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Effects of Thermoradiation on Bacteria

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A ^{60}Co source was used to determine the effects of thermoradiation on *Achromobacter aquamarinus*, *Staphylococcus aureus*, and vegetative and spore cells of *Bacillus subtilis* var. *globigii*. The rate of inactivation of these cultures, except vegetative-cell populations of *B. subtilis*, was exponential and in direct proportion to temperature. The D_{10} (dose that inactivates 90% of the microbial population) value for *A. aquamarinus* was 8.0 Krad at 25°C and 4.9 Krad at 35°C. For *S. aureus*, D_{10} was 9.8 and 5.3 Krad at 35 and 45°C, respectively. Vegetative cells of *B. subtilis* demonstrated a rapid initial inactivation followed by a steady but decreased exponential rate. The D_{10} at 25°C was 10.3 Krad, but at 35 and 45°C this value was 6.2 and 3.8 Krad, respectively. Between 0 and 95 Krad, survival curves for *B. subtilis* spores at 75°C showed slight inactivation, increasing in rate at and above 85°C. The D_{10} values for spores at 85 and 90°C were 129 and 92 Krad, respectively. Significant synergism between heat and irradiation was noted at 35°C for *A. aquamarinus* and 45°C for *S. aureus*. The presence of 0.1 mM cysteine in suspending media afforded protection to both cultures at these critical temperatures. On the other hand, cysteine sensitized *B. subtilis* spores at radiation doses greater than 100 Krad. The combined effect of heat and irradiation was more destructive to bacteria than either method alone.

A number of reports have been published in the last few years describing the effect of heat on the radiation sensitivity of bacteria. Several investigators (4-6, 19) established that pre-irradiation sensitized spores of *Bacillus subtilis* to heating, whereas preheating had no effect on the radiation resistance of *Clostridium botulinum* spores (15). The use of heat and radiation in simultaneous combination (thermoradiation) could be of value in medical and pharmaceutical industries as well as in food processing (11, 13, 20, 33). Over certain ranges of temperature and dose rate, the process causes a much greater reduction in a given bacterial population than that which could be anticipated if first heat and then radiation were applied (30). The effectiveness of the combined treatment may even increase due to the possibility of synergism (25, 29). Kempe (16) reported that the use of a one-third radiation sterilization dose reduced the heat treatment required to sterilize a substance to about one-fourth. The radiosensitivity of some bacteria increased as a function of rise in temperature, and the rate of their destruction was significantly greater when ionizing and thermal energies were applied simultaneously rather than consecutively (18). Purdie et al. (24) reported that NO_2 sensitized *B. megaterium* spores at a low dose rate but not at a high dose rate and that the response of oxic spores (O_2) was unaffected by increasing the dose rate in the range of 0.1 to 10 Krad/μs pulses of electrons. Other investigators used organic (22) and inorganic sensitizers (26, 27, 32) to increase the radiation sensitivity of these spores. Grecz et al. (10) showed that during irradiation the resistance of *C. botulinum* 33A spores decreased progressively with increasing temperature. Briggs and Yazdany (3) stated that thermoradiation as a sterilization process is less damaging to the materials but would probably be unable to maintain accepted standards of freedom from contamination. Emborg (9) showed that at 100°C the radiation response of *B. subtilis* spores was unaffected by the high temperature and concluded that such treatment may have little practical application in sterilization of medical equipment. In view of these conflicting reports, we decided to examine the effects of thermoradiation on spore and vegetative cells of some bacteria and the role of synergism.

MATERIALS AND METHODS

Organisms and preparation of cell suspensions. The organisms used were *Achromobacter aquamarinus* (ATCC 14400), vegetative cells and spores of *B. subtilis* var. *globigii* (ATCC 9372), and *Staphylococcus aureus* (ATCC 6538 P). All cultures were propagated in Difco tryptic soy broth (TSB) except for *A. aquamarinus*, which was grown in TSB supplemented with 3% sodium chloride. Sterile flasks of TSB were inoculated with a 2% test culture and

