

Thermal Resistance of Spores from Virulent Strains of *Bacillus anthracis* and Potential Surrogates

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ABSTRACT

The objective of this study was to determine the thermal resistance of spores of *Bacillus anthracis* and potential surrogates. The heat resistance of spores suspended in buffer (pH 7.0 or 4.5), milk, or orange juice was determined at 70, 80, and 90°C. *D*-values for *B. anthracis* strains Sterne, Vollum, and Pasteur ranged from <1 min at 90°C to ~200 min at 70°C and were lower under acidic than under neutral conditions. The *D*-values for *B. anthracis* spores fell within the range obtained for spores from eight strains of *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus mycoides*, and *Bacillus subtilis*. However, there were significant differences ($P < 0.001$) among the *D*-values of the strains. The *z*-values in pH 7.0 buffer and milk averaged ~10.5°C and were not significantly different among strains ($P < 0.05$). The *z*-values in pH 4.5 buffer and orange juice averaged 12.9 and 13.9°C, respectively, significantly ($P < 0.05$) higher than those obtained in milk or in pH 7.0 buffer. The significance of this difference was driven by large differences among a few strains. The *z*-values for *B. anthracis* strain Pasteur were twice as high in the acid media than in the neutral media. This study confirms that *B. anthracis* spores are not unusually heat resistant and that spores from validated *Bacillus* species are appropriate surrogates for thermal resistance studies.

An incident of politically motivated food bioterrorism occurred in the United States in 1984 when members of the Rajneesh cult inoculated salad bars in Oregon with *Salmonella* Typhimurium to decrease voter turnout (5). In 1999, the U.S. Government Accounting Office warned that the United States remained unprepared to respond to acts of food bioterrorism (10). Because bacterial spores survive desiccation and transport, maintain viability for long periods, and are resistant to inactivating agents, they make better bioagents than do *Salmonella* cells. One of the most feared sporeformers, *Bacillus anthracis*, was dispersed in chocolate candies by the Japanese during state-sponsored acts of food bioterrorism in the 1930s and 1940s and by the apartheid government of South Africa in the 1980s (27). In 2001, *B. anthracis* was sent through the U.S. mail, and became notorious as an agent of inhalation anthrax (2).

Anthrax is usually considered a veterinary disease (19), and human anthrax is primarily associated with workers in the hide and wool industries. There have been recent veterinary anthrax outbreaks in Spain, Greece, Turkey, Albania, and the United States (13). Human anthrax can present as cutaneous, inhalation, or gastrointestinal forms (8). Gastrointestinal or foodborne anthrax begins with anorexia and nausea followed by vomiting and abdominal pain. Diarrhea, ascites, ulceration, sepsis, and fatal meningitis may also result. Foodborne anthrax is rare, has been associated with the consumption of undercooked infected cattle, and is endemic to tropical and subtropical areas. It has a high case-

fatality rate and can cause death within 2 to 3 days of consumption of contaminated items (27).

Genetically, *B. anthracis* is a member of the *Bacillus cereus* group, which also includes *Bacillus thuringiensis* and *Bacillus mycoides* (11). Because of the close genetic relationship among these species, spores from this group, especially those from *B. cereus*, have been suggested for use as surrogates for anthrax spores. The degree of relatedness among members of the *B. cereus* group is controversial and depends on the discrimination criteria applied. Amplified fragment length polymorphism and PCR analyses have revealed little variability among *B. anthracis* isolates but significant differences between *B. anthracis* and *B. cereus* (14). In contrast, sequence analysis of 16S rRNA revealed identical sequences for 86 *B. anthracis* strains and <1% difference among *B. anthracis*, *B. cereus*, and *B. thuringiensis* (26). This finding and multicriteria genetic analysis (12) have led to proposals that *B. cereus* group members be classified as a single species.

There are limited data on the thermal resistance of *B. anthracis* spores. Neither low-temperature long-time (65°C for 30 min) nor high-temperature short-time (72°C for 15 s) pasteurization reduces viability of spores from the attenuated vaccine strain *B. anthracis* Sterne (23, 24). Murray's 1931 thermal death point studies of 17 *B. anthracis* strains (21) can be used, assuming linearity, to calculate approximate D_{90} values (time required at 90°C to reduce the viability of a population 10-fold) of 2.5 to 7.5 min in physiological saline. Buffer and peptone at pH 7.0 gave similar results. However, decreasing the pH from 7.0 to 4.0 decreased the thermal death time four- to sixfold. Results of

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B. anthracis spore inactivation studies suggest that 5 min at 100°C inactivates 10⁸ spores/ml and that 20 minutes at 90°C inactivates 10⁶ but not 10⁸ spores/ml (28). No *z*-values (temperature required to change the *D*-value 10-fold) have been reported for virulent strains, although there are large amounts of data on the heat resistance of *B. cereus* spores (3, 4, 9, 17, 18, 24). Novak et al. (23) found that the attenuated vaccine strain *B. anthracis* Sterne had thermal resistance characteristics similar to those of *B. cereus* ATCC 9818, but no direct comparisons of heat resistance have been conducted between spores from virulent *B. anthracis* and spores from other *Bacillus* species. Because spore thermal resistance properties are influenced by strain, sporulation conditions, and heating medium in both *B. anthracis* (7) and *B. cereus* (9, 17, 18), it is important that such comparisons be made using spores generated under the same conditions. The objective of our research was to compare under the same sporulation conditions the heat resistance of spores from three *B. anthracis* strains with that of spores from seven other *Bacillus* strains representing four other species. The influence of the heating medium was also examined.

