APPLIED MICROBIOLOGY, Sept. 1974, p. 406-410

Vol. 28, No. 3
Printed in U.S.A.

Thermoradiation Inactivation of Naturally Occurring Bacterial Spores in Soil

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Received for publication 10 May 1974

Samples of soil collected from the Kennedy Space Center near the spacecraft assembly facilities were found to contain microorganisms very resistant to conventional sterilization techniques. The inactivation kinetics of the naturally occurring spores in soil were investigated by using dry heat and ionizing radiation, first separately and then simultaneously. Dry-heat inactivation kinetics of spores was determined at 105 and 125 C; radiation inactivation kinetics was determined for dose rates of 660 and 76 krads/h at 25 C. Simultaneous combinations of heat and radiation were then investigated at 105, 110, 115, 120, and 125 C, with a dose rate of 76 krads/h. Combined treatment was found to be highly synergistic, requiring greatly reduced radiation doses to accomplish sterilization of the population.

For some time, it has been recognized that the heat stability of bacterial spores may be altered considerably by the manipulation of cultural conditions during sporulation (10). Recent studies of the dry-heat inactivation kinetics of spore populations in soil have shown that the heterogeneous soil populations exhibited higher dry-heat resistance levels than subcultured survivors obtained at corresponding intervals on the survivor curves (2); subsequent to this study, a naturally occurring spore population in soil collected at the Kennedy Space Center, Florida, was found to be exceptionally resistant to dry heat at 125 C (1). Since the organisms indigenous to local soils could comprise part of the bacterial flora of industrial manufacturing and assembly areas, it was recognized that these highly resistant spore populations were possible contaminants of spacecraft surfaces (12, 13, 14, 19). This report describes efforts to develop a more effective sterilization procedure applicable to labile articles, such as extraterrestrial life-detection spacecraft.

MATERIALS AND METHODS

Preparation of soil specimen. Soil samples were taken in three different locations at the Kennedy Space Center, Florida, and were mixed. The composite sample was spread in a thin layer on sterile paper, covered, and allowed to dry for 48 h at room temperature. This dry soil (2,771 g) was then processed dry through a stainless-steel sieve series (W. S. Tayler Co., Cleveland, Ohio) down to a 0.124-mm screen size to remove rocks, shell particles, and plant particles. A series of rinses in 95% ethyl alcohol (6) and then a final sieving with a 0.043-mm screen resulted in 4,900 ml of soil-spore suspension in 95% ethyl alcohol. Serial dilutions in sterile deionized water were plated with Trypticase soy agar (BBL), supplemented with 0.1% soluble starch and 0.2% yeast extract, and were incubated aerobically for 1 week at 35 C (3). The viable concentration was 2.7×10^5 organisms per ml, and the approximate weight of soil in the suspension was 0.03 g/ml.

The soil suspension described was used as the inoculum for all the experiments, and the suspension was maintained at 4 C during storage. Prior to use, the soil-spore suspension was insonated (9, 11) for 2 min in an ultrasonic bath (TURCO 750-W output with a cavitation intensity of 1.75 W/cm^2) to break up clumps within the ethyl alcohol suspension, and it was continuously agitated with a stirring bar during inoculation to prevent settling. The samples were prepared by applying 0.1 ml of the suspension onto the surface of 0.038-mm thick, biological-grade aluminum foil disks, 32 mm in diameter. The samples were then allowed to air dry until the ethanol evaporated. When dry, the inoculated disks were assembled on aluminum strips (38 by 200 by 0.51 mm); four sample disks were placed on each strip, a single clean foil disk was placed over each sample, and then another aluminum strip was placed on top and held firm with wire clamps. This assembly of the disks clamped between two strips permitted considerable handling and suspending of the assembly in a vertical position without loss or damage to the sample disks. The assembled sample strips were then placed in a desiccator over Drierite (W. A. Hammond Drierite Co.) in a

vacuum for 15 h prior to exposure to the sterilization environment. All of the inoculation and assembly operations were performed in a class-100 laminar airflow clean room (7, 21, 22).

Exposure methods. The thermal environment was provided by a recirculating air temperature chamber (Delta Design model 1060 w/type III controller) having a volume of 0.016 m^3, with a rail arrangement in the door to hold the aluminum strips; temperature was controlled and recorded to an accuracy of ±0.2 C. The radiation environment was provided by Sandia Laboratories Gamma Irradiation Facility which contained remote handling equipment to introduce and remove the source, including visual, physical, and electrical access with necessary safety controls. The ^{60}Co source was introduced in a corner of the cell (2.13 by 2.44 by 2.59 m), and the dose rates ranged from 10^6 to 4×10^3 rads/h depending on the location of the sample within the cell.

