

Pulsed Microwave Induced Bioeffects

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Invited Paper

Abstract—High-power pulsed microwave radiation, when applied to solutions containing dissolved carbon dioxide (or bicarbonate), hydrogen peroxide, and the soluble organic semi-conductor diazolumelanin, generates sound, pulsed luminescence, and electrical discharge. Microbes exposed to these phenomena experienced damage comparable to short-time, high-temperature insults, even though the average and measurable localized temperatures were insufficient to cause the observed effects.

Index Terms—Anthrax, bacillus, high-power, microwaves, pulse.

I. INTRODUCTION

PREVIOUSLY, we have reported the sensitization of anthrax bacteria (*Bacillus anthracis*) to killing (a 5-log reduction in viable cells) by continuous-wave 2450-MHz radiation [1]. This effect was accomplished by growing the bacteria on culture medium that induces the synthesis of the organic semi-conductor diazolumelanin (DALM) and treating them with sodium bicarbonate and hydrogen peroxide [1]. The temperature of the exposure was maintained at 37 °C, well below the thermal kill temperature of anthrax, either its vegetative or spore forms [see Fig. 1(a) and (b)]. Furthermore, we have shown that HL-60, a human leukemia cell line, can be induced to produce DALM and show enhanced absorption of continuous microwave radiation [2]. Last, we have conferred this property on several cell lines—bacterial, mouse, and human—by introduction of a plant nitrate reductase gene fragment into their genomes [3]–[5]. The nitrate reductase gene is responsible for the biosynthesis of DALM [6]. This polymer demonstrates thermochemiluminescence, continuous and pulsed, when peroxidizing solutions containing bicarbonate, or other sources of carbon dioxide, are exposed to microwave radiation, continuous or pulsed, respectively [7]. Here we examine damage to anthrax spores with high-power pulsed mi-

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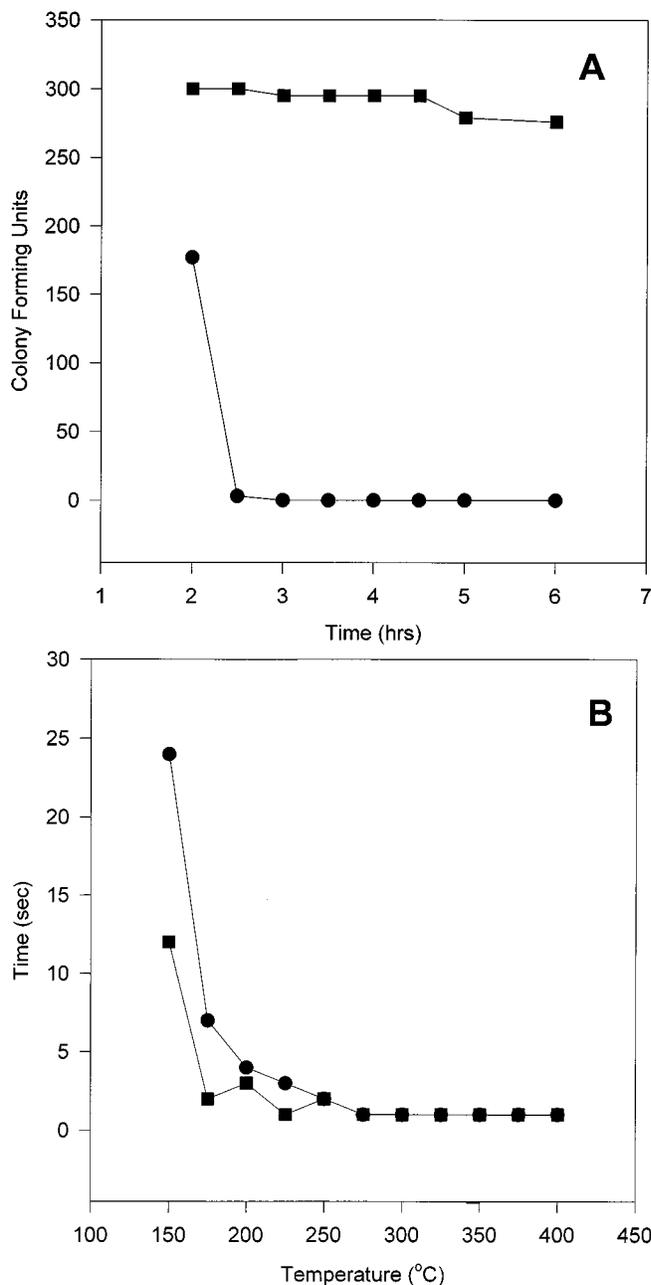


Fig. 1. Thermal sensitivity of killing and growth inhibition of *Bacillus anthracis* (anthrax bacteria) dry spores assayed on blood agar and 4X3AT agar. (A) Colony-forming units surviving on blood agar after exposure to 60 °C (squares) or to 100 °C (circles) for the respective times. (B) Colony-forming units surviving on blood (circles) or 4X3AT (squares) agar for the respective time and temperature.



