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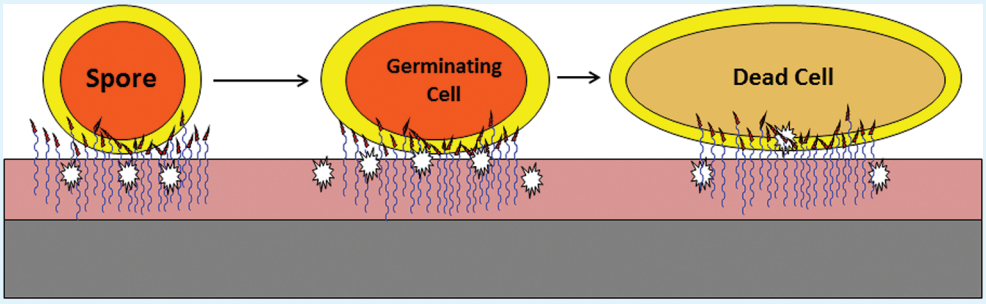
Coatings Capable of Germinating and Neutralizing *Bacillus anthracis* Endospores

Preston A. Fulmer and James H. Wynne*

Chemistry Division, Code 6100, Naval Research Laboratory, 4555 Overlook Avenue SW, Washington, D.C. 20375, United States



ABSTRACT: Endospores are formed by various bacterial families, including *Bacillus* and *Clostridium*, in response to environmental stresses as a means to survive conditions inhospitable to vegetative growth. Although metabolically inert, the endospore must interact with its environment to determine an optimal time to return to a vegetative state, a process known as germination. Germination has been shown to occur in response to a variety of chemical stimuli from specific nutrient germinants including amino acids, sugars and nucleosides. This process is known to be mediated primarily by the GerA family of spore-specific receptor proteins which initiates a signal transduction cascade that results in a return of oxidative metabolism in response to germinant receptor interactions. Herein, we report the development of a novel coating system capable of germinating *B. anthracis* endospores, followed by rapid killing of the vegetative bacteria by a novel incorporated amphiphilic biocide. The most effective formulation tested exhibited an ability to germinate and kill *B. anthracis* endospores and vegetative bacteria, respectively. The formulation reported resulted in a 90% reduction in as little as 5 min, and a 6 log reduction by 45 min.

KEYWORDS: *endospores, germination, coatings, self-decontaminating, antimicrobial surfaces, Bacillus anthracis, surfaces*

INTRODUCTION

With the increase in antibiotic resistant microbes, interest in the production of novel functional materials and self-decontaminating surfaces has become an area of research that has seen a surge of activity in recent years.[1−7] Of particular interest are coatings with the capabilities to decontaminate bacterial endospores. Endospores are formed by various bacterial families, including *Bacillus* and *Clostridium*, in response to environmental stresses as a means to survive conditions inhospitable to vegetative growth. The formation of endospores has been extensively studied and includes several processes: asymmetric cell division of the bacterial cell into a mother cell and prespore, engulfment of the prespore by the mother cell, cortex formation, protein coat deposition, and in the case of *B. anthracis*, production of an additional glycoprotein exosporium.[8−12] It is the multilayered nature of these spores that allow for their survival in extremely hostile conditions.[13]

Although inert metabolically, the endospore must interact with its surrounding environment to determine an optimal time to germinate, and return to a vegetative (growing) state. Germination of *B. anthracis* endospores has been studied sporadically over the past sixty years.[14] *B. anthracis* spores possess an outer coat that is nearly impervious to temperature, drought, harsh chemicals, mild radiation, many sporicides, and UV radiation. They frequently remain dormant for decades, yet can germinate in minutes under ideal conditions.[14] *B. anthracis* spore germination does not rely on a single signal; relying on a redundant germination mechanism ensures it will not germinate until conditions are ideal for survival. Small molecules, known as germinants, have been known to trigger the germination process. These triggers can take the form of a variety of chemical stimuli from specific nutrient germinants, including amino acids, sugars and nucleosides.[14−19] L-alanine in the case of *Bacillus subtilis*, L-proline in the case of *Bacillus megaterium*, and inosine in the case of *Bacillus cereus* are known to be effective germinants.[14,19] In 1949, Hills et al.[14,17,18] demonstrated that *B. anthracis* germination was influenced by L-alanine, tyrosine and adenosine. Likewise, in 1987 Titball and Manchee[19] showed that L-alanine initiated *B. anthracis* endospore germination, whereas D-alanine inhibited germination. Germination has been shown to be mediated primarily by a family of spore-specific receptor proteins.[20] Interaction between the GerA family of receptors and their specific germinants initiates a signal transduction cascade that results in a return of ion transport across the spore's inner membrane, spore cortex