Moisture content of the air in the temperature chamber was controlled by a system (8) which passed pressurized air through a saturator in a warm water bath and then through a condenser coil and water trap in a cold-water bath. The air was allowed to expand into a coil in the warm-water bath prior to entry into the temperature chamber. Adjustments of the air pressure and the temperature of the cold-water bath provided a controlled relative humidity for the experiments of 30 ± 1% measured at 25 C. Relative humidity measurements of the air were made at the input to the temperature chamber with a dew-point indicator, and continuous measurements were provided by lithium chloride sensors and a strip chart recorder. The rate of airflow into the temperature chamber was controlled to 0.236 liter per s, and the air pressure in the chamber was regulated at approximately 23.2 kg/m^2 by adjustment of a bleeder valve on the chamber.

For each radiation experiment, the temperature chamber was placed in the Gamma Irradiation Facility cell at the appropriate distance from the ^{60}Co source for the desired dose rate, and the chamber was positioned so that the sample strips assembled with the foil disks were vertical and the faces of the strips were perpendicular to the direction of the gamma rays. The temperature chamber controller, temperature recorder, and humidity control system were located outside the cell with the necessary cable connections passing through the cell wall. A block diagram of the equipment setup is shown in Fig. 1. Silver phosphate or cobalt glass dosimeters, depending on the dose range, were placed on selected sample strips to verify the computed dose rates.

Recovery methods. Each sample strip, when removed from the temperature chamber, was wrapped in sterile aluminum foil and returned to the class-100 clean room facility for recovery operations. A 20- to 30-min time period was required to transport the samples from the remote Gamma Irradiation Facility area.

Each of the samples from the strips was placed in a separate 50-ml beaker containing 10 ml of sterile 0.1% Tween 80 (BBL Polysorbate 80) in distilled water. The samples were then insonated for 2 min to remove the organisms from the foil disks. Care was exercised in placing the foil disks into the beakers, to assure separation of the inoculated disk from the cover disk and to complete wetting and submersion of both disks. The insonation was accomplished with the beakers immersed in the ultrasonic water bath to a level just above the recovery fluid level in the beaker. Occasional agitation of the beakers kept the disks separated and prevented the disks from cold welding.

Appropriate 10-fold serial dilutions of the recovery fluid for each sample were made in sterile deionized water and plated in duplicate with supplemented Trypticase soy agar. Prior to pour-plating, petri dishes were prepared by pouring a thin layer of agar medium to underlay the specimen. After pour plates of the dilutions were solidified, sterile agar medium was carefully overlaid on each plate to retard spreading bacterial growth. Plates were counted after a 1-week incubation at 35 C, and each data point represents the mean value from four replicate sample foil disks.

RESULTS

Experimentation with the naturally occurring spores in soil was directed primarily toward the response to dry-heat treatment, radiation treatment, and the combination of dry heat and gamma radiation (thermoradiation) (20).

The results of the first base-line experiment to determine dry-heat resistance of the organisms in soil at 125 C are shown in Fig. 2. The viable population, beginning at 2×10^4 organisms per disk (zero heat treatment), underwent a rapid reduction to about 10^2 organisms per disk during the first treatment period. The initial drop, found to be characteristic of naturally occurring organisms in soil (2), was followed by a second logarithmic phase of destruction. The second phase represents a very heat-resistant subpopulation of approximately 1% of the original sample. Except for radiation inactivation, this biphasic order of death was noted in all dry-heat and thermoradiation experimentation. The second phase on the logarithmic part of the survivor curve was used as a basis of comparison for various treatments. By using the method of least squares, the data were fit with a straight line so that slopes or D values (time at temperature or the radiation dose required to reduce the viable population by 90%) might be compared. The 95% confidence intervals are also shown. The D_{125C} value of the resistant subpopulation was 29 h (Fig. 2). This value is roughly 50 to 100 times the D_{125C} value for *Bacillus subtilis* var. *niger* spores (4), an organism commonly used as a biological indicator for dry-heat sterilization cycles.