Fig. 2. Single captured frames from integrating charged couple device (left) and nonintegrating black and white (inset flash) video cameras of discharges from DALM solution absorbing 1.25-GHz radiation ($6\text{-}\mu\text{s}$ pulsewidth, 10 pulses/s, 2-MW peak forward power).

crowave radiation in peroxidizing solutions of biosynthesized DALM and the mechanism thereof. The absorption is very small for microwave frequencies but is predicted to rise rapidly in the infrared and visible wavelengths. However, by increasing the number of redox activated molecules that are susceptible to microwave absorption, the probability of enhanced microwave absorption is increased. This enhancement can be accomplished by simultaneous exposure to microwave and shorter wavelength radiation (into the visible and ultraviolet). The thermally activated delayed fluorescence demonstrated by DALM, when coated onto epoxy polymer, is supportive of this mechanism [9]. It is induced by simultaneous exposure to 366-nm wavelength (UVA) light and microwave radiation. The delayed fluorescence is observed by a reexposing of the DALM film to pulsed UVA and observing the afterglow. The intensity of the delayed fluorescence is proportional to the period of exposure or energy of radio-frequency exposure. Heating the film and exposing it to UVA simultaneously can also generate the effect, but a higher level of conventional than microwave heating is necessary [9].

II. EXPERIMENTS

A. Optimizing Focal Absorption of Pulsed Microwave Radiation

We previously reported the generation of light, sound, and electrical discharges in DALM solutions by exposure to pulsed microwave radiation [7]. A variety of mixtures were used, but the process was not optimized. The chemical structure of DALM and its relationship to its microwave radiation absorptive properties is an object of intense research and remains unresolved at this time [10]. In order to determine the basis for variable susceptibility of DALM preparations to the generation of these responses, organically synthesized DALM was prepared and was

nitrate. Because nitrate reduction, under aerobic conditions, is necessary to the biosynthesis of DALM, nitration was considered an essential process for optimizing the pulse response.

Nitration was achieved by mixing a solution containing 1.38 g of sodium nitrite in 20-ml water with 2.27 g of 30% hydrogen peroxide plus 0.33 ml of concentrated sulfuric acid in 20 ml of water at ice bath temperatures. As soon as the solution turned yellow, 1.12 g of sodium hydroxide in 20 ml of water was added to the previous mixture. Lyophilized synthetic DALM (2.5 g) was dissolved in 10 ml of the peroxy nitrite solution (99 mM) formed as described above. The mixture was allowed to react overnight at room temperature. It was then dialyzed against cold deionized water with several changes and freeze-dried. Five milliliters of the nitro-DALM solution was placed in a 15-ml polystyrene conical tube and was activated by addition of saturated sodium carbonate and luminol solution with 3% hydrogen peroxide added.

As previously described for other DALM solutions, the nitro-DALM peroxidizing solution was exposed to 10 pulses/s ($6\text{-}\mu\text{s}$ pulse duration at 1/2 pulse height) of 1.25-GHz radiation with a peak incident power of 2-MW forward power [7]. The sound and light produced were recorded with a RCA BK-6B M-11 017A microphone and low-light cameras (ITT model #F4577 black-and-white camera and an Optronics DEI-470 integrating color camera), respectively. The nitro-DALM began pulsing with sound and light almost immediately and with greater intensity than that of the original DALM (1:100 dilution of crude biosynthetic extract). Fig. 2 displays captured frames of the video from the Optronics and ITT cameras showing single-pulse results. The spark discharge extended from a point in the meniscus (the point is not at the same location for each discharge) into the gas head space of the tube but did not extend above the open mouth of the tube. Fig. 3 shows a typical temperature profile (measured with Vitek)

