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**“Green” Oxidation Catalysis for Rapid Deactivation of Bacterial Spores\*\***

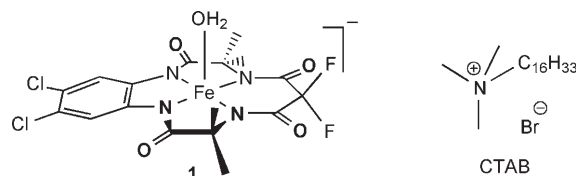
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Under environmental stress, the vegetative cells of certain Gram-positive bacteria such as lethal *Bacillus anthracis* form metabolically dormant spores, which can survive indefinite periods of starvation and desiccation as well as withstand UV radiation, chemical assault, and elevated temperatures.<sup>[1]</sup> Spore hardness derives from two structurally complex and chemically robust encapsulating layers: the proteinaceous coat<sup>[2]</sup> and the peptidoglycan cortex.<sup>[3]</sup> These protective barriers envelop the inner core compartment that contains dehydrated cytoplasm and the cellular components until nutrients are encountered in the environment to stimulate spore germination.<sup>[4]</sup> Additionally, the protective walls include two membranes: the inner membrane surrounding the core and the outer membrane lying between the cortex and coat.<sup>[5]</sup>

*B. anthracis* spores are considered to be among the most difficult biological warfare and terrorism (BWT) agents to destroy.<sup>[6,7]</sup> Better lines of defense against such agents are crucially needed. Ideally, decontamination technologies would rapidly deactivate BWT pathogens in diverse scenarios while being nontoxic, user friendly, materials compatible, and environmentally benign.<sup>[8]</sup> An effective chemical decontamination system presumably must breach the aggregate protective casing swiftly to irreversibly damage critical components. Several oxidation-based chemical approaches have been successfully investigated for the deactivation of bacterial

spores, including chlorine dioxide,<sup>[9]</sup> vaporized hydrogen peroxide,<sup>[9]</sup> oxone,<sup>[10]</sup> and the activation of hydrogen peroxide by bicarbonate.<sup>[11]</sup> These processes involve stoichiometric quantities of the respective oxidants and exhibit varied levels of efficacy and materials compatibility.

Herein, we describe a relatively innocuous catalytic sporicidal system. Minute concentrations of the Fe–tetra-amido macrocyclic ligand ([Fe(taml)]) activator<sup>[12,13]</sup> **1** (Scheme 1) activate hydrogen peroxide or *tert*-butyl hydro-



**Scheme 1.** The principal [Fe(taml)] activator used in this study (**1**), and the cationic surfactant cetyltrimethylammonium bromide (CTAB).

peroxide (TBHP) in aqueous solutions of simple composition under ambient conditions to deactivate spores of the non-infectious anthrax surrogate *Bacillus atrophaeus* (ATCC 9372). The process incorporates numerous desirable properties including the attainment of a 10<sup>7</sup>-fold reduction in the viable bacterial spore population in 15 minutes—an important criterion in the pursuit of a superior spore decontamination technology.<sup>[8,14]</sup>

The resistance of *Bacillus* spores to enzymatic and chemical treatments has been attributed partly to the presence of protein disulfide cross-linkages in the spore coat.<sup>[5,11,15,16]</sup> The 1/H<sub>2</sub>O<sub>2</sub> system ruptures the disulfide bond of cysteine, > 90 % of which was converted into cysteic acid within 1 h (pH 10.0, 25 °C) compared to < 5 % with H<sub>2</sub>O<sub>2</sub> alone. This result suggested the system would fracture the coat and render other spore components vulnerable to destructive chemistries.

*B. atrophaeus* bacteria were cultured at 32 °C (12 h) in brain heart infusion (BHI) broth and were then transferred to BHI agar plates containing MnCl<sub>2</sub> (0.002 %). The plates were incubated at 32, 37, and 42 °C for approximately 4 weeks to obtain spores at different temperatures. The mixture of spores and unaltered vegetative bacterial cells on the BHI plates were suspended in sterile distilled water and heated (80 °C, 20 min) to kill vegetative cells, thus leaving only viable spores.

The *B. atrophaeus* spores were dispersed well in a buffered solution (0.1 M Na<sub>2</sub>CO<sub>3</sub>/0.1 M NaHCO<sub>3</sub>, pH 9.9 ± 0.1) containing the cationic surfactant cetyltrimethylammonium bromide (CTAB), a quaternary ammonium compound (QAC). QACs are known to be antimicrobials because of their ability to disrupt microbial cellular membranes,<sup>[15,17]</sup> but are not independently sporicidal. However, we found that CTAB significantly enhances the sporicidal activity of [Fe(taml)]/peroxide toward *B. atrophaeus* spores. CTAB was used at 0.03 %, in slight excess of its critical micelle concentration.