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and coat layer degradation, DNA damage repair, and consequently a return of oxidative metabolism.[21−25]

Although several studies on the efficacy of various germinants have been performed in solution, an exhaustive literature search revealed no study reporting the efficacy of endospore germination on a coating surface. Additionally, our group previously reported on the effective antimicrobial properties of several compounds, particularly quaternary ammonium salts (QAS) and phenolic biocide compounds that were formulated to retain activity within a variety of coatings.[26−32] Although not sporicidal, these compounds have demonstrated surface segregation capabilities as well as effectiveness against many vegetative bacteria. Identification of an effective germinant package for incorporation into these coatings in conjunction with the antimicrobial compounds would greatly increase their efficacy against endospores and endospore forming bacteria. The development of such a novel coating system capable of germinating *B. anthracis* endospores, followed by rapid killing of the resulting vegetative bacteria through incorporation of an effective biocide that is capable or presenting itself at the air-coating interface would be extremely beneficial in high risk environments. It is envisioned that such a coating would be beneficial for high risk buildings and hardware.

EXPERIMENTAL SECTION

Bacteria and Media. *Bacillus anthracis* (Sterne) was used for all bacterial germination and biocidal experiments. Luria−Bertani (LB) media (Difco Laboratories, Detroit, MI), prepared as per the manufacturer's specifications, was used as a bacterial growth and dilution medium for preparation of bacteria. *Bacillus anthracis* spores were prepared using a *Bacillus* sporulation media as previously described.[33] Spores were enumerated using phase contrast microscopy as *Bacillus anthracis* endospores appear as bright refractory bodies. Spore preparations were then treated at 65 °C for 30 min to ensure killing of all remaining vegetative bacteria, adjusted to 10^9 CFU/mL, and stored at 4 °C until use according to standard microbiological standards. Sterile stock solutions of individual L-amino acids (Sigma-Aldrich, St. Louis, MO), nucleosides (adenosine, guanosine, and inosine) (Sigma-Aldrich, St. Louis, MO), casamino acids (hydrolyzed casein) (Difco Laboratories, Detroit, MI), and yeast extract (YE) (Difco Laboratories, Detroit, MI) were prepared for use in germinant studies. All work was conducted in a BSL-2 laboratory and hood using standard BSL-2 practices.

Germinant Testing. Germinant evaluation was first conducted in solution. A 10 μL aliquot of a 1×10^9 CFU/mL stock solution of *Bacillus anthracis* spores was added to 1 mL germinant solution containing amino acids, nucleosides, casamino acids, and/or YE at specified concentrations. At either 30 or 120 min, tubes were heated to 65 °C for 30 min (to ensure all vegetative bacteria are killed) followed by a serial 1:10 dilution in LB. The serially diluted LB solutions were incubated at 37 °C for 18 h and the highest dilution showing turbidity was recorded (low values represent most favorable results). All data reported are an average of triplicate experiments and are rounded to one significant digit.

Coating Preparation. Films were prepared by the combination of 0.80 g of Hydrothane (Cardiotech LTD, UK) with 25 mL of freshly distilled tetrahydrofuran and stirred for 4 h, at which time the polymer completely dissolved. Hydrothane was selected as the control resin for this study because of its availability and relative purity as compared to other commercial resin systems. To the dissolved Hydrothane, a solution consisting of 0.008 g of each additive dissolved in 1 mL of deionized water (di-H_2O) was added dropwise, resulting in a final loading of 1 wt % with respect to polymer solids. The final solution was allowed to stir for an additional 30 min, and films were solvent-cast by the delivery of a 1 mL solution via a pipet to a precleaned glass microscope slide. The glass slide was stored overnight in a sterilized, covered Petri dish to slow the rate of evaporation and generate a uniform coating. The resulting coatings were rinsed with 5 mL of di-H_2O to remove any nonincluded additive prior to subsequent examination and microbial evaluation.