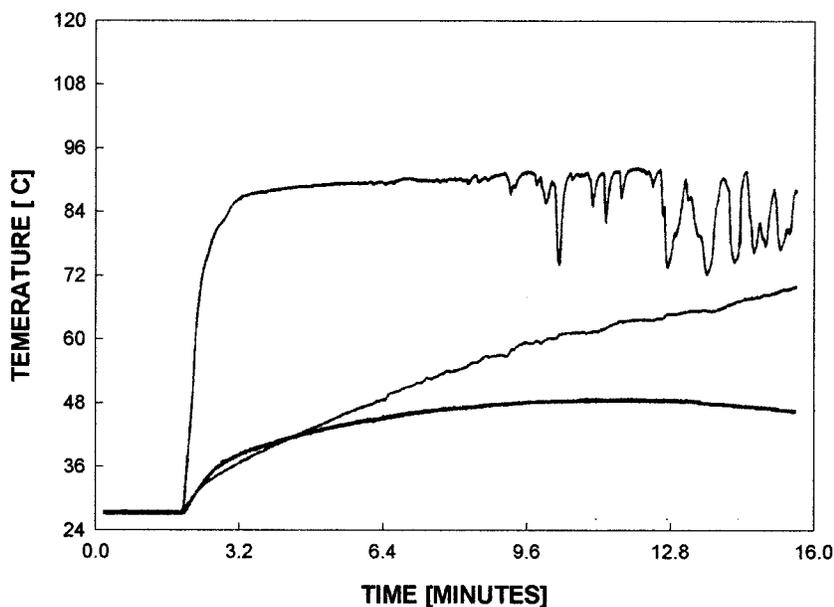


Fig. 3. Time/temperature profile of a pulsed microwave (1.25 GHz, 6- μ s pulsewidth, 10 pulses/s, 2-MW peak power) exposed 5-ml sample of DALM. The top curve is the air temperature above the meniscus, the middle curve is the temperature of the meniscus, and the bottom curve is the temperature of the liquid at the bottom of the tube being exposed.

nonperturbing high-resistance thermal probes) of such an exposed tube. The solution in the bottom of the tube reached a thermal steady state, but the temperature of the meniscus appeared to linearly increase over the time of the exposure. The air immediately above the meniscus rapidly became about 40 °C–50 °C hotter than steady-state temperature of the solution and then fluctuated wildly with the discharges. None of these temperatures was sufficient to kill anthrax spores in the time frame of the exposure [Fig. 1(a) and (b)].

B. Determining Thermal Sensitivity of Anthrax Spores

The *Bacillus anthracis* (BA; Sterne strain) spore vaccine¹ was centrifuged, the supernatant decanted, and the button washed with chilled deionized water. Dilute powdered milk solution was made with chilled deionized water to a concentration of 26 mg of milk solids in 1 ml of solution and filtered through a 0.2- μ m filter. The BA button was resuspended in 1 ml of the sterile milk solution. Then, 50 μ l of this suspension were diluted in 450 μ l of physiological phosphate buffered saline (PBS) and used as the source for colony forming unit (CFU) assays. The solutions were transferred with a 1- μ l calibrated loop to blood or 4X3AT agar plates for incubation at 37 °C for 18–24 h. They were then counted up to 300 colonies per plate. For thermal exposures, silicone-coated [BDH Silicone Products, Repelcote (VS) solution, coated according to manufacturer directions] 5.75 in Pasteur pipettes were used. These pipettes were washed with deionized water, autoclaved, and oven dried. Each pipette was charged at the tip with 3 μ l of well-mixed skim milk/BA suspension. They were then frozen. The frozen samples were lyophilized for four to five days. They were stored under vacuum at room temperature when not in use. The lyophilized spore samples were exposed to various temperatures for various times by placing them

in the heating block of an electrothermal² melting-point apparatus. Each set of exposures included a control, which was not heated. The tubes were assayed for CFU's by recovering the exposed material in 450 μ l of sterile PBS and plating to the blood or 4X3AT agar, using the 1- μ l loop or 50 μ l of the suspension. The plates were incubated as described above.³ The 4X3AT agar was custom made by the same manufacturer using 55 g of trypticase soy agar base, 6 g of potassium nitrate, 50 mg of luminol, and 160 mg of three-amino-L-tyrosine HCl per liter of sterile deionized water.

Fig. 4 shows that the 4X3AT medium displays no CFU's at about 225 °C near 1 s of exposure to heat, and the growth on blood agar drops to zero in 1–2 s at about 250 °C. This slight phase shift in the kill curve for BA on the two media allows for pinpointing the apparent temperature to which the BA was exposed during the pulsed microwave exposure. Figs. 5 and 6, for comparison, show the surviving CFU's for *Bacillus thuringiensis var. kurstaki*⁴ and *Bacillus globigii var. niger*,⁵ respectively, exposed under the same conditions in the melting-point apparatus and cultured as the BA was.