The initial *B. atrophaeus* spore population was about 10<sup>8</sup> colony-forming units per mL (CFU mL<sup>-1</sup>). The spores were treated with [Fe(taml)]/peroxide (H<sub>2</sub>O<sub>2</sub> or TBHP) and CTAB

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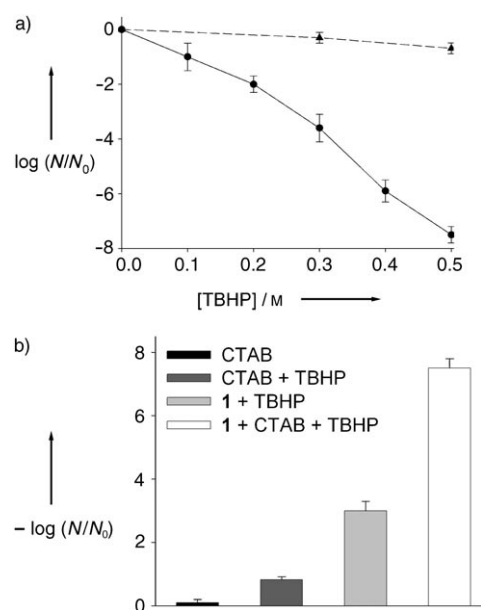
at 25°C in pH 10.0 buffer. The reactions with  $\text{H}_2\text{O}_2$  were quenched with catalase (from *Aspergillus niger*, Sigma). In reactions with TBHP, residual TBHP was removed by centrifugation ( $14000 \times g$ , 60 s) of the reaction mixtures and the spore pellets were washed twice with sterile phosphate-buffered saline (PBS, pH 7.4). Spore suspensions, both treated and untreated, were diluted serially ( $10^1$ – $10^7$ -fold dilutions in PBS, pH 7.4), and a 100  $\mu\text{L}$  aliquot from each tube was plated on BHI agar plates. Colonies were counted after incubating the plates overnight at 37°C from which the reduction in the population of viable spores accompanying deactivation was determined. The plates were also observed for 72 h to monitor the growth of any new colonies.<sup>[18]</sup>

Spores of *B. atrophaeus* (grown at 32°C) were treated with  $\text{H}_2\text{O}_2$  and CTAB at pH 10.0 in the absence and presence of **1** for 1 h. The presence of **1** improved the deactivation factor from  $<2 \log_{10}$  kill with  $\text{H}_2\text{O}_2$  (0.5 M, 1.7%) to a  $3 \log_{10}$  (or 99.9%) kill (**1**, 10  $\mu\text{M}$ ;  $\text{H}_2\text{O}_2$ , 0.5 M, 1.7%; CTAB, 0.03%). This could be improved to a  $5 \log_{10}$  kill (1 h) by increasing the concentration of  $\text{H}_2\text{O}_2$  (0.85 M, 3%); the control with 0.85 M  $\text{H}_2\text{O}_2$  resulted in an approximately  $4 \log_{10}$  kill. At higher peroxide concentrations, the kill rate increased. Higher peroxide concentrations also increase concerns over the safety of human exposure and materials compatibility. Increasing the concentrations of **1** did not enhance spore killing, a feature that we believe derives from the appreciable catalase-like activity of **1**. Therefore, we explored *tert*-butyl hydroperoxide (TBHP) as an alternative oxidant, since it is less hydrophilic than  $\text{H}_2\text{O}_2$  and could thus result in an increased peroxide concentration at the spores. It is less susceptible to catalase-like decompositions than  $\text{H}_2\text{O}_2$ ,<sup>[19]</sup> and in vitro studies have been found to show substantial DNA-cleaving activity.<sup>[20]</sup> TBHP is also one of only two organic peroxides that the U.S. Department of Transportation certifies for tank car shipment.<sup>[14]</sup>