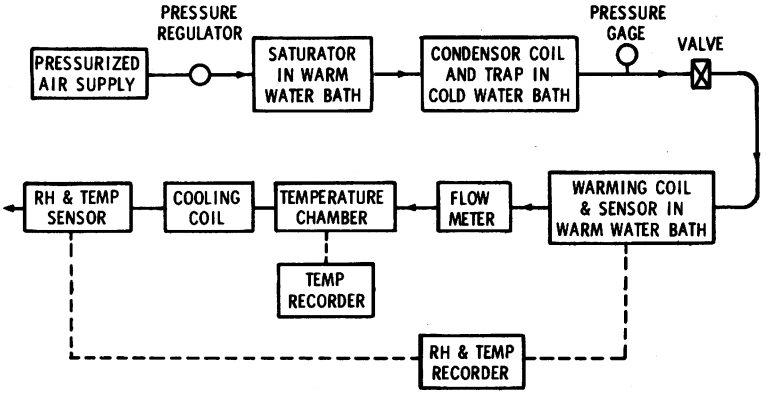

FIG. 1. *Equipment setup for thermoradiation experiment with controlled humidity. RH, Relative humidity.*

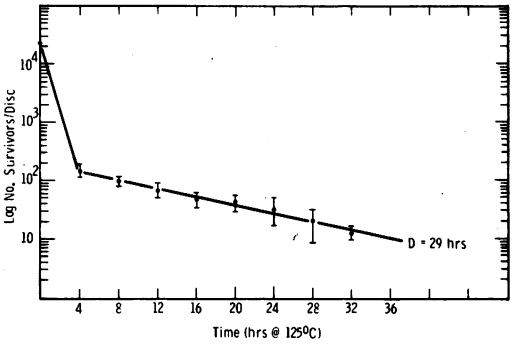

FIG. 2. *Dry-heat inactivation of naturally occurring spores in soil.*

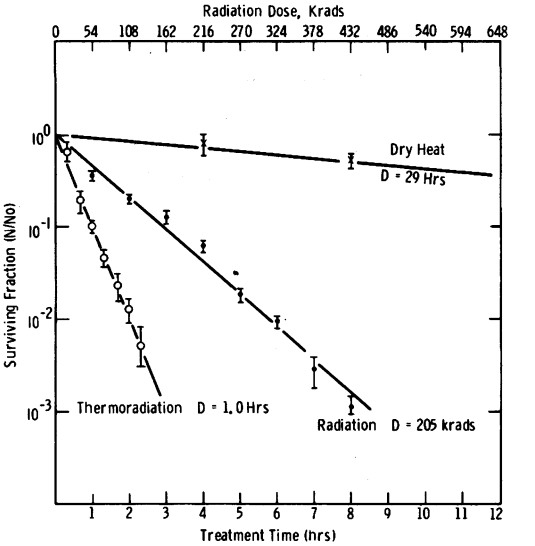

FIG. 3. *Comparison of thermoradiation, radiation, and dry-heat inactivation of naturally occurring spores in soil.*

The radiation resistance of the naturally occurring spores in soil was then determined. Samples were exposed at room temperature (25 C) to gamma radiation from the ^{60}Co source. Temperature and moisture-conditioned air was supplied to the sample chambers at a flow rate which provided one air change per minute. The results of irradiation are shown in Fig. 3. In this figure, the data from dry-heat inactivation at 125 C (Fig. 2) were repeated to simplify comparisons of the inactivation rates with various treatments. The second curve shows results of radiation at room temperature with a dose of 54 krads/h, and the D value was found to be 205 krads (3.8 h). Although the dose rate was 76 krads/h, the samples were actually exposed to the source for only 45 min out of each hour of treatment. The 15 min of down time was required to remove the samples from the Gamma Irradiation Facility cell. The third curve on Fig. 3 is the result of simultaneously combining dry heat and radiation. The thermoradiation D value was found to be approximately 1 h as compared to 3.8 h for radiation alone or 29 h for heat alone. For an example of the synergism obtained, the singular effects of heat and radiation, when added, would reduce the population by 1 log after 3 h of treatment. Thermoradiation, for the same time at temperature and total dose, reduced the population by 3 logs. If the slopes of these examples are examined, the singular effects of heat and radiation, considered in an additive sense, would result in a D value of 3.37 h. The thermoradiation D value, 1 h, is less than one-third that of the additive effects. This level of synergism means that sterilization of this spore population could be accomplished in one-third of the time at temperature with one-third of the normal dose.