In order to generate a preparation of single spores rather than conglomerated spores for BT and BG, special procedures were developed. One-half gram of the commercial preparation of BT Javelin was placed into a 15-ml centrifuge tube, 6 ml of chilled deionized water was added, and the suspension was thoroughly vortexed. It was then sonicated for 3–5 s. Next, 6 ml of chilled ethyl acetate was added to the suspension and the tube was shaken vigorously for 1 min. The suspension was then centrifuged in a clinical centrifuge for 15 min. The suspended plug

² Model 9300; Electrothermal Engineering Limited, Southend-on-Sea, Essex, U.K.

³ The blood agar was commercially obtained from Remel, Lenexa, KS.

⁴ BT, a close relative of anthrax bacteria that kills insects; Javelin, Sandoz Agro, Inc., Des Plaines, IL.

⁵BG; provided by Dr. R. Liebert, U.S. Army Dugway Proving Ground, UT.

¹ Thraxol, Mobay Corp., Animal Health Division, Shawnee, KS.

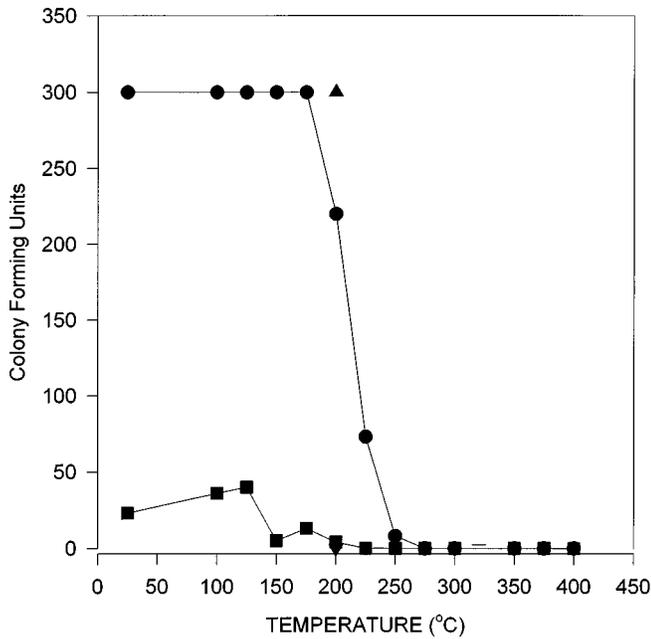


Fig. 4. Anthrax bacterial growth on 4X3AT (square) and blood (circle) agars following 1-s exposures of dry spores to various temperatures. The single triangles indicate high-power, pulsed microwave data fitted to the thermal response data for growth on blood (triangle pointing up) and 4X3AT (triangle pointing down) agars, respectively.

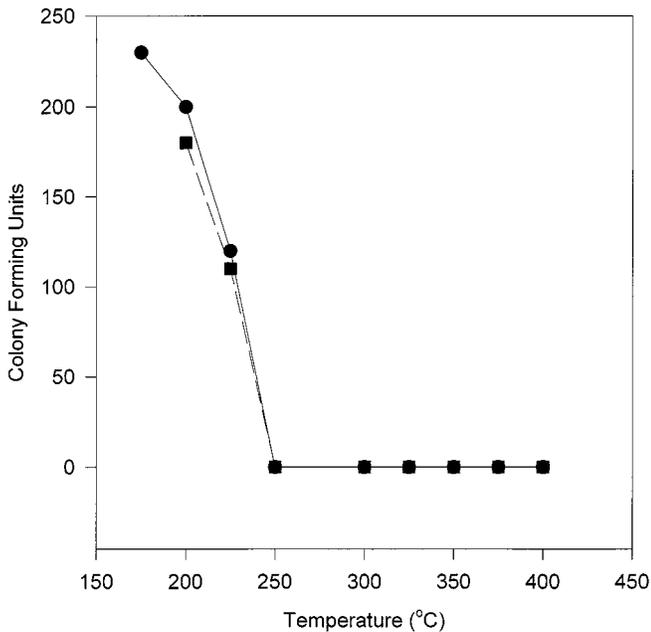


Fig. 5. *Bacillus thuringiensis* var. *kurstaki* growth on 4X3AT (squares) and blood (circles) agars following 1-s exposures of dry spores to various temperatures.

