each other.

3.3 | 6-Log$_{10}$ reduction times for different batches of *Bacillus* spores

Six-log$_{10}$ reduction times were estimated by generating regression kill lines for spore suspensions of 4 *Bacillus* species against GTA, using viable plating by membrane filtration. Two batches of each species were produced, and for each batch, each dilution was plated in triplicate and each test was repeated 3 times. Figure 3 indicates the wide range of GTA 6-log$_{10}$ reduction times observed for the tested spores, from a minimum of approximately 7 min up to a maximum of nearly 2.5 hr. Differences in 6-log$_{10}$ reduction times were significantly different at a 95% confidence level for all species except for between *G. stearothermophilus* and *B. atrophaeus*.

3.4 | Correlation between estimated 6-log$_{10}$ kill times and mean fluorescence intensities

Mean fluorescence intensity data were compared with data from Time-Kill assays for spores from each species to investigate the possible correlation between GTA kill times and bound-AF488-generated fluorescence. Figure 4 shows that as fluorescence decreases, kill time increases (R^2 = 0.78, typical values for statistically positive "strong" correlations range from 0.5 to 1.0).

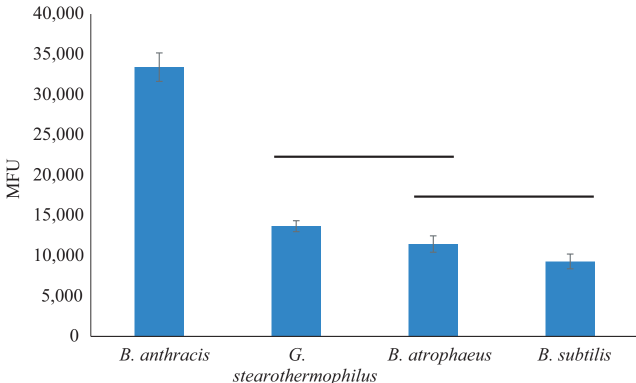

FIGURE 2 Fluorescence profiles of spores from various *Bacillus* species. Each column indicates the mean fluorescence units (MFU) generated by each spore species. Each bar represents the mean of 3 replications. Error bars represent ±1 standard error. Horizontal lines connect means that are not statistically different from each other at a 95% confidence level

3.5 | Inhibition of AF488 binding by prior treatment of spores with GTA

The ability of GTA to block AF488 from binding to spores was tested to determine the similarity of reactive sites used by GTA and AF488. Figures 5 and 6 show the mean fluorescence values generated by *B. anthracis* and *B. subtilis* spores (selected as extremes of the fluorescence data set) after treatment with GTA for various contact times. Relative to the untreated control, there was a 1.19 log$_{10}$ (~94%) drop in fluorescence for *B. anthracis* after a 5-min treatment with GTA. Fluorescence did not decrease significantly with longer contact times. The same trend was observed with *B. subtilis*: After the initial contact time, mean fluorescence did not significantly decrease beyond the 0.85 log$_{10}$ (~86%) loss of fluorescence relative to unstained spore controls.

3.6 | Spore coat thickness

The coat thickness of the spores used in this study was measured by electron microscopy. Figure 7 shows electron micrographs of spore sections for each species. Figure 8 shows comparisons of the thicknesses of spore coats from *Bacillus* species. Coat thickness was measured at six points on 12 spores from thin-section electron micrographs using ImageJ™ imaging software. Mean coat thicknesses were then compared using ANOVA with Tukey HSD. *B. anthracis* had statistically thinner spore coats ($p < 0.05$) than the other tested spores, while *B. subtilis* had the thickest coat. Coat thicknesses were slightly different between *B. atrophaeus*, *G. stearothermophilus*, and *B. subtilis*, although the differences were statistically insignificant ($p > 0.05$).

3.7 | Correlation between spore coat thickness and 6-log$_{10}$ reduction estimates

Figure 9 shows the relationship between spore coat thickness and 6-log$_{10}$ Reduction time for each spore species. As spore coat thickness increased, kill time also increased (R^2 = 0.89), a correlation even stronger than that of reduction times and fluorescence.

4 | DISCUSSION

A comparison of the 6-log$_{10}$ reduction time values for selected *Bacillus* spores confirms that not all spores share the same sensitivity to GTA (March et al., 2012). Mild variations existed in kill times between different batches of identical spore species, but these were not statistically significant. These data are consistent with previous observations seen with *Mycobacterium bovis* and GTA (Robison et al., 1996).

