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A Comparison of the Effects of Microwave Irradiation and Heat Treatment of T4 and T7 Bacteriophage

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In an attempt to elucidate the mechanism of sterilization via microwave irradiation, a comparison of irradiated and heated T4 and T7 coliphages was executed. Samples of T4 and T7 were subjected to 20 seconds of microwave irradiation using a 2,450 MHz microwave oven. Additionally, samples of T4 and T7 were heated to 83° C for 25 minutes. The effects of the different treatments were evaluated via survival assays and agarose gel electrophoresis. The heat-treated phage clearly exhibited decreased survival, but the irradiated phage did not. Digestion of the irradiated and heated phage with endonuclease KpnI followed by agarose gel electrophoresis was not successful for determination of the mechanism of phage inactivation.

The effectiveness of microwave irradiation as a means of sterilization has been acknowledged for several decades (http://www.rfsafe.com/article536.html). But, the underlying mechanism for sterilization has not yet been elucidated. At present, two putative mechanisms for the inactivation of microbes via microwave irradiation exist. One proposal suggests that thermal energy is the sole cause of inactivation, whereby heat denatures enzymes, nucleic acids and disrupts membranes (http://vm.cfsan.fda.gov/~comm/ift-micr.html). The other mechanism proposes that inactivation occurs due to non-thermal effects of selective heating, electroporation, cell membrane rupture or magnetic field coupling (http://vm.cfsan.fda.gov/~comm/ift-micr.html). The selective heating model suggests that microbes are heated to a greater extent than the surrounding medium, subsequently killing them. Electroporation, in which the formation of membrane pores due to electron potential across the membrane, causes leakage from microbes resulting in death. Rupture of the cell membrane may be the result of voltage drop across the membrane. And finally, the coupling of electromagnetic energy with essential cellular components disrupts internal activities and leads to cell lysis (http://vm.cfsan.fda.gov/~comm/ift-micr.html). There are lines of evidence which support both the thermal and non-thermal theories. Evidence that microwave irradiation was indeed different from heating was demonstrated when samples of DNA from PL-1 bacteriophage subjected to each treatment was compared. Evaluation using electron microscopy showed DNA from irradiated samples was severely fragmented while DNA from heated samples was not. Additionally, gel electrophoresis showed that the DNA from the microwaved bacteriophage had generally disappeared (4). A double tube system, which allows the input of microwave energy to be coupled with cooling water to remove thermal energy, has also been tested (http://vm.cfsan.fda.gov/~comm/ift-micr.html). Inactivation of *Enterobacter aerogenes*, *Escherichia coli (E. coli)*, and *Listeria innocua* in various consumable fluids at sub-lethal temperatures was not observed when the double tube system was used. It was therefore concluded that microwave inactivation of microbes required additional factors, such as pH or heat stress (http://vm.cfsan.fda.gov/~comm/ift-micr.html).

Previous attempts have used microwave irradiation of T4 and T7 phage at a reduced temperature to investigate the mechanism of sterilization (6).This experiment was designed to further investigate the controversial mechanisms of microwave irradiation inactivation of microbes. The intent was to examine the differences between microwave irradiation at low temperature and heat treatment at lethal temperature as a continuation of this investigation. It was speculated that both microwave irradiation and heat treatment would cause the phage capsid to rupture, resulting in the release of bacteriophage DNA.

MATERIALS AND METHODS

Bacteria and phage. Wildtype *E. coli* B23 was used as the host strain for bacteriophages T4 and T7 in this experiment. Bacteria and phage were provided as a gift from Dr. William Ramey of the University of British Columbia Department of Microbiology and Immunology.

Phage stock preparation and phage titring. An overnight culture was prepared by inoculating 10 ml of Luria broth (tryptone (1.0 g), sodium chloride (1.0 g), yeast extract (0.5 g), distilled water (to 100 mL)) with a single colony of *E. coli* B23. The culture was grown with gentle aeration at 37° C for approximately 18 hours. The phage was amplified using a standard plaque overlay technique (5). First, plates of bottom agar (tryptone (9.7 g), sodium chloride (6.0 g), sodium citrate (1.5 g), glucose (0.98 g), agar (11.25 g), and distilled water (to 750 ml)) were warmed by placing them in the 37° C incubator for at least 30 minutes. Top agar (tryptone (1.96 g), sodium chloride (1.2 g), sodium citrate (0.3 g), glucose (0.45g), agar (1.13 g),