incubated at 37°C on a Burrell wrist-action shaker kept at 150 cycles/min. Incubation times, chosen to obtain viable cells in middle log phase, were determined from previously established growth curves for each test culture and were 8 h for *A. aquamarinus* and *S. aureus* and 5 h for vegetative cells of *B. subtilis*. The cells of the test culture were then harvested by centrifugation at 4°C, washed three times, and resuspended in sterile saline to approximately 10^{10} cells/ml. Clean spore crops of *B. subtilis* var. *globigii* were prepared by using the method of Powers (23) and allowing 11 days of incubation at 37°C on a thin layer of tryptic soy agar (TSA) in 500-ml bottles. The spores were harvested, washed with sterile saline, and resuspended in same. The saline cell suspensions of the test cultures were then frozen statically in a dry ice-acetone bath (−78°C) for 1 to 2 min, and the frozen suspensions were stored at −34°C for not more than 1 week. It was found that the number of normal viable organisms in cell suspension decreased upon prolonged storage, probably due to metabolic injury. The frozen cells were allowed to thaw at 25°C for 10 min and were then resuspended in sterile sodium phosphate buffer (0.066 M, pH 7.2) or in saline as deemed necessary. The cell suspension was shaken vigorously with the aid of a Vortex mixer, and 5-ml aliquots were distributed to vials for cooling and heating as well as for thermoradiation treatments.

Effect of various environments. The effect of thermordiation on the survival of cells suspended in various environments was also examined. The environments tested were: saline; 0.1 mM DL-cysteine in saline; sodium phosphate buffer, pH 7.2; and 0.066 M and 0.1 mM DL-cysteine in the sodium phosphate buffer. For the cysteine experiments, 4.5 ml of the cell suspension was placed in vials, and at exactly 40 min before irradiation, 0.5 ml of stock cysteine solution (pH 7.0) was added to these vials to yield the desired molar concentration.

Analytical procedures. Since irradiation and heating (or cooling) were conducted simultaneously, we decided to examine the effects of each treatment alone on the percent survival of the test cultures. A 10-min holding temperature at 25°C for all vegetative cells and 30 min for the spore cells was used as the point of divergence (i.e., control). It should be pointed out that no effort was made to alter the gas phase during treatments of the cell suspensions.

A ^{60}Co source (Gammacell 200), 215.7 rad/s, was utilized to irradiate the cell suspensions, which were kept in sealed vials. The vials were placed in a water bath maintained at the desired temperature (±0.5°C), and all the experiments were designed to permit the heating of the vegetative-cell suspensions for a total of 10 min, including the time of irradiation. The spore suspension was heated and irradiated for a total time of 30 min. However, the time of irradiation varied, depending on the dose received, and did not exceed the times previously indicated. Before and after each treatment, the number of viable cells was determined by the pour plate technique. For *B. subtilis* and *S. aureus*, TSA was used, whereas for *A. aquamarinus*, 3% sodium chloride was added to the TSA. Cells surviving the treatment were indicated by counting colony-forming units after 3 days of incubation at 37°C and determining the survival fraction (N/N_0), where N_0 represents the number of vegetative or spore cells per milliliter of suspension kept at 25°C (control), and N denotes that number after each treatment at the specified temperature. The D_{10} (dose that inactivates 90% of the microbial population) values were determined from survival curves by inspection. Curves were based on data collected from three to five repeated experiments, and no attempt was made to alter the atmospheric conditions during any treatment.

RESULTS

Effect of thermoradiation. The inactivation curves for *A. aquamarinus* and *S. aureus* cells as a function of increased temperature or combined heat and irradiation at different temperatures are shown in Fig. 1. It is evident that heating *A. aquamarinus* cells above, but not below, 25°C resulted in their inactivation. However, irradiation at either 25 or 35°C destroyed most of the population exponentially, and the synergistic effects between irradiation and heat at 35°C were also observed. The inactivation of this culture by thermoradiation increased in direct proportion to the increase in temperature as evidenced by the decrease in the D_{10} value from 8.0 to 3.2 Krad at 25 and 45°C, respectively. *S. aureus* cells were also affected by heating for 10 min at temperatures above 35°C, and the rate of cell destruction was very rapid between 45 and 55°C. The log survival curves as a function of thermoradiation were all exponential and in direct proportion to the temperature. The D_{10} value (Table 1) was 9.8 Krad at 35°C, decreasing to 5.3 and 3.0 Krad as the temperature increased to 45 and 55°C, respectively. At 45°C the synergistic effect of heat and radiation on both cultures was evident since inactivation was more rapid than would be expected if heat and radiation effects were merely additive.