Coating Tests. *Germination.* A 10 μL aliquot of spore suspension was added to the prepared coating surfaces containing a germinant formulation. A coverslip was placed on top of the spores deposited to prevent contamination and to promote wetting over a uniform area. Coatings were then allowed to incubate at room temperature for 2 or 24 h, swabbed vigorously with two sterile swabs to remove remaining spores, and resuspended in 0.85% sterile NaCl.[34] The resulting mix was heated to 65 °C for 30 min to ensure all vegetative bacteria are killed, followed by a serial 1:10 dilution in LB and 18 h incubation at 37 °C. The highest dilution showing turbidity was recorded (7 log spores - highest dilution showing turbidity = log spores remaining).Low values represent most favorable results. All data reported are an average of triplicate experiments and are rounded to one significant digit.

Viable CFU Reduction. As previously described, a 10 μL aliquot of spore suspension was added to the prepared coating surfaces containing both a germinant formulation and biocide, and a coverslip added to ensure even wetting of the surface and to prevent contamination. Coatings were incubated at room temperature. The coverslip was then removed using sterile forceps, and both coating and coverslip were swabbed vigorously to remove bacteria at indicated time intervals and resuspended in media, followed by a serial 1:10 dilution in LB and incubated 18 h at 37 °C.[34] Results are given as log kill (high values represent most favorable results). All data reported are an average of triplicate experiments and are rounded to one significant digit. Lethene media (Difco Laboratories, Detroit, MI) was used as a growth media for all biocidal experiments for its ability to deactivate any biocide transferred from the coating.

Multiple Challenge. As previously described, a 10 μL aliquot of either spore suspension or log phase growth *B. anthracis* (~1×10^9 CFU/mL) was added to the prepared coating surfaces containing both a germinant formulation and biocide, and a coverslip was added to ensure even wetting of the surface and to prevent contamination. Coatings were incubated at room temperature, swabbed vigorously to remove bacteria after 2 h, and resuspended in LB, followed by a serial 1:10 dilution in LB and incubated 18 h at 37 °C. Slides were then cleaned with 70% ethanol to ensure removal of any residual spores or vegetative bacteria. The exposure process was then repeated an additional 5 times over the course of 2 weeks. Results are given as log kill (high values represent most favorable results). All data reported are an average of triplicate experiments and are rounded to one significant digit.

Antimicrobial Kinetics. As previously described, a 10 μL aliquot of spore suspension was added to the prepared coating surfaces containing both a germinant formulation and biocide, and a coverslip was added to ensure even wetting of the surface and to prevent contamination. Coatings were incubated at room temperature, swabbed vigorously to remove bacteria at indicated time intervals, and resuspended in media, followed by a serial 1:10 dilution in LB and incubated 18 h at 37 °C. Results are given as log kill (high values represent most favorable results). All data reported are an average of triplicate experiments and are rounded to one significant digit.

RESULTS AND DISCUSSION

Germinant Testing. To ascertain the performance of germinant formulations under uniform conditions prior to incorporation into coatings, we first conducted experiments in solution. As previously noted, a number of compounds have been reported to have effects on endospore germination, both positive and negative. To further elucidate the effects on germination, an extensive set of formulations were evaluated, and the results are summarized in Table 1.

The solution studies were examined after 30 min and 2 h acknowledging that it would likely be more difficult to germinate on a surface, we elected not to examine the solution

Table 1. Solution Data for Nutrients and Germinants Showing Number of Log Spores Remaining after 7 log Challenge[a,b]