and distilled water (to 150 ml)) was microwaved at high power until completely melted. Small tubes were immediately filled with 3 ml of molten top agar and placed in a 50° C water bath. Stock of bacteriophage T4 and T7 was serially diluted with tryptone sodium glucose (TSG) broth (tryptone (0.05 g), sodium chloride (0.3 g), yeast extract (0.7 g), D-glucose (0.13 g), and distilled water (to 100 ml)) to a final dilution of 10^{-7}. Two hundred microliters of overnight culture *E. coli* B23 was added to the tubes of top agar, followed by 100 µl of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} diluted phage. The contents were mixed by phage-style mixing, then directly poured onto plates of bottom agar. Plates were gently tilted whilst pouring top agar to ensure even coverage. The plates were allowed to solidify for approximately 40 minutes, then inverted and incubated at 37° C for 18-20 hours. The top agar from plates showing confluent lysis was gently scraped into separate sterile centrifuge tubes using a sterilized glass rod. Approximately 2 ml of TSG was used to rinse the remaining top agar and also deposited into the corresponding centrifuge tube. One hundred microliters of chloroform was added and the tubes were incubated for 30 minutes at room temperature with periodic mixing. The tubes were then incubated at 4° C for 72 hours and then centrifuged at 11,000g for 10 minutes at room temperature. The supernatants were collected and stored in a screw cap tube with 200 µl chloroform at 4° C. The amplified phage suspensions were titred by performing serial dilutions to 10^{-8} then plated using the above described plating method. The titre of T4 was 4.7 x 10^{10} pfu/ml and the titre of T7 was 5.4 x 10^{9} pfu/ml.

Microwave irradiation. A sterile system to prevent the temperature of the phage suspension from rising during irradiation was prepared. Small (10 ml) centrifuge tubes were filled halfway with water then inserted into large (30 ml) centrifuge tubes and autoclaved. The tubes were placed in the -20° C freezer overnight to allow the water to freeze. The cavity in-between the tubes was filled with 0.5 ml of undiluted amplified phage suspension. The tubes were placed in a beaker containing cold water and microwaved at high power for 20 seconds using a 2,450 MHz microwave oven (Samsung Electronics, Mississauga, Ontario, Canada). The irradiated phage suspension was recovered using a sterile Pasteur pipette.

Heat treatment. Sterile small test tubes were filled with 0.5 ml of undiluted amplified phage and placed in an 83° C water bath. The samples were heated for 25 minutes.

Survival assay. The percent of phage surviving the heat and irradiation treatments was determined by the plaque overlay method as described above (5).

Restriction digest. Samples of heated, irradiated and untreated phage were subjected to digestion with restriction endonuclease KpnI. As the DNA concentration in the phage suspensions was not known, two sets of digestions were performed. One set was prepared using 18 µl of undiluted amplified phage suspension, 2 µl of REact4™ buffer (16304-016, Invitrogen) and 0.5 µl of KpnI (15232-036, Invitrogen). The other set was prepared using 36 µl of undiluted amplified phage suspension, 2 µl of REact4™ and 1.0 µl KpnI. The samples were briefly spun down then placed in a 37° C water bath for 80 minutes. Samples were held on ice until electrophoresis.

Electrophoresis. A sufficient volume of 6x sample buffer (bromophenol blue (0.025 g), xylene blue (0.025 g), 50% glycerol (6 ml), 50x TBE buffer (1.2 ml) and distilled water (2.8 ml)) was added to the samples digested with KpnI and the untreated controls. The samples were loaded onto a 0.4 % agarose gel in 1x tris-borate buffer (20x recipe: tris-base (216 g), EDTA (14.89 g), boric acid (110 g), distilled water (to 1 litre) to a final pH of 8.3). HindIII digested λ phage (15612-013, Invitrogen) was used as the DNA ladder. Five microliters of undiluted ladder, 6 µl of 10^{-1} diluted ladder, 7 µl of 10^{-2} diluted ladder and 7 µl of 10^{-3} diluted ladder were included in the gel to compensate if the gel needed to be overexposed during visualization. Electrophoresis continued for 30 minutes at 120 volts and then the gels were stained in an ethidium bromide bath (0.2 µg/ml). DNA fragments were viewed using a UV-transilluminator and photographed. As the DNA had migrated too short a distance to be interpreted, electrophoresis of both gels was continued at 20 volts overnight (approximately 14 hours).