The curves for *B. subtilis* vegetative and spore cells (Fig. 2) showed no significant effect upon heating (10 min) the vegetative cells at any temperature below 35°C, but at 45 and 55°C, 40 and 90%, respectively, of the cell population was destroyed. The inactivation curves followed the same general pattern noted for *A. aquamarinus* cells heated at the same temperature and for the same time. The effect of heating the spores for 30 min revealed that a temperature of 75°C exhibited no visible effect, whereas at 85 and 90°C, 3 and 60%, respectively, of the cell population was destroyed. The data summarizing the effect of thermoradiation on vegetative and spore cells of *B. subtilis* are also shown in Fig. 2. It is evident that the

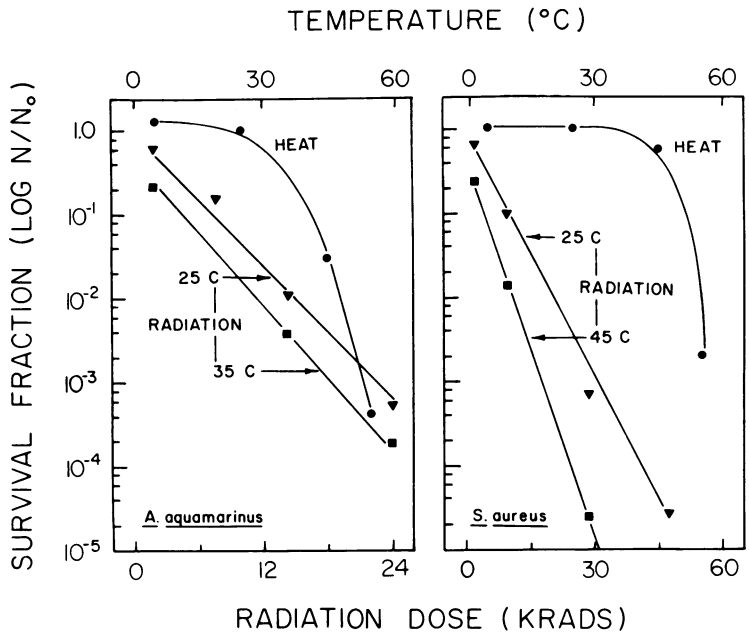


FIG. 1. *Effect of heat alone and combined heat and irradiation on log survival fraction (log N/N_0) of A. aquamarinus and S. aureus. N_0 represents the number of nonirradiated cells per milliliter of suspension at 25°C, and N denotes the number of cells per milliliter of suspension after treatment at specified temperatures.*

TABLE 1. *Effect of thermoradiation on D_{10} values for cells of test organisms*[a]

Irradiation temp (°C)	*A. aquamarinus*	*S. aureus*	*B. subtilis*	
			Vegetative	Spores
5	8.0	13.1	10.1	–[b]
15	8.0	13.0	10.3	–
25	8.0	9.8	10.3	158
35	4.9	9.8	6.2	158
45	3.2	5.3	3.8	158
55	–	3.0	–	158
75	–	–	–	158
85	–	–	–	139
90	–	–	–	94

[a] The D_{10} (dose [in kilorads] required to inactivate 90% of the initial cell population) values were determined from the curves relating survival fraction ($\log N/N_0$) to radiation dose. N_0 represents the number of cells per milliliter of suspension of nonirradiated cells at 0 Krad and 25°C, and N denotes the number of cells per milliliter of suspension after irradiation at specified temperatures.

[b] –, Not determined.

inactivation curves for the cells had an initial high rate of destruction followed by a steady but slow rate of cell inactivation. The synergistic effect of heating and irradiation on the vegetative and spore cells was evident, particularly at 45°C and 6 Krad for the former cells and at 90°C and 160 Krad for the latter. The D_{10} values (Table 1) for the vegetative cells due to irradiation at 25, 35, and 45°C were 10.3, 6.2, and 3.8 Krad, respectively, whereas at 85 and 90°C the D_{10} values for *B. subtilis* spore cells were 129 and 92 Krad, respectively.