The differences in spore susceptibilities seen in Figure 3 suggest a continuum of spore GTA susceptibility ranging from high

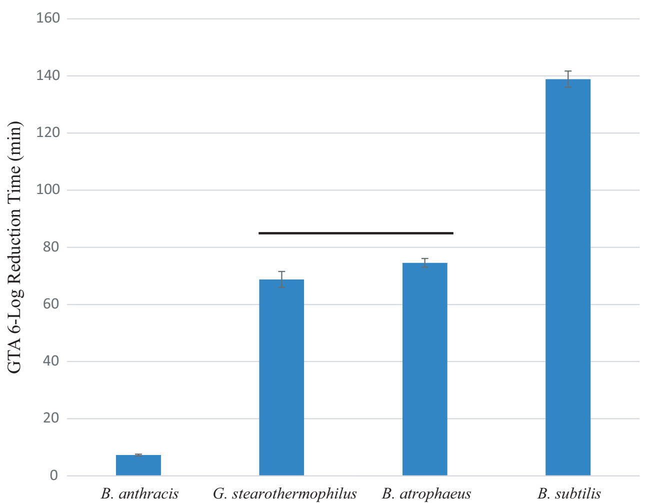

FIGURE 3 Comparison of estimated glutaraldehyde 6-$\log_{10}$ reduction times for spores from 4 different species. Horizontal lines connect means that are not statistically different at the 95% confidence level. Each column represents the mean of 6 replications. Error bars represent ±1 standard error

(*B. anthracis*) to low (*B. subtilis*), with statistically significant differences at the extremes. It has been well-established that there are substantial differences in GTA kill times between *B. anthracis* and *B. subtilis* spores (Gorman, Scott, & Hutchinson, 1984; Rubbo, Gardner, & Webb, 1967). Several studies have additionally shown that *G. stearothermophilus* and *B. atrophaeus* have similar kill times to those presented in this paper (using various methods), although this is the first time all 4 of these spores have been tested identically in the same study (Block, 2000).

The elevated mean fluorescence generated by *B. anthracis* spores seen in Figure 2 strongly suggests a relative abundance of available primary amines, while *G. stearothermophilus* and *B. atrophaeus* had fewer, and *B. subtilis* the least, with statistically significant differences between *B. subtilis* and *G. stearothermophilus* but not between *B. atrophaeus* and either *B. subtilis* or *G. stearothermophilus*. Because it is known that both GTA and AF488 bind to primary amines, and considering the results of the spore fluorescence-quenching assay (Figures 5 and 6), it is probable that increased levels of fluorescence generally correspond with increased susceptibility to GTA (Bowes & Cater, 1968; Chisalita et al., 1971; Quiocho & Richards, 1966).

The ability of GTA to block AF488 binding is consistent with what is known about how AF488 and GTA bind similar functional groups (Bantan-Polak, Kassai, & Grant, 2001; Bowes & Cater, 1968; Chisalita et al., 1971; Haugland, 1996; Quiocho & Richards, 1966). GTA was able to block fluorescence from AF488 labeling relative to a control, which suggests that AF488 binds to identical or comparable sites in relation to GTA. Some residual fluorescence (Figures 5 and 6) may indicate fluorophore that was also able to enter but not exit the spore, or fluorophore that was able to bind other functional groups that are present on, or near the surface of these spores (Molecular Probes, 2012; Okuda, Urabe, Yamada, & Okada, 1991).

Considering that the differences in mean fluorescence in *B. subtilis* with respect to *B. atrophaeus* and *G. stearothermophilus* spores were not as pronounced as the differences in kill times for *B. subtilis* with respect to *B. atrophaeus* and *G. stearothermophilus*, it is likely that in more highly resistant spores, other unknown mediating factors are contributing to GTA resistance. Nevertheless, when mean fluorescence was plotted against 6-$\log_{10}$ reduction times (Figure 4), a strong linear relationship between fluorescence and kill time was shown.