RESULTS

Comparison of the effects of microwave irradiation and heat treatment on T4 phage. Untreated T4 bacteriophage, and T4 subjected to microwave irradiation or T4 subjected to heat treatment were assayed for differences in survival using standard plaque assay (5). The titre of the untreated samples was 4.9 x ^{10} pfu/ml. The titre of the phage irradiated for 20 seconds was 3.8 x ^{10} pfu/ml. The titre of phage heated at 83° C for 25 minutes was 1.0 x ^{10} pfu/ml. The different treatments were also analyzed via KpnI endonuclease digestion followed by electrophoresis. Electrophoretic migration of T4 samples was visualized after running for 30 minutes at 120 volts and after running overnight at 20 volts. Microwave irradiation for 20 seconds results in the same banding pattern as no treatment when digested with KpnI (lanes 2, 3, 5, 6, Fig.1). The irradiated and untreated samples which were not subjected to KpnI digestion (lanes 8, 9, Fig. 1) show the same pattern but contain more DNA in the low molecular weight bands following the dye front. The heat treated samples show a long smear (lanes 1, 4, 7, Fig.1).

Continuing electrophoresis overnight resulted in the smaller molecular weight bands running off the gel, although the large molecular weight bands are more clearly defined (Fig. 2). While the restriction digest did not appear to be successful, there was sufficient phage DNA to be visualized using agarose gel electrophoresis stained with ethidium bromide.

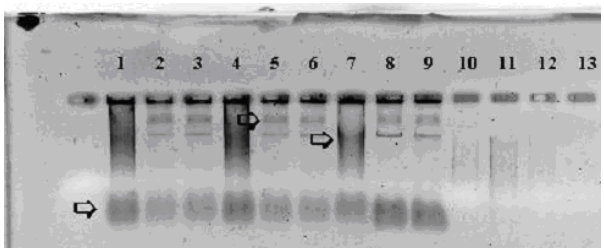

FIG. 1 Electrophoresis of irradiated, heated and untreated samples of T4 bacteriophage in 0.4 % agarose at 120 volts for 30 minutes and stained with ethidium bromide. The samples are heated T4 digested with 1 µl KpnI (lane 1), untreated T4 digested with 1 µl KpnI (lane 2), irradiated T4 digested with 1 µl KpnI (lane 3), heated T4 digested with 0.5 µl KpnI (lane 4), untreated T4 digested with 0.5 µl KpnI (lane 5), irradiated T4 digested with 0.5 µl KpnI (lane 6), heated T4 without KpnI digestion (lane 7), untreated T4 without KpnI digestion (lane 8), irradiated T4 without KpnI digestion (lane 9), undiluted λ phage digested with HindIII (lane 10), 10^{-1} diluted λ phage digested with HindIII (lane 11), 10^{-2} diluted λ phage digested with HindIII (lane 12), 10^{-3} diluted λ phage digested with HindIII (lane 13).

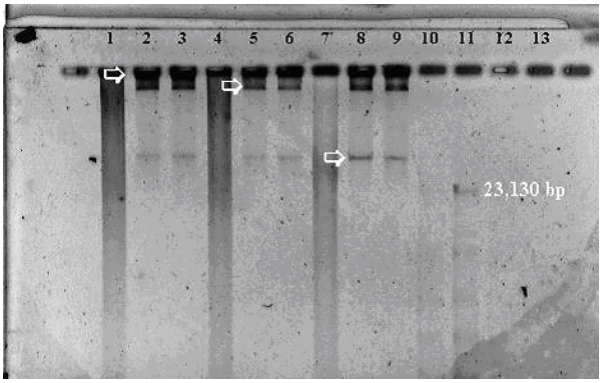

FIG. 2 Electrophoresis of irradiated, heated and untreated samples of T4 bacteriophage in 0.4 % agarose at 120 volts for 30 minutes followed by 20 volts for 14 hours and stained with ethidium bromide. The samples are heated T4 digested with 1 µl KpnI (lane 1), untreated T4 digested with 1 µl KpnI (lane 2), irradiated T4 digested with 1 µl KpnI (lane 3), heated T4 digested with 0.5 µl KpnI (lane 4), untreated T4 digested with 0.5 µl KpnI (lane 5), irradiated T4 digested with 0.5 µl KpnI (lane 6), heated T4 without KpnI digestion (lane 7), untreated T4 without KpnI digestion (lane 8), irradiated T4 without KpnI digestion (lane 9), undiluted λ phage digested with HindIII (lane 10), 10^{-1} diluted λ phage digested with HindIII (lane 11), 10^{-2} diluted λ phage digested with HindIII (lane 12), 10^{-3} diluted λ phage digested with HindIII (lane 13).