germinant	0.5 h	2 h
alanine + YE	5(0)	2(0)
alanine +2% YE	5(0)	2(0)
alanine +5% YE	5(0.6)	2(0)
alanine +10% YE	5(0.6)	2(0)
alanine + adenosine + YE	5(1)	0(0)
alanine + ATP + YE	7(0.6)	7(0.6)
alanine + ATP + 5% YE	7(0.6)	4(0)
alanine + guanosine + YE	3(0.6)	3(0.6)
alanine + inosine + YE	6(0)	2(0.6)
alanine + inosine +2% YE	6(0)	1(0)
alanine + inosine +5% YE	5(0.6)	1(0)
alanine + inosine +10% YE	5(1)	1(0)
alanine +3,4-dihydroxy alanine + YE	4(0.6)	4(0)
alanine + asparagine + YE	2(0)	2(0.6)
alanine + glutamic acid + YE	3(0.6)	1(0)
alanine + histidine + YE	2(0)	1(0)
alanine (400 mM)+ methionine (400 mM)+ YE	3(0.6)	2(0)
alanine + methionine + YE	1(0)	1(0)
alanine + methionine +2% YE	5(0.6)	3(0.6)
alanine + methionine +5% YE	4(0.6)	3(0)
alanine + methionine +10% YE	4(0.6)	3(0.6)
alanine + methionine + ATP + 5% YE	6(0.6)	2(0)
alanine + methionine + ATP + 10% YE	6(0)	3(0)
alanine + phenylalanine + YE	2(0)	1(0)
alanine + polylysine + YE	5(0)	4(0.6)
alanine + serine + YE	3(0.6)	2(0)
alanine + threonine + YE	5(0.6)	3(0)
alanine + tryptophan + YE	5(0.6)	5(0.6)
alanine + tyrosine + YE	2(0)	1(0)
alanine + polytyrosine (MW 16,500) + YE	3(0.6)	2(0)
alanine + polytyrosine (MW 40,000) + YE	4(0)	2(0)
alanine + polytyrosine (MW 40,000) + 2% YE	4(0)	2(0)
alanine + polytyrosine (MW 40,000) + 5% YE	4(0.6)	2(0)
alanine + polytyrosine (MW 40,000) + 10% YE	3(0)	2(0)
3,4-Dihydroxy alanine + YE	5(0.6)	4(0)
alanine methyl ester hydrochloride + YE	7(0.6)	7(0)
alanine methyl ester hydrochloride +2% YE	7(0.6)	7(0)
alanine methyl ester hydrochloride +5% YE	7(0.6)	7(0)
alanine methyl ester hydrochloride + methionine + YE	7(0.6)	7(0)
N-tert-butoxycarbonyl-L-alanine + YE	7(0.6)	7(0)
N-tert-butoxycarbonyl-L-alanine + methionine + YE	7(0.6)	7(0)
N-tert-butoxycarbonyl-L-alanine +2% YE	7(0.6)	7(0.6)
N-tert-butoxycarbonyl-L-alanine +5% YE	7(0.6)	7(0)
asparagine + YE	5(0.6)	4(0)
glutamic acid + YE	5(0.6)	3(0.6)
histidine + YE	3(0)	3(0.6)
methionine + YE	2(0)	2(0)
phenylalanine + YE	2(0)	1(0)
phenylalanine +2% YE	7(0.6)	3(0.6)
phenylalanine +5% YE	6(0.6)	2(0)
phenylalanine +10% YE	5(0)	2(0)
proline + YE	5(0)	4(0)
serine + YE	4(0)	4(0)
threonine + YE	4(0)	3(0)
tyrosine + YE	4(0)	3(0)
valine + YE	4(0)	3(0)
polylysine + YE	5(0)	5(0.6)
polyproline + YE	4(0)	3(0)
polytyrosine (MW 16 500) + YE	5(0.6)	5(0.6)
polytyrosine (MW 40 000) + YE	5(0)	4(0)
polytryptophan + YE	4(0)	3(0)
adenosine + YE	5(0.6)	4(0.6)
guanosine + YE	5(0.6)	5(0.6)
inosine + YE	3(0)	2(0)
inosine + casamino acids 1%	4(0.6)	3(0)

[a]Amino acid and nucleoside concentration is 200 mM and YE is 1% unless otherwise noted. [b]() denotes standard deviation.

results past the 2 h time frame. The majority of amino acids employed were selected from the extensive previous literature reports. Polymeric amino acids were also included in the evaluation to determine effect on activity due to the additional linkage, which could potentially interfere with the binding ability. Also, by increasing molecular weight, it was believed that the mobility within the coating system would be diminished, which would potentially limit contact with the individual spores at the surface, yet also prevent eventual leaching over time. Blocked (protected) amino acids were included in the solutions testing to determine if the reactivity of such molecules would be diminished in a germination evaluation. These protected species could mimic the effect that tethering of the individual amino acid would demonstrate in a solutions environment without the steric bulk of the backbone to which it would eventually be tethered.