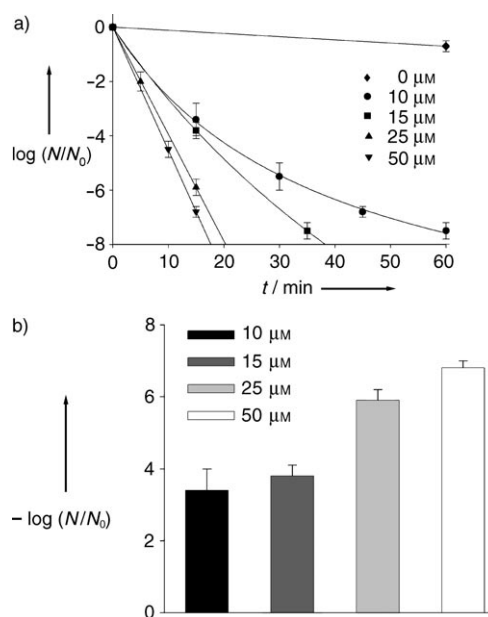
The dependence of spore deactivation on TBHP concentration was studied in the presence of CTAB with and without **1** (Figure 1a). A  $7.8 \log_{10}$  kill was achieved in 1 h with **1** (10  $\mu\text{M}$ ), TBHP (0.5 M), and CTAB (0.03%; Figure 1b). Treatment of the spores with **1**/TBHP but without CTAB resulted in an approximately  $3 \log_{10}$  reduction (Figure 1b). The  $5 \log_{10}$  enhancement of the sporicidal activity of **1**/TBHP by CTAB is noteworthy. In contrast with the  $\text{H}_2\text{O}_2$  case, higher concentrations of **1** were found to accelerate spore deactivation (Figure 2a). Increasing the concentration of **1** from 10 to 15  $\mu\text{M}$  resulted in a  $7.8 \log_{10}$  reduction in just 35 min. At **1** = 25  $\mu\text{M}$ , added in 3 aliquots over 10 minutes, a  $6 \log_{10}$  reduction was achieved in 15 minutes. At **1** = 50  $\mu\text{M}$ , added in 5 equal aliquots over 10 minutes, with the same amount of TBHP (0.5 M) and increased CTAB (0.05%), a  $7 \log_{10}$  kill in 15 minutes was achieved (Figure 2b).

Spores prepared at higher temperatures exhibited greater resistance to deactivation, a phenomenon attributed to subtle differences in the coat proteins.<sup>[21]</sup> These results are presented in Table 1.

[Fe(taml)] activators show highest reactivity at pH 10.0, the pH value used for all the prior results. However, decontamination under near-neutral pH conditions could be useful for protecting sensitive materials when rapid decon-



**Figure 1.** a) Deactivation of *B. atrophaeus* spores in 1 h at various concentrations of TBHP in the presence (●) of **1** (10  $\mu\text{M}$ ) and without **1** (▲). b) Bar chart showing spore deactivation in 1 h with different components of the [Fe(taml)]/peroxide system using **1** (10  $\mu\text{M}$ ) and TBHP (0.5 M). All the experiments were conducted in the presence of 0.03% CTAB.  $N_0$  = initial number of spores,  $N$  = number of surviving spores at a given time, and  $N/N_0$  = fraction of surviving spores at the same time.



**Figure 2.** a) Time dependence of the deactivation of *B. atrophaeus* spores with various concentrations of **1**. b) Bar chart showing levels of deactivation achieved in 15 min with different concentrations of **1**. The experiments were conducted in the presence of 0.5 M TBHP and 0.03% CTAB (except in the case of 50  $\mu\text{M}$  [Fe(taml)], where the CTAB concentration used was 0.05%).

tamination might be less critical. At pH 8.0 (0.1 M  $\text{KH}_2\text{PO}_4$ ), a  $7 \log_{10}$  kill of *B. atrophaeus* spores (prepared at 32°C) was attained in 5 hours by employing **1** (50  $\mu\text{M}$  added in 5 aliquots) and 0.5 M TBHP with CTAB (0.03%).

**Table 1:** Rate of deactivation of *B. atrophaeus* spores prepared at 32, 37 and 42 °C.

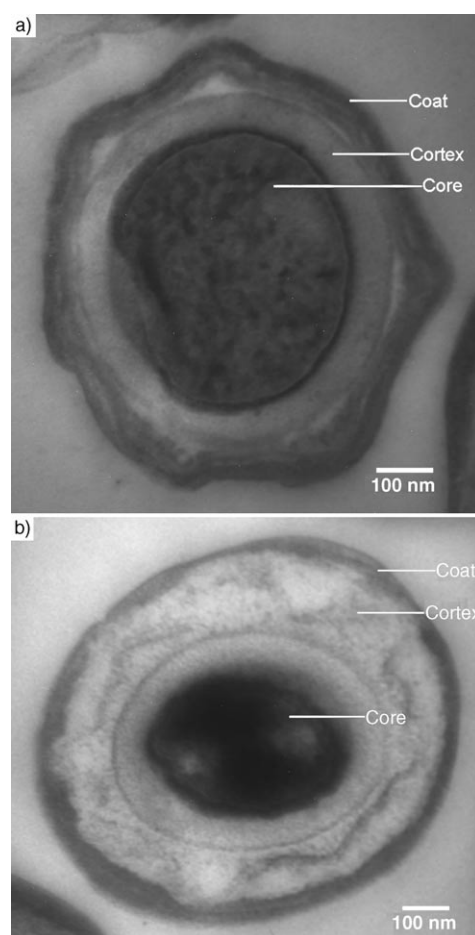
| <i>B. atrophaeus</i> spores prepared at | Reduction in the number of spores  |                       |                       |
|---|------------------------------------|-----------------------|-----------------------|
|   | 15 min                             | 60 min <sup>[c]</sup> | 90 min <sup>[c]</sup> |
| 32 °C                                   | 7 log <sub>10</sub> <sup>[a]</sup> | 7.8 log <sub>10</sub> | –                     |
| 37 °C                                   | 7 log <sub>10</sub> <sup>[b]</sup> | 5.5 log <sub>10</sub> | 7 log <sub>10</sub>   |
| 42 °C                                   | –                                  | 4.5 log <sub>10</sub> | 5.5 log <sub>10</sub> |