Spore kill times increased with increasing spore coat thickness. This is consistent with other studies that have also noted the differences in spore coat thickness between *B. subtilis* and

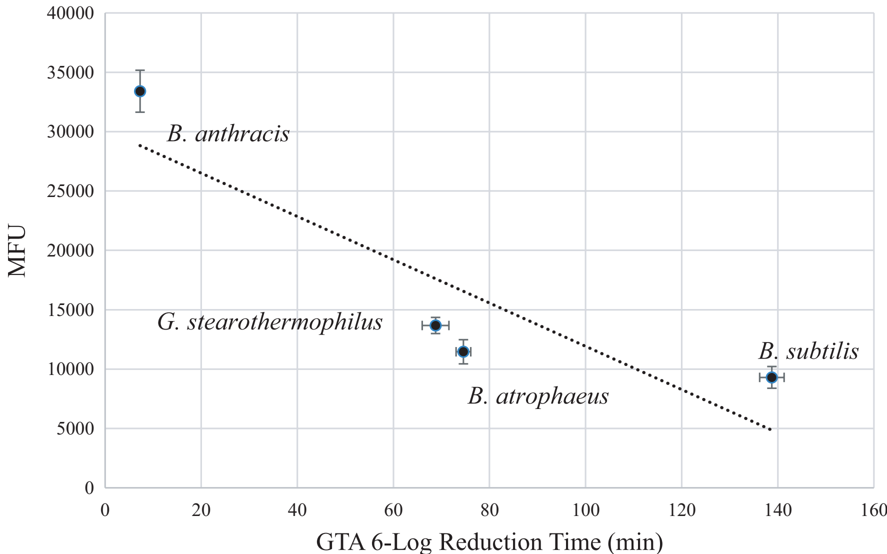

FIGURE 4 Mean fluorescence versus 6-$\log_{10}$ reduction time estimates for spores from various species. Each circle represents the mean of 6 reduction time replications and 3 fluorescence replications. Error bars represent ±1 standard error. (R^2 = 0.78)

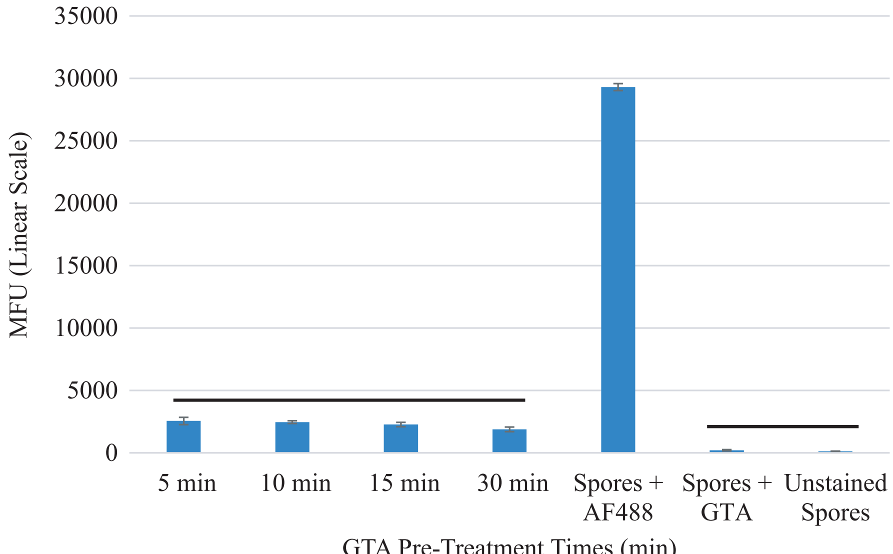

FIGURE 5 Reduction of *Bacillus anthracis* spore fluorescence when pretreated with GTA for 5–30 min before staining with AF488. Spores +AF488 were not pretreated with GTA. Spores +GTA were not subsequently stained with AF488. Unstained, untreated spores were included as a control. Horizontal lines connect means that are not statistically different from each other at a 95% confidence level. Each bar represents the mean of 3 replications. Error bars represent ±1 standard error

B. anthracis and suggested it as a contributing factor in *B. subtilis*' remarkable resistance to GTA, as well as many other disinfectants (Driks, 2002; Henriques, Moran, & Charles, 2007; Jenkinson, Sawyer, & Mandelstam, 1981; Lai et al., 2003; Moir, 1981). Together with the fluorescence data, this would suggest that increasing spore coat thickness is associated with a decrease in the number of primary amine targets available for GTA binding, or at least that it significantly increases the amount of time required for GTA to penetrate the spore coat and reach its lethal targets.