The nutrient employed in this study was yeast extract (YE) because of its previous history. A variety of other nutrients could have been employed; however, to limit the scope, we performed preliminary evaluations and found YE to be the best and also compatible not only with our aqueous solutions studies but also with the control resin employed. Unless otherwise noted, a one percent (wt %) additive of each ingredient listed in Table 2 was added to the control resin system. Also, only L-amino acids were employed as a result of literature reports and confirmation in our laboratory that L-amino acids promoted spore germination whereas D-amino acids inhibited germination and promoted sporulation.

Table 2. Germination of Spores on Surfaces Containing the Indicated Formulation Showing Log of Spores Remaining after 7 log Challenge[a,b]

germinant	solution 0.5 h	solution 24 h	coating 2 h	coating 24 h
alanine + adenosine + YE	5(0.6)	0(0)	4(0.6)	4(0)
alanine + inosine +2% YE	6(0.6)	1(0.6)	3(0)	3(0)
alanine + inosine +5% YE	5(0.6)	1(0)	5(0.6)	4(0.6)
alanine + inosine +10% YE	5(0.6)	1(0.6)	5(0)	5(0)
alanine + glutamic acid + YE	3(0)	1(0)	5(0)	3(0)
alanine + histidine + YE	2(0)	1(0)	5(0)	5(0.6)
alanine + methionine +YE	1(0)	1(0.6)	1(0)	0(0)
alanine + phenylalanine +YE	2(0.6)	1(0.6)	6(0.6)	5(0)
alanine + tyrosine + YE	2(0.6)	1(0.6)	6(0.6)	6(0.6)
phenylalanine + YE	2(0)	1(0)	7(0.6)	7(0.6)

[a]Amino acid and nucleoside concentration is 200 mM. YE is 1% unless otherwise noted. [b]Reported values are average of triplicates with standard deviation values in ().

As previously suggested in other reports, the better performing germination formulations in solutions consisted of alanine in combination with a ring structure, either in the form of a nucleoside or an additional amino acid. There were notable exceptions to this, however, most notably in the formulations containing alanine paired with amino acids with longer R-groups (glutamic acid and methionine), and the germinant formulation containing phenylalanine alone.

Germination on Coating Surface Evaluation. On the basis of the results obtained in the solution evaluation, it was concluded that only those ingredients affording at least a 6 log germination of spores (1 log or less remaining) should proceed to the subsequent step in this study and be incorporated into the model resin system for surface evaluation. As a result, the best ten combinations resulting from the solution testing were down-selected and formulated into the model resin system, as shown in table 2. Hydrothane was employed in this study as the model resin system. This polyurethane hydrogel was selected because of its exceptional versatility, resin purity and unique water absorption characteristics. This urethane hydrogel is capable of absorbing 5−25 wt % water, which was extremely beneficial in the germination process.

After incorporation of additives into the coating system, nearly all formulations exhibited a significant reduction in their ability to germinate spores, as one would expect, because of encapsulation within the polymer as compared to solutions data. There was one notable exception, the alanine, methionine and YE formulation not only retained its activity, but exhibited an increase in spore germination at 24 h. On the basis of these germination results, the three formulations exhibiting the greatest germination effectiveness in the control coating were selected for biocide incorporation.

Antimicrobial Inclusion in Coating. To examine the compatibility of the germination additives with an antimicrobial additive, we selected three effective biocides based on performance in previous reported studies.[29,31] In addition to efficacy, the biocide compounds were selected due to the polar ether segments, which promote resin compatibility along with nonpolar alkyl moieties to promote orientation and presentation at the air−surface interface. Two quaternary ammonium salts (**1** and **2**)[29] were selected because of their broad spectrum activity, in addition to the more lethal phenolic molecule (**3**),[31] and these structures are depicted in Figure 1.

Coating containing these antimicrobials alone without nutrients and germinants did not reduce the spore count at 24 h. Compounds **1**−**3** were incorporated into the coatings containing each of the three down-selected germinant packages, respectively. Results are depicted in Figure 2A. Biocide **1** was most effective across all germinant packages, exhibiting 3−6 log reductions. Alanine, methionine, and YE showed the most activity, exhibiting the greatest log reduction for all biocides evaluated. The combination of the most active biocide with the most effective germinant package (compound **1**, alanine, methionine and YE) demonstrated a decontamination of 6 logs of *B. anthracis* spores on the surface. Although **3** had been effective in previous studies, it was not as effective as compounds **1** or **2** under these conditions.