Figure 3 depicts comparative data for the three test cultures exposed to different radiation doses as a function of increased temperature. Again, a holding time of 10 min for vegetative cells and 30 min for spores at a temperature of 25°C was used as control (N_0). The irradiation of the cells over a range of 25 to 35°C was clearly critical to the survival of vegetative cells of all the test cultures, whereas a temperature of 85 to 90°C was very effective against the spore cells. Beyond these aforementioned temperatures, a rapid rate of destruction and a highly synergistic effect were noted. It is of interest that at an irradiation dose of 19 Krad and above, the shapes of the survival curves for *B. subtilis* vegetative cells at any temperature were similar to those of spore suspensions. This was due to the presence of approximately 0.5% spores in the suspension of vegetative cells.

Effect of various environments. Since the cells of *A. aquamarinus, S. aureus,* and *B. subtilis* spores were sensitive to the combined effects of radiation and heating at 35, 45, and 90°C, respectively, it was decided to examine

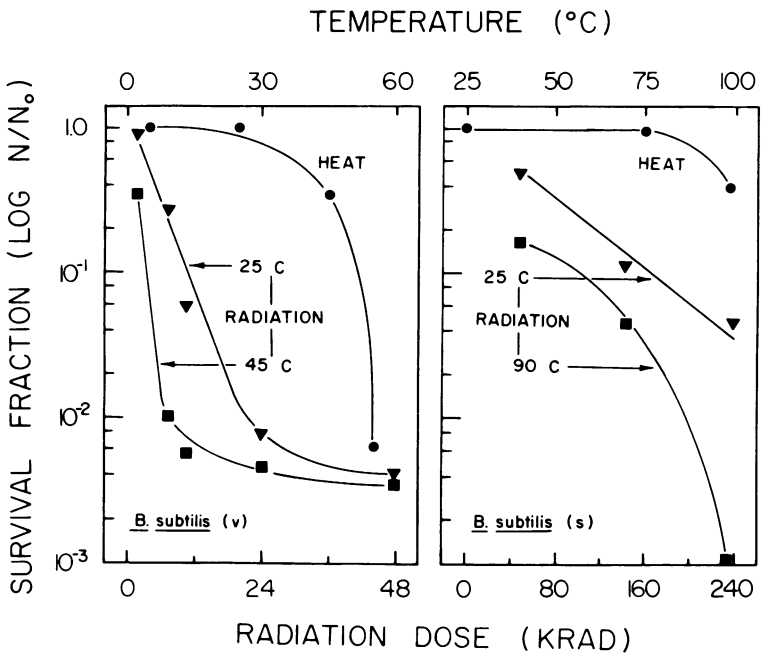


FIG. 2. *Effect of heat alone and thermoradiation on B. subtilis var. globigii vegetative (v) and spore (s) cells.*