was loosened with a sterile stick and poured off the spore button with the supernatant. The button was washed three times with chilled deionized water and centrifuged each time. After decanting the final wash, one calibrated loop ($1 \mu\text{l}$) of the spore button was placed in 1 ml of the sterile skim milk solution. The procedure for BG was modified from that of BT. One gram of BG powder was placed in a 15-ml centrifuge tube, and the tube was filled with deionized water. The suspension was vortexed and then sonicated for 5 s. The suspension was then filtered with

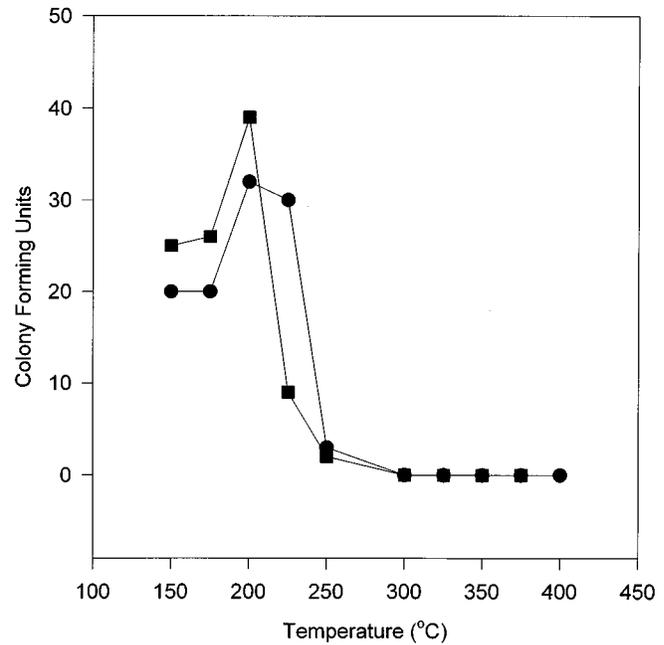


Fig. 6. *Bacillus globigii* var. *niger* growth on 4X3AT (circles) and blood (squares) agars following 1-s exposures of dry spores to various temperatures.

a sterile paper filter and funnel pulled through by vacuum. The filtrate was placed in another sterile 15-ml centrifuge tube and centrifuged for 15 min at maximum speed in a 45° fixed head in a clinical centrifuge. A vertical streak of spores formed along the length of the tube, and a button of debris was deposited in the bottom. The supernatant was poured off and the button was removed with a pipette. The streak was washed and centrifuged three times with chilled deionized water. After the final wash, the supernatant was removed and a 1-l loopful of the streak was removed and resuspended in 1 ml of sterile deionized water. The samples were stored at 2°C – 8°C until used. The media did not show differential growth for BT and BG like that seen with BA but did display about the same thermal kill profile as for BA.

C. Pulsed Microwave Effects on Anthrax Spore Viability

For pulsed microwave exposure, 0.5 ml of BA spore suspension was placed into $0.2\text{-}\mu\text{m}$ -filter⁶ centrifuge tubes. The spores were then centrifuged onto the filter (16000 g for 15 min). The tubes were refilled with 1.5 ml of a reaction mixture consisting of 0.9 ml of saturated sodium bicarbonate/luminol solution, 0.1 ml of 1 : 10 biosynthetic DALM, 0.6 ml of 1 : 10 diazoluminol, 0.33 ml of saturated sodium bicarbonate/luminol solution, and 0.33 ml of 3% hydrogen peroxide. All the dilutions were made in the saturated sodium bicarbonate/luminol solution. The final dilution of the DALM was 1 : 1000. A detailed description of the reaction mixture has been previously published [6], [7]. The filter, with the BA spores, was inserted into the tube to the level just below the meniscus of the fluid. The exposures were as described above for the nitro-DALM. They were started at 3 min and 22 s after placing the reaction mixture in front of the microwave wave guide. The microwave exposure was for 13 min and 28 s. Therefore, the total radiation exposure was for 48 ms (a duty factor of 0.000 06). The temperature of the sample was

⁶ Microfilterfuge, Rainin Instrument Co., Inc., Woburn, MA.