Additionally, phase contrast microscopy offered additional evidence for the proposed activity on the described coating system. When *B. anthracis* endospores where placed on the control Hydrothane coating containing no nutrients or biocide, the spores remained indefinitely, even when stored under 100% relative humidity (Figure 3A). When the optimized nutrient package was formulated into the coating and spores applied,

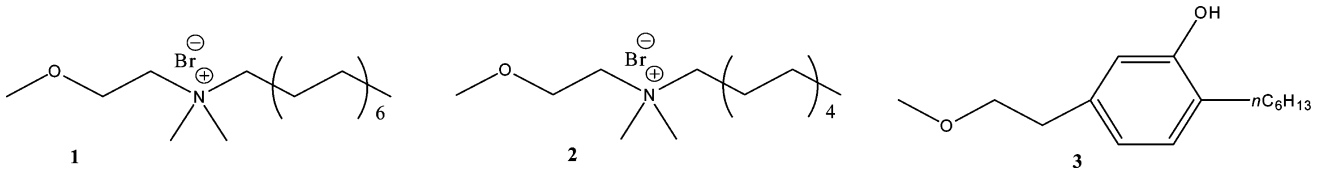

Figure 1. Structures of biocides included in urethane coating.

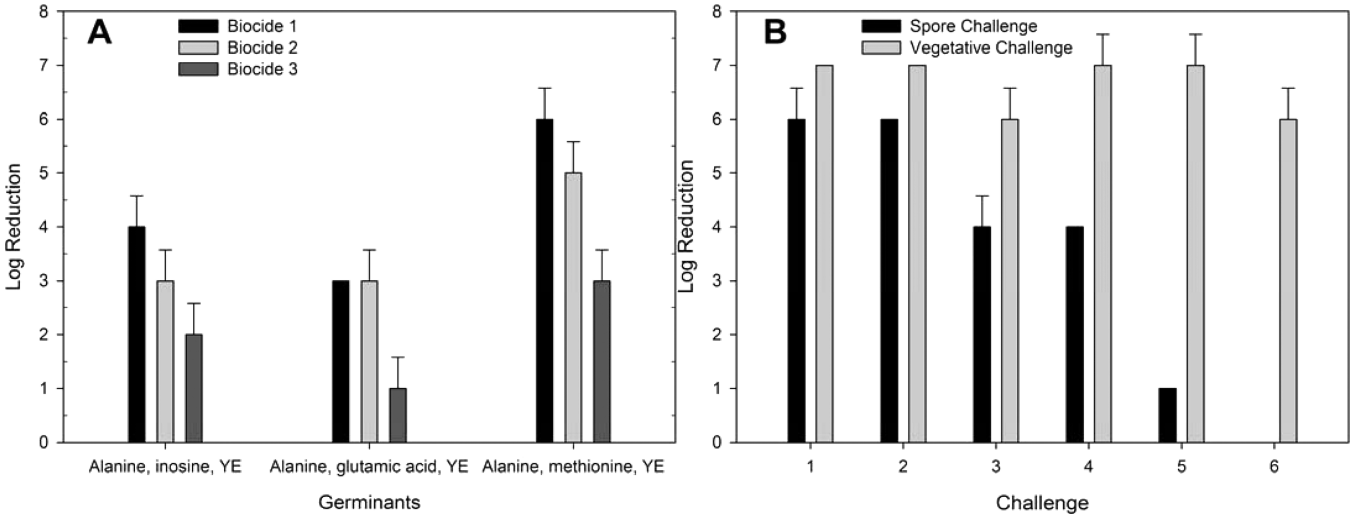

Figure 2. (A) Results of biocide and germinant incorporation into Hydrothane coatings. (B) Results of multiple challenge of biocide **1**, alanine, methionine, YE combination in Hydrothane. Results are shown as log reduction (higher number is better) after 7 log challenge. All additives loaded at 1 wt %.