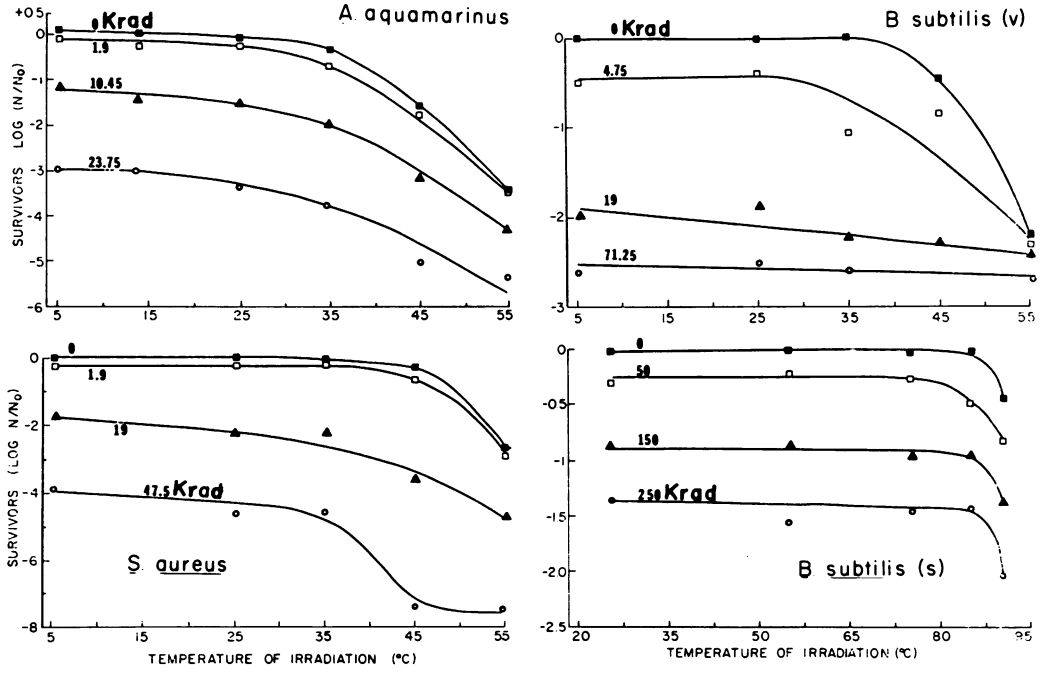


FIG. 3. *Comparative survival of test cultures as a function of temperature alone and irradiation dose (in kilorads) at different temperatures.*

the effect of the suspended media on the survival of these organisms at these critical temperatures. The results obtained disclosed several unexpected observations (Fig. 4). For example, all the curves for *A. aquamarinus* cells heated at 35°C and simultaneously irradiated at different doses in various environments were exponential and basically the same except in saline, where an initial rapid rate of inactivation was noted. A D_{10} value for these cells in saline was 4.9 Krad, whereas this value was 8.4 Krad for all other environments (0.10 and 0.77 mM cysteine in saline, 0.066 M phosphate buffer, 0.10 mM cysteine in phosphate buffer). This indicated the sensitizing effect of saline alone and the protective effect of the other environments.

Two main inactivation curves resulted from irradiating *S. aureus* cells at 45°C. One represents 0.10 mM cysteine in saline, and the second denotes all other environments. Again, the protective effect of cysteine in saline was evident (a D_{10} value of 6.0 Krad for this curve compared with 4.3 Krad for all other environments). It was also observed that both curves exhibited two different exponential slopes, one between 0 to 15 Krad, indicating a rapid rate of destruction, and the other from 15 to 45 Krad, denoting a slower rate of inactivation.

Regression curves representing log survival fraction for *B. subtilis* spores during heating at 90°C in saline or phosphate buffer (with or without 0.10 mM cysteine) all were exponential, paralleled each other, and had a D_{10} value ranging from 130 to 158 Krad. The survival curve for spores in saline containing 0.10 mM cysteine deviated considerably from the previous curves, particularly above 100 Krad, where the synergistic effect of heating at 90°C led to rapid inactivation of spores. The average D_{10} value for this curve was 109 Krad.