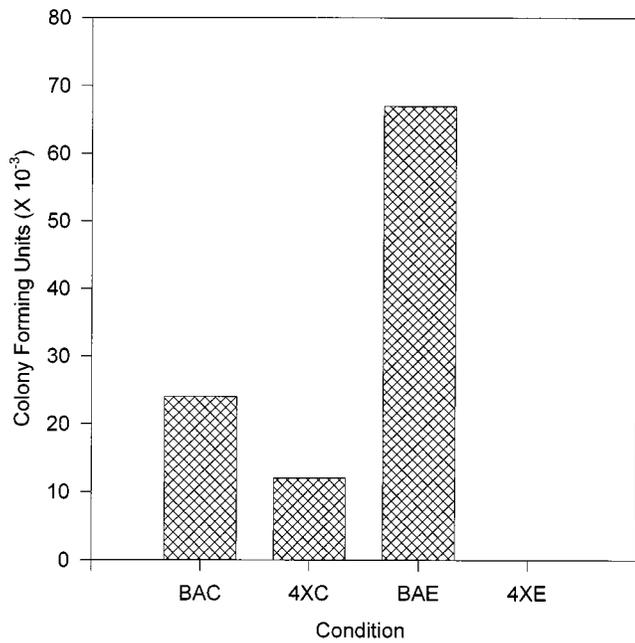


Fig. 7. Recoverable colony forming units of anthrax bacteria following exposure of spores to pulsed microwave radiation (1.25 GHz, 6 –s pulsewidth, 10 pulses/s, 2-MW peak power). BAC and 4XC are sham exposed spores recovered onto blood and 4X3AT agars, respectively. BAE and 4XE are microwave exposed spores recovered onto blood and 4X3AT agars, respectively.

continuously monitored with a nonperturbing, high-resistance temperature probe (Vitek). The temperature began at 25.3 °C and reached a high point, at the end of the exposure, of 64 °C. During the exposure, light and sound pulses were produced that correlated with the microwave pulses. Spores were recovered from the filter by placing it in a 50-ml centrifuge tube containing 5-ml PBS and vortexing extensively. This solution was titered ten- to 1-million-fold for colony count determination. The spore suspension samples were transferred to 4X3AT or blood agar with a 1- μ l calibrated loop. The samples (exposed and sham exposed) were assayed as described above.

Fig. 7 displays the CFU counts for sham exposed (to reaction mixture but no radiation) and exposed samples. The respective survivability points for the pulsed microwave exposure, when assayed on the 4X3AT agar and blood agar media and imposed on the heat-kill curve in Fig. 4, indicate an apparent effect comparable to that of heating for 1 s at about 200 °C. This effect greatly exceeds that expected for the 64 °C maximum observed in the meniscus during the experiment.

D. Biological Mechanism of Pulsed Microwave Effect on Anthrax Bacteria

Fig. 8(a) and (b) indicates that when carbon dioxide is added to the growth conditions (Marion Scientific CO₂ gas generator placed in a zip-lock bag with the culture medium plates during incubation), the growth of BA on 4X3AT plates was greatly enhanced. The CFU's rose to the level of those on blood agar, and the blood agar showed no enhancement in growth with the addition of CO₂. Neither *Bacillus globigii* var. *niger* nor *Bacillus thuringiensis* var. *kurstaki* displayed this differential growth between 4X3AT and blood agars (Figs. 5 and 6). Furthermore, when BA is grown at an elevated temperature (42 °C) for ten