Additional experiments were performed to

determine the characteristics of thermoradiation treatment at temperatures below 125 C. For this, a constant dose rate, sampling time, and moisture environment were maintained throughout, with temperature as the only variable. The D values of the resistant subpopulations were found to be 57 min at 125 C, 60 min at 120 C, 77 min at 115 C, 89 min at 110 C, and 95 min at 105 C (Fig. 4). The interesting aspect of this series of experiments was the manner in which the shape of the inactivation curve changed. As the temperature was lowered, the sharp initial drop usually experienced at 125 C was lessened, requiring a longer exposure to reach the second phase of inactivation.

To illustrate the temperature-dose relationship, the D values presented in Fig. 4 were plotted as a function of temperature in Fig. 5. For example, at 105 C with a 95-min D value, the radiation dose per log population reduction would be 121 krads per log. As the temperature was elevated (constant dose rate), the D value dropped to 57 min with a total dose of 72 krads per log population reduction, or roughly 60% of the radiation required at the lower temperature.

DISCUSSION

Naturally occurring bacterial spores in soil present a very realistic and yet difficult contamination problem when dealing with a labile article, such as a spacecraft destined to undergo a terminal sterilization cycle prior to launch.

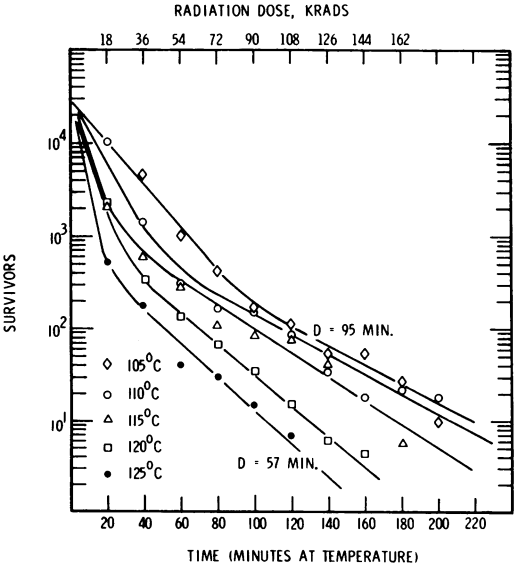

FIG. 4. *Thermoradiation inactivation of naturally occurring spores at various temperatures.*

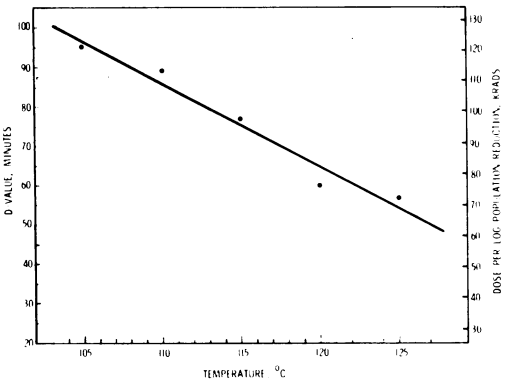

FIG. 5. *D value versus temperature at a dose rate of 76 krads/h.*

Although many microorganisms are resistant to either heat or radiation, the naturally occurring bacterial spores in soil are resistant to both. As such, the spores in soil have been a realistic adjunct to past studies of heat and/or radiation resistance based primarily on the standard test spore of *Bacillus subtilis* var. *niger*. The synergistic behavior of dry heat and ionizing radiation on the spores in soil was found to be in agreement with observations of the inactivation kinetics of other bacterial spores, bacteriophages, proteins, viruses, and yeasts (5, 15, 17, 18); however, the synergism exhibited with the naturally occurring organisms appeared to be more pronounced than with *Bacillus subtilis* var. *niger* tests (a factor of 3.25 versus 2.6, respectively [16]).

To persons or organizations dealing with the sterilization of labile articles, thermoradiation offers several advantages over conventional sterilization cycles. When actual microbial populations contaminating a particular article are defined (these are naturally occurring populations directly from environments associated with the article), laboratory tests can easily be designed to identify the most effective regions of synergism. By manipulating either or both of the synergistic components, sterilization cycles can be adjusted to optimize treatment efficacy and minimize the degradation of the article being sterilized. Thermoradiation in many instances also has the distinct advantage of being less sensitive to environmental parameters than either heat or radiation when applied separately (21).

ACKNOWLEDGMENTS

The services provided by Sandia Laboratories and the Phoenix Laboratories Center for Disease Control were in support of the planetary quarantine requirements of the National Aeronautics and Space Administration under contracts W-12,853 and W-13,062, respectively.

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