5 | CONCLUSION

Alkaline glutaraldehyde disinfection kinetics have a strong positive correlation to the number of available primary amines on bacterial endospores, suggesting that primary amine binding plays an important role in GTA disinfection mechanisms. Increasing spore coat thickness shows a strong positive correlation to spore resistance to GTA, which when coupled with fluorescence data, suggests that increasing spore coat thickness decreases the number of primary amine targets available for successful GTA disinfection and

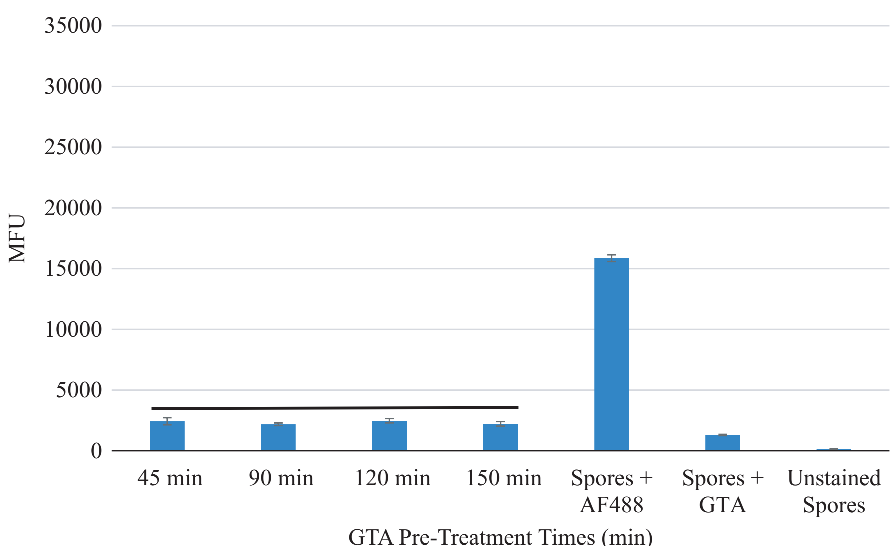

FIGURE 6 Reduction in *Bacillus subtilis* spore fluorescence when pretreated with GTA for 45–150 min before staining with AF488. Spores +AF488 were not pretreated with GTA. Spores +GTA were not subsequently stained with AF488. Unstained spores were included as a control. Horizontal lines connect means that are not statistically different from each other at a 95% confidence level. Each bar represents the mean of 3 replications. Error bars represent ±1 standard error

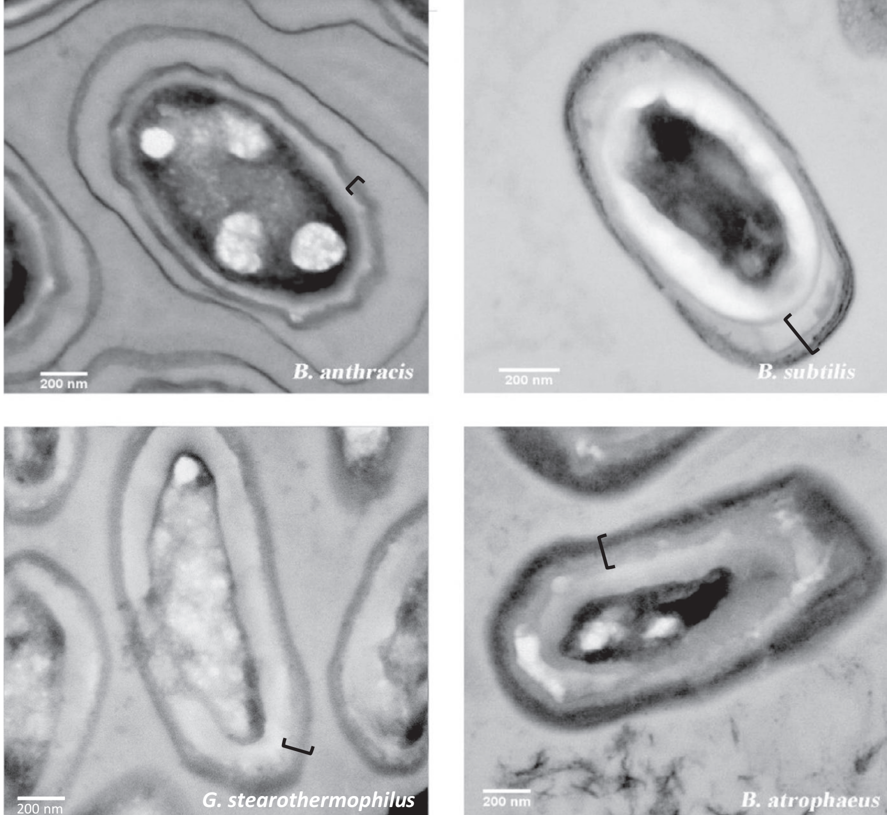

FIGURE 7 Representative transmission electron micrographs of the different spore species used in this study. Spore coat thicknesses are indicated by black brackets. Magnifications are 65,000–150,000×.