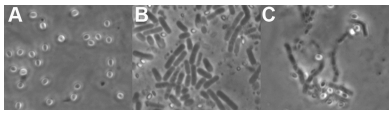

Figure 3. Phase contrast microscopy showing *B. anthracis* endospores on (A) no germination on control coating, (B) germination and healthy cell division, (C) germination and subsequent killing of vegetative cells after 2 h contact time.

within minutes, germination and cell division was observed without killing of the vegetative bacteria (Figure 3B). When the coating was formulated with the optimized nutrient package in addition to the amphiphilic biocide (**1**), germination and subsequent killing of the vegetative cells resulted. The image in figure 3C documents the lack of viable bacteria on the coating surface after 2 h. It should be noted that a very few spores remain after 2 h; however, these proved to be defective, as they did not germinate on recovery and subsequent solution germination attempts.

Multiple Challenge Tests. To evaluate the effectiveness of the newly formulated sporicidal coating, we conducted a multiple challenge experiment and is summarized in Figure 2B. It demonstrated a 6-log reduction after challenges 1 and 2; however, the coating quickly became less effective with subsequent challenges. For the third and fourth challenge, the coating only neutralized *B. anthracis* spores by 4 logs, followed a dramatic reduction to one log reduction upon challenge five, and no reduction was observed for the sixth challenge. To gain insight and attempt to determine the reason for the reduction in performance upon subsequent challenges, we subjected the coating to the vegetative bacterial challenge of *B.anthracis.* For all six challenges, there was no appreciable diminished performance. On the basis of this observation, it was concluded that the reduction in effectiveness against spores after multiple challenges was most likely due to the gradual depletion of nutrients from within the coating system, leading to a loss of ability to germinate spores. It should be noted that relative humidity was consistent in all surface evaluations and consequently viewed as a noncontributing factor in this study.

Antimicrobial Kinetics. To assess the kinetics of spore germination and killing, an experiment was undertaken to provide additional insight into the observed surface activity. For this study, the best performing biocide and germinant combination (compound **1**, alanine, methionine, and YE) were incorporated into the same Hydrothane coating; results are summarized in Table 3.

Table 3. Kinetics Data of *B. anthracis* Spore Reduction on Urethane Coating after 7 log Challenge[a]

time (min)	log of spores remaining on coating containing **1**
0	7(0)
5	6(0)
10	5(0.6)
15	4(0.6)
20	3(0)
25	2(0)
30	2(0.6)
45	1(0)
60	1(0)
120	0(0)

[a]All results reported were the average of triplicate and are rounded to one significant digit, standard deviation in ().

The data show that this biocide germinant combination promotes rapid decontamination of *B. anthracis* spores. Coatings containing biocide **1**, alanine, methionine, and YE exhibited a single log reduction after only 5 min, a 2 log reduction after 10 min, and a 6 log reduction after 1 h. The final log reduction requires a 2 h incubation period. There are two potential explanations for this:[1] Although the ammonium salts are not considered to be consumed in the lysing of the vegetative bacteria, it does leave behind subsequent waste on the coating surface which naturally hinders the availability of the functional surface and thus limits contact with the viable bacteria.[2] This also gives rise to the idea that the rate-limiting step for neutralizing a surface contaminated with *B. anthracis* spores is the germination process. As these data have demonstrated, the ability to neutralize vegetative bacteria once no longer in the spore form is a relative facile process. Additionally, the optimized coating system has demonstrated effectiveness against *B. globigii*.

CONCLUSIONS

Despite the numerous reports of spore germination in solution studies, no published report demonstrates the germination of spores on the surface of a coating. Therefore, it was desirable to incorporate a small amount of additive into an existing coating to induce spore germinating without affecting the properties of the coating. We began this study by performing a solution study to examine the viability of those nutrients and germinants reported. This performance allowed for a down-select of the most effective germinant combination for incorporation into a model resin system.

Herein we report the development of a novel coating system that incorporates both a germinant package and a biocide capable of reducing the number of viable CFUs of *B. anthracis* spores by at least 6 logs within 45 min. Because of the inability of this biocide to kill nongerminated spores, it is clear that the activity of this coating proceeds in a stepwise manner, beginning with germination of spores followed quickly by the death of more vulnerable vegetative bacteria by the incorporated biocide.

AUTHOR INFORMATION

Corresponding Author

*E-mail: james.wynne@nrl.navy.mil.

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