MATERIALS AND METHODS

Bacterial cultures and media. *B. cereus* ATCC 9818, ATCC 4342, and ATCC 7004, *B. thuringiensis* ATCC 13366 and ATCC 10792, *B. mycoides* ATCC 21929, and *Bacillus subtilis* ATCC 6633 were purchased from the American Type Culture Collection (ATCC; Manassas, Va.). The vaccine strain *B. anthracis* Sterne was a gift from Dr. Darcy Haynes (Center for Food Safety and Applied Nutrition, College Park, Md.). The fully virulent strains of *B. anthracis* (Vollum and Pasteur) were obtained from the Centers for Disease Control and Prevention (CDC; Atlanta, Ga.). Cells were cultured on nutrient broth (NB; Becton Dickinson, Sparks, Md.), maintained at 4°C on nutrient agar as working stocks, and renewed monthly for a maximum of 6 months (at which point new working stocks were generated from the spores produced from the original cultures).

Spore preparation. Spores were prepared as previously described (20). Cultures were inoculated into NB and incubated overnight at 30°C, and 1 ml of culture was then transferred into 10 ml of NB. Five milliliters of this culture was incubated overnight (30°C) and inoculated into 100 ml of NB in a 500-ml Nalgene baffled flask sealed with a filtered closure (BugStopper, Whatman, Clifton, N.J.) to allow oxygen transmission but prevent aerosol release. Cultures were incubated at 30°C with shaking (200 rpm) and monitored for sporulation with phase contrast microscopy. Spores were harvested when 90% of the cells had sporulated. Oak Ridge style Nalgene centrifuge tubes were secured with caps containing O-rings to prevent aerosols and centrifuged at 10,000 × *g* for 10 min at 4°C in a sealed biosafety rotor. The rotor was opened in a class IIa biosafety cabinet, and the supernatant was carefully removed. The spores were resuspended in 25 ml of cold sterile distilled water containing 0.01% lysozyme (Sigma, St. Louis, Mo.) and refrigerated at 4°C for 48 h. The spores were then centrifuged twice at 5,000 × *g* for 20 min at 4°C and then resuspended in sterile distilled water. The final spore preparation, which contained >95% phase-bright spores, was stored at 4°C.

Determination of thermal resistance. Experiments were conducted by adding by 0.1 ml of spores to 0.9 ml of buffer, milk,

or orange juice in 3-ml glass ampoules (Wheaton Glass, Millville, N.J.), which were then sealed at the neck over a Bunsen burner. The Clark and Lubs buffers used by Murray (21) were prepared at pH 7.0 and 4.5 according to Dawson et al. (6). Commercially available ultrahigh-temperature pasteurized milk containing 2% milk fat and commercially pasteurized orange juice (pH 3.8) without pulp were obtained in a local supermarket.

The ampoules were completely submerged in a water bath at 70, 80, or 90°C. At appropriate intervals, a single vial was removed, cooled in an ice-water bath, and transferred to the class IIa biosafety cabinet, where a microbiologist wearing cotton gloves over latex gloves opened the ampoules with plastic ampoule openers. The spore suspensions were serially diluted in 1% peptone water (Difco, Becton Dickinson) and then plated in duplicate on tryptic soy agar (Difco, Becton Dickinson). The plates were then incubated overnight at 30°C. Each experiment was conducted in duplicate.

Plates having between 30 and 300 colonies were counted. Data were recorded and graphed as log CFU per milliliter versus time using the least squares linear regression function in Microsoft Excel. The linear portion of the graph was used to calculate the *D*-value based on the negative inverse of the slope, and the *D*-values of the duplicate experiments were averaged. The *z*-values were determined by graphing the log of the mean *D*-value against temperature, performing linear regression, and taking the inverse of the slope.

Statistical analysis. The influence of strain, substrate, and temperature on *D*-values was evaluated with an analysis of variance (ANOVA; JMP, SAS Institute Inc., Cary, N.C.) after a Johnson (15) transformation of the data to produce a normal distribution. The *z*-values were normally distributed and analyzed using the ANOVA procedure for Duncan's multiple range test. All statements regarding significance were based on a probability of ≥95%.

Safety and security considerations. All procedures involving *B. anthracis* were conducted by immunized personnel working in a select agent laboratory registered with the CDC in compliance with the USA Patriot Act. All culture manipulations were performed inside a class IIa biosafety cabinet using protocols approved by the Rutgers University Biosafety Committee.

RESULTS AND DISCUSSION

Preliminary experiments verified that the spore suspensions reached the test temperature within 15 s (data not shown). Neither shoulders nor tails were observed in the spore survival curves. At least four points were used to calculate *D*-values, which were generally within 15% of each other for duplicate experiments. The average *r*² values for the linear regressions used to determine the *z*-values were >0.97.

Thermal resistance characteristics. Spores of the three *B. anthracis* strains