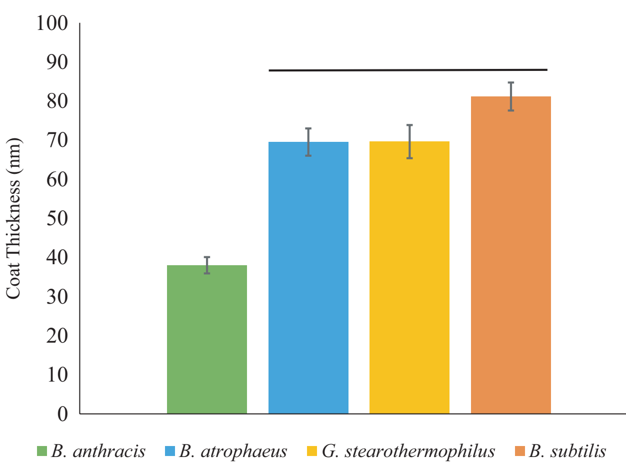

FIGURE 8 Comparison of spore coat thicknesses for different spore species: *Bacillus anthracis* (green), *B. atrophaeus* (blue), *Geobacillus stearothermophilus* (yellow), and *B. subtilis* (orange). Horizontal lines connect means that are not significantly different at a 95% confidence level. Each bar represents the mean measurements of 12 spores. Error bars represent ±1 standard error

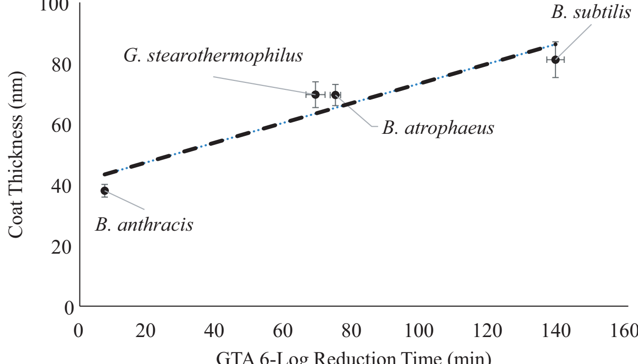

FIGURE 9 Spore coat thicknesses plotted against GTA 6-$\log_{10}$ reduction times for various spore species. Points on the plot indicate the average coat thickness and estimated 6-$\log_{10}$ reduction times for a given spore. Each circle represents the mean of 6 reduction time replications and 12 spore measurement measurements. Error bars represent ±1 standard error. (R^2 = 0.89)

that another important aspect of the GTA disinfection mechanism in spores is the ability to penetrate through the spore coat and reach additional important targets. These data provide valuable insights into GTA disinfection mechanisms that may better inform its proper application and use as a disinfectant in biomedical settings where it plays an essential role in the prevention of infection, disease, and the further spread of problematic microbes.

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CONFLICTS OF INTEREST

None declared.

AUTHOR CONTRIBUTION

Jacob Kent Player: Conceptualization (equal); Data curation (lead); Formal analysis (equal); Funding acquisition (equal); Resources (equal); Writing-original draft (equal); Writing-review & editing (lead). **Justen Thalmus Despain:** Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Investigation (lead); Methodology (equal); Resources (equal); Writing-original draft (lead); Writing-review & editing (supporting). **Richard A. Robison:** Conceptualization (equal); Data curation (supporting); Funding acquisition (equal); Investigation (supporting); Methodology (equal); Project administration (lead); Resources (equal); Supervision (supporting); Writing-review & editing (supporting).

ETHICS STATEMENT

None required.

DATA AVAILABILITY STATEMENT

The data sets generated and analyzed during the current study are available in the figshare repository at https://doi.org/10.6084/m9.figshare.12401951

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