Comparison of the effects of microwave irradiation and heat treatment on T7 phage. Untreated T7 bacteriophage, and T7 subjected to microwave irradiation or T7 subjected to heat treatment were assayed for differences in survival using standard plaque assay (5). The titre of the untreated samples was 8.6 x ^{8} pfu/ml. The titre of the phage irradiated for 20 seconds was 1.5 x ^{9} pfu/ml. The titre of phage heated at 83° C for 25 minutes was 1.0 x ^{7} pfu/ml. These samples were also treated with KpnI, followed with agarose gel electrophoresis. Electrophoretic migration was visualized after running the gel overnight at 20 volts (Fig. 3). Very little T7 DNA was visible. Irradiated T7 phage, not subjected to KpnI endonuclease treatment, contains a dense, high molecular weight band (Fig. 3, lane 9). It is possible that this band represents the entire 39 kb T7 genome. Unfortunately this band migrated more slowly than the highest molecular weight band in the lanes containing HindIII digested λ DNA. The highest molecular weight marker present in the ladder is 23,130 bases. It is clear that band in lane 9 is larger than 23,130 bases, but it is not safe to speculate how much larger via extrapolation. Bands are not visible in lanes 1 through 8.

DISCUSSION

Sterilization via microwave irradiation is speculated to occur either entirely by heat-induced damage or by non-thermal factors. Commercial microwave ovens utilize electromagnetic waves, typically at a frequency of 2,450 MHz (http://vm.cfsan.fda.gov/~comm/ift-micr.html). The heating theory for sterilization proposes that the microwaves cause vibrations at the atomic level to occur within the phage, resulting in their conversion to thermal energy (http://vm.cfsan.fda.gov/~comm/ift-micr.html). Thermal energy manifests as heat which capable of denaturing capsid proteins, essential enzymes, and nucleic acid if temperatures are high enough. This theory is supported by experiments with *E. coli* (1,2) and *Bacillus subtillis* (2). A group investigating microwave heating of food and microwave inactivation of foodborne pathogens found that thermal factors were responsible. They assert that conflicting evidence for non-thermal factors may have been due to previous difficulties with obtaining precise temperature measurements (3).

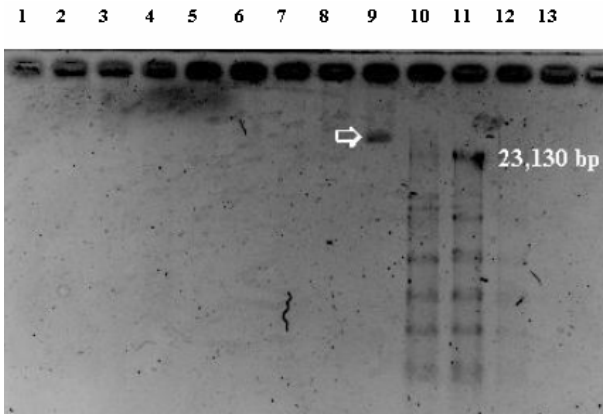

FIG. 3 Electrophoresis of irradiated, heated and untreated samples of T7 bacteriophage in 0.4 % agarose at 120 volts for 30 minutes followed by 20 volts for 14 hours and stained with ethidium bromide. The samples are heated T7 digested with 1 µl KpnI (lane 1), untreated T7 digested with 1 µl KpnI (lane 2), irradiated T7 digested with 1 µl KpnI (lane 3), heated T7 digested with 0.5 µl KpnI (lane 4), untreated T7 digested with 0.5 µl KpnI (lane 5), irradiated T7 digested with 0.5 µl KpnI (lane 6), heated T7 without KpnI digestion (lane 7), untreated T7 without KpnI digestion (lane 8), irradiated T7 without KpnI digestion (lane 9), undiluted λ phage digested with HindIII (lane 10), 10^{-1} diluted λ phage digested with HindIII (lane 11), 10^{-2} diluted λ phage digested with HindIII (lane 12), 10^{-3} diluted λ phage digested with HindIII (lane 13).