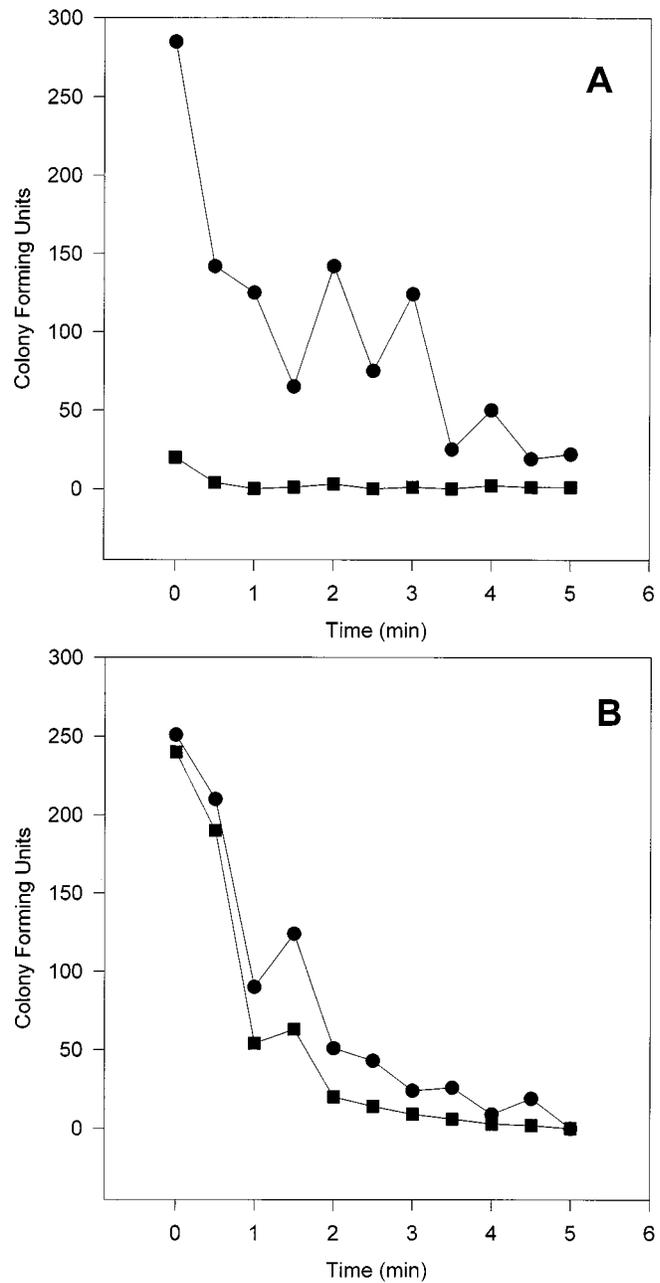


Fig. 8. Effect of the presence of carbon dioxide on the recovery of anthrax colony forming units on 4X3AT (squares) and blood (circles) agars after exposure of the dry spores to 125 °C for various lengths of time. (A) is without carbon dioxide present and (B) is with carbon dioxide.

days and passaged to new medium every 24 h, the stimulation of growth by CO₂ is lost along with the pXO1 plasmid (data not shown). Because a transactivating factor for gene expression, sensitive to CO₂ levels, appears to reside on the pXO1 plasmid for toxin production [16], the CO₂ enhanced growth on 4X3AT medium appears to be controlled by this plasmid. The plasmid resides in Sterne strain as well as pathogenic strains of BA. Even though growth on the 4X3AT agar was restricted after either sufficient thermal or microwave exposure, polymerase chain reaction amplification of a target sequence on the pXO1 plasmid, however, revealed that it was still present in these treated bacteria (data not shown).

III. CONCLUSION

The comparison of the pulsed microwave effects on the viability of anthrax spores compared to standard thermal insult indicate that the spores perceive much higher temperatures than were measured during the exposures. The results indicate thermal damage to the plasmid, but not its thermal destruction, which occurred with the short-time, high-temperature (200 °C–225 °C) exposures and the pulsed microwave exposures. These results strongly suggest that the plasmid expression was affected and that the same thermal mechanism was operational in both types of insult.

The physical evidence, other than direct temperature measurements, also supports the ultrashort-time, very high-temperature mechanism for pulsed microwave effects. The electrical discharges above the liquid surface, and the pulsed light and sound that correlated with the microwave pulses support this mechanism. Even though the measured temperatures never reached 200 °C, the fact that the gas temperature above the meniscus was at least 30 °C hotter than the meniscus and 50 °C hotter than the bulk of the fluid in the tubes suggests that a hot gas was produced and ejected from the fluid. This gas was obviously not in thermodynamic equilibrium with the surrounding fluid. Also, if the temperature of the bacterial spores did approach 200 °C, as indicated by the assay responses, then the bacteria must have been in contact with a hot gas, not the aqueous solution that could not have exceeded 100 °C and remained a liquid. Therefore, gas must have been trapped or generated at the surface of the bacterial spores and transferred some of its energy to the bacterial spores. The physical and biological evidence presented here suggests that a sonoluminescent-like mechanism could be at work. The hot plasma involved could be generated by the intense thermal gradient, pulsed microwave induced sound (shock) wave, cavitation, and collapse of gas. The chemical reaction on or in the DALM molecules facilitated this process. A very high, very localized temperature effect on the spores is compatible with such a mechanism.

At this time it is not known how the redox chemistry of DALM brings about the process. What is known is that DALM is a nitration product of luminol and three-amino-L-tyrosine and that the latter is an analog of a natural amino acid tyrosine. Tyrosine and other biomolecules are nitrated in animal and human tissues [12]–[17]. Therefore, nitrated proteins, lipids, catecholamines, and nucleic acids could possibly participate in an energy focusing reaction comparable to the one involving DALM.

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Dr. Kiel was elected to the American College of Veterinary Microbiologists in 1984. He became a Fellow of the American Association for the Advancement of Science in 1995 and of the Air Force Research Laboratory in 1998. He received the U.S.A.F. Basic Research Award in 1994 for his work in radio-frequency radiation bioeffect mechanisms. His inventions won him the 1991 R&D 100 Award and the 1992 Federal Laboratory Consortium Award for Excellence in Technology Transfer. His scientific team was named a Star Team by the Air Force Office of Scientific Research in 1993. He was named an Air Force Association Scientist of the Year in 1994.



Jill E. Parker was born in Sussex, U.K., and received the B.Sc. (Hons.) in Biochemistry from the University of Sheffield in 1963 and the Ph.D. degree in "Human Biochemical Genetics" from King's College, University of London, in 1966.

She held Postdoctoral positions at the Galton Laboratory, University College, London University and at the University of Texas at Austin in the Department of Zoology and Department of Microbiology. Her research focused on obtaining the protein sequence of human Transferrin and a Flavodoxin from *Desulphovibrio gigas*. In the Microbiology Department, she ran a centralized tissue culture facility to produce hybridomas for many departments. In 1982, she joined Dr. Barbara Bowman's group in the Department of Cellular and Structural Biology at the University of Texas health Science Center, San Antonio, and determined, by nucleic acid sequencing, the sequence of human Haptoglobin. In 1985, she transferred to the Microbiology Department working with Dr. Wendell Winters, and became a Fellow under a United States Air Force training program. Her research focused on the bioeffects of exposure to low level electromagnetic fields (ELF) and radiofrequency radiation (RFR). In 1987, she was recruited to Civil Service at Brooks AFB in what is now the Human Effectiveness Directorate. She works with Dr. Jonathan Kiel in the Biomechanisms and Modeling Branch as a molecular biologist. Her current research focuses on characterization and sequence of key metabolic genes from *Bacillus anthracis* (B. a.) and the utilization of these genes in the rapid identification of B. a. from its near relatives.



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Satnam P. Mathur (M'88) was born in Dholpur, India, on October 17, 1943. He received the diploma in electronics and radio engineering (with honors) from BTE, Bombay, India, in 1966, the M.A.Sc degree in electrical engineering from the University of Windsor, ON., Canada, in 1969, and the Ph.D. degree in electrical engineering from Michigan State University, East Lansing, in 1974.

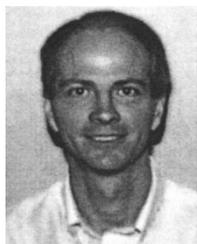
From 1966 to 1967, he was a Scientific Assistant in the Microwave Division at the Tata Institute of Fundamental Research, Bombay. From 1974 to 1975, he was with the School of Radar Studies, Indian Institute of Technology, New Delhi, India. From 1982 to 1987, he was an Assistant Professor and from 1982 to 1987 a Professor of electrical engineering at the Delhi College of Engineering, Delhi University, Delhi, India. From 1988 to 1989, he was a Research Associate at the University of Utah, Salt Lake City, where he worked on FDTD methods applied to microwave energy deposition in biological systems. Since 1989, he has been with McKesson BioServices, Brooks AFB, TX, where he is engaged in microwave bioeffect research. His current research interests are microwave dosimetry and FDTD modeling of UWB exposure systems. He is an author of *Electronic Devices, Applications, and Integrated Circuits* (Delhi, India: Umesh, 1980).



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radio frequency radiation bioeffects.



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Dr. Mason is a member of the Bioelectromagnetics Society and the Society for Neuroscience.



Eric A. Holwitt taught chemistry at the U.S. Air Force Academy from 1980 to 1984. While at the Armed Forces Radiobiology Research Institute, he developed methods for synthesizing radiation products of DNA. He has also studied the interaction of radioproductive compounds with DNA. Other projects he has participated in are developing a biosynthetic polymer to destroy hydrazine and demonstrating the radiosensitivity of an Air Force patented material. During this time, he served as a UN weapons inspector with UNSCOM in Iraq and,

for the last three years, has been working on chemical and biological defense issues for the Air Force. He has co-authored 15 publications and has two patents. Besides research, he popularizes science throughout San Antonio, TX, performing chemistry magic shows for schools and civic groups.