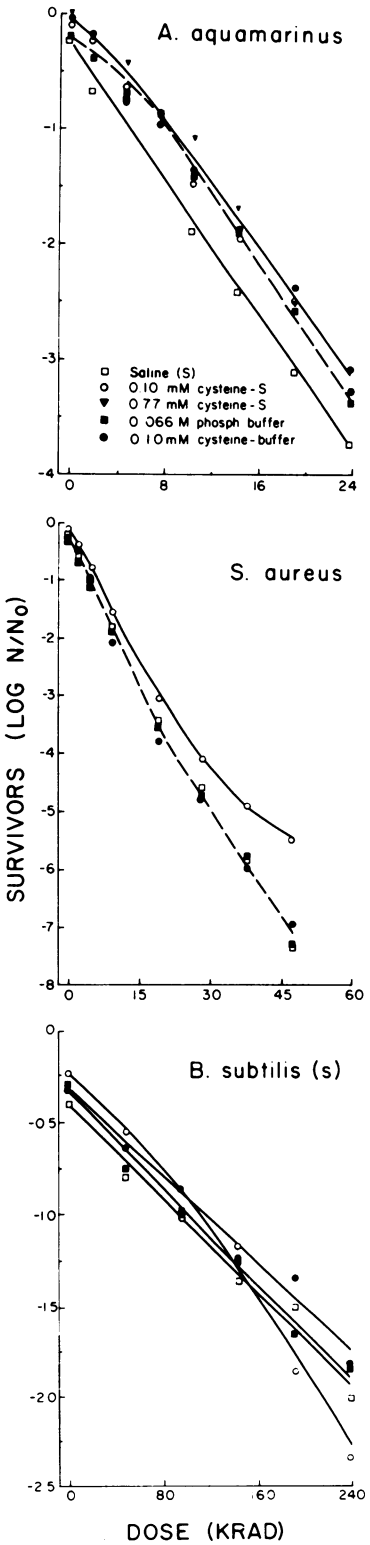
DISCUSSION

Irradiation, in the megarad range, of suspensions of both vegetative bacteria and spores usually results in their inactivation. However, the magnitude of this inactivation depends on the type of organism, irradiation dose, temperature, pH, presence of radioprotectors or sensitizers, and preheating (7, 31). Our data showed that the test cultures exhibited different sensitivities to sublethal heating above 25°C and to the combination of heat and irradiation. Many investigators reported that this sublethal heating may lead to metabolic injury of the cells as evidenced by the intercellular degradation of ribosomes (28) and the release of D-alanine esters of teichoic acid (14). A significant decrease in the D_{10} value from 8.0 to 4.6 Krad was noted for *A. aquamarinus* irradiated at 25 and 35°C, respectively, as compared with *S. aureus* and *B. subtilis*, where only slight or no changes were detected. This may indicate the presence of many highly sensitive sites in the *A. aquamarinus* cells that are accessible to radiation damage, such as their nucleic acid content. It is documented that percent base pairs (guanine and cytosine) in the deoxyribonucleic acid of gram-negative bacteria are usually much higher than those observed in gram-positive organisms. Lewis et al. (17) reported a D_{10} value of 9.4 Krad for an *Achromobacter* sp. and concluded that the radio survival curves for this culture represent a summation of two distinct processes, the radiation-induced damage of the cells and the post-irradiation recovery of the injured cells.

The presence of cysteine protected *A. aquamarinus* (at 35°C) and *S. aureus* (at 45°C) during irradiation below 24 Krad. This effect was also noted on spore cells during heating and irradiation at 90°C and below 100 Krad, but above this value cysteine exhibited a sensitizing effect. The radioprotective effect may be due to competition for hydroxyl radicals between the sulfhydryl group of the cysteine molecule and the different components of the spore coat, resulting in the formation of cystine and water. The cystine may also take up active hydrogen produced as a result of radiolysis of the water resulting in the regeneration of cysteine (12). The sensitizing effect, on the other hand, may be due to competition of cysteine with the disulfide bridges of cystine present in greater proportion in the spore coat protein, leading to cell inactivation. Petkau and Chelack (21) established that the radioprotective activity of cysteine was progressively destroyed by ionizing radiation greater than 28.8 Krad. Eldjarn and Pihl (8) reported that some chemicals could act as both radiosensitizers and radioprotectors through formation of more or less free radicals, thus increasing or decreasing the sensitivity of the target site.

During thermoradiation of *B. subtilis* vegetative cells, an initial high rate of temperature-dependent inactivation was followed by a steady but slow rate of destruction, the latter due to the presence of a very low concentration of spores. Briggs (2) reported similar results using *B. licheniformis*, *B. megaterium*, and *B. cereus* after simple heat treatment and stated that this trend is usually associated with systems containing organisms of two different resistances, e.g., a mixture of vegetative cells and spores.

Various authors have shown that the combined effect of heat and irradiation not only



may be additive but also may result in synergism (25, 29, 31). This synergistic effect was more pronounced at 35°C for *A. aquamarinus*, at 45°C for both *S. aureus* and *B. subtilis* vegetative cells, and at 90°C for the *B. subtilis* spores. It was noted that the cell inactivation due to thermoradiation at these temperatures but not at others was more pronounced than expected if heat and radiation effects were simply additive. The behavior of synergism in different microbial systems as a function of temperature has been discussed by several investigators (1, 8, 25). Sivinski et al. (30) showed that the degree of synergism during thermoradiation may be related to the nucleic acid content of the organism. It could be that the major advantage of using thermoradiation lies with the fact that one can achieve a higher inactivation rate per unit of energy expended by selecting the proper radiation dose and temperature according to the system to be treated and its susceptibility to heat.

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FIG. 4. *Effect of thermoradiation on survival of test cultures in different environments: A. aquamarinus heated to 35°C; S. aureus kept at 45°C; and B. subtilis var. globigii (spores) subjected to 90°C.*

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