The results of this experiment were not the same as those obtained in a similar experiment (6). In the previous experiment, a time course of phage inactivation was performed which demonstrated that 20 seconds of irradiation with a 2,450 MHz microwave was sufficient to achieve virtually 0 % survival. Using the same model of microwave 77 % survival was achieved when samples of T4 were irradiated for 20

seconds, while the results of the T7 survival assay indicated that survival exceeded 100%. The procedures for microwaving the phage were similar in both experiments. The temperature of phage suspension was kept low by utilizing a tube-within-a-tube system whereby the inner tube contained ice in both experiments. But, as I was unable to aseptically obtain the temperature of phage suspension after microwaving, I placed the samples in a beaker of cold water during the microwave procedure. The previous study had performed irradiation of the samples contained in a empty beaker. Thus, I inadvertently increased the depth the 2,450 waves needed to penetrate without compensating for duration.

Fig. 1 and 2 show the electrophoretic mobility of T4 samples. Digestion with endonuclease KpnI would produce 7 fragments of genomic DNA in samples whose capsid had ruptured, releasing phage DNA (7). Microwave irradiation or heat treatment was expected to rupture the bacteriophage capsid. The untreated phage would not be digested with KpnI as the DNA would be protected within the intact capsid. Lanes that show a long smear may represent T4 genome, fragmented during the heat treatment (lanes 1, 4, 7 Fig.1, Fig. 2). Of the heat treated samples, KpnI digested (lanes 1, 4, Fig. 1, Fig. 2) and undigested samples (lane 7, Fig. 1, Fig. 2) look similar, except the undigested sample shows less high molecular weight density. This may be due to the action of KpnI cleaving DNA at some of its 7 recognition sites present in the T4 genome (7). The high molecular weight bands (lanes 2, 3, 5, 6, 8, 9 Fig. 2) could represent the entire 168 kb T4 genome. These high molecular weight bands have migrated far more slowly than the largest λ-HindIII fragment , which was 23,130 bases. If this band is undigested T4 genome, it reveals that the KpnI restriction digest was unsuccessful and unfortunately, a control consisting of intact T4 genome was not included in the gel. The method of endonuclease digestion (5) suggested using a maximum of 18 µl of phage suspension, with 2 µl appropriate buffer and 0.5 µl restriction enzyme. Due to concern of low phage DNA concentration, the digest was also performed using twice as much phage suspension and enzyme. Perhaps the phage suspension should have been concentrated prior to endonuclease treatment.

The results obtained using T7 phage were poor. The titre of amplified T7 was 5.4 x 10^9 pfu/ml. This value is significantly lower than the amplified T4 titre of 4.7 x 10^{10} pfu/ml. Another difficulty arose from the presence of agar remaining in the amplified T7 phage suspension. The survival assay via plaque overlay revealed greater survival for the irradiated phage than the untreated T7, while the heated phage exhibited very poor survival. The difference in survival for irradiated and untreated phage may reflect an error in technique. A simple filtration to remove the agar should have been performed to eliminate the problem of inaccurate pipetting due to agar interference. The inability to see DNA bands or smears from the T7 samples (Fig. 3) is most likely due to a low concentration.

Unfortunately, procedural difficulties encountered early in the experiment prevented sufficient time be available to repeat various aspects of the experiment. It would have been beneficial to perform a time course of phage inactivation by irradiation, since 20 seconds was not sufficient when the samples were microwaved in a container of water. Also, determining the DNA concentration of the phage suspension prior to KpnI treatment may have lead to better digestion. Observing the agarose gels when the sample dye was about to migrate off of the gel may have shown some smaller molecular weight bands in the T4 gel. The T4 survival assay showed that irradiation for 20 seconds resulted in a decrease in survival, while the T7 survival assay did not. Further investigation into the mechanism of sterilization by microwave is warranted, as this technology provides an economical and rapid technique with potential for widespread application.

FUTURE EXPERIMENTS

Future investigations into the effects of microwave irradiation on T4 and T7 bacteriophage should concentrate on first purifying the phage. Several procedures for phage purification are available (5). The poor results obtained for T7 samples indicate these steps are necessary. Phage samples should also be subjected to various durations of irradiation to determine the time required for inactivation, as 20 seconds did not appear to be sufficient. It would be interesting to investigate the effects that combined irradiation and heating have on bacteriophage.

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