[a] **1** (50 μM), TBHP (0.5 M), and CTAB (0.05 %). [b] **1** (60 μM), TBHP (0.5 M), and CTAB (0.05 %). [c] **1** (10 μM), TBHP (0.5 M), and CTAB (0.03 %).

The role of oxygen-based free radicals as probable biocides is well documented in the literature and bactericidal/sporicidal activities of TBHP through a free-radical mechanism have been reported.<sup>[22,23]</sup> An HPLC analysis of the supernatant solution from the suspension containing deactivated spores revealed the presence of acetone (and dipicolinic acid, see below). Control experiments revealed that reaction between **1** and TBHP produce significant amounts of acetone. The formation of acetone suggested that *tert*-butoxyl radicals (*t*BuO•) form during the reaction: one path of their decomposition involves the formation of the methyl radical and acetone.<sup>[24]</sup> *tert*-Butoxyl radicals have been reported to yield bactericidal activity.<sup>[24]</sup> This bactericidal activity is in addition to that of the “membrane-active” CTAB.

In the late stage of sporulation pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) accumulates within the highly dehydrated spore core.<sup>[25]</sup> The HPLC quantitation of DPA in the supernatant solution from deactivated spores (prepared at 32 °C) was estimated to be 10 ± 2 % of the total spore core depot, based on the amount of DPA released from otherwise untreated spores on heating at 100 °C for 20 minutes.<sup>[26]</sup> The deactivated spores still appeared bright under a phase-contrast microscope. Heating these spores at 85 °C (a sublethal temperature for healthy spores) for 30 minutes led to release of the remaining DPA from the spore core (85 ± 5 %). Such release of DPA from the core has been attributed to damage incurred to the inner membrane during oxidative treatment.<sup>[27]</sup> The structural changes induced in *B. atrophaeus* spores before and after treatment with **1**/TBHP and CTAB are revealed in the transmission electron micrographs<sup>[28]</sup> in Figure 3. After treatment with **1**/TBHP and CTAB, the outermost spore layer appeared less punctate, the core was appreciably compressed, and the cortex layer was enlarged.

This work shows that [Fe(taml)]/peroxide oxidation can rapidly deactivate bacterial spores. The inherent robustness of the [Fe(taml)] activators under oxidizing conditions and their high catalytic activity with peroxides allow relatively simple compositions to render the bacterial spores incapable of germination and reproduction. The incorporation of CTAB plays a significant role in enhancing the sporicidal activity of **1**/TBHP relative to **1**/H<sub>2</sub>O<sub>2</sub>, as the membrane-disruptive action of CTAB apparently potentiates the biocidal activity produced by **1**/peroxide. Preliminary toxicity tests indicate that [Fe(taml)] complexes and their decomposition products are relatively nontoxic.<sup>[13]</sup> Thus, these studies demonstrate that [Fe(taml)]/peroxide systems show considerable promise

**Figure 3.** TEM images of *B. atrophaeus* spores a) before and b) after treatment with **1**/TBHP (10 μM/0.5 M) and CTAB (0.03 %) for 1 h.

for providing a superior decontamination technology for real-world applications.

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- [28] Spores were fixed in 2% glutaraldehyde, followed by 1% osmium tetroxide buffered with PBS. After washing the spores with distilled water and ethanol solutions of increasing strength (50%, 70%, 95%, and 100%), they were placed overnight in a 1:1 mixture of LR White resin and propylene oxide. The mixture was then replaced with 100% LR White resin and the spores were sliced into sections (100 nm thick). Thin sections were stained with 1% uranyl acetate and Reynold’s lead citrate, and viewed on a Hitachi 7100 transmission electron microscope. Digital images were obtained using AMT Advantage 10 CCD Camera System and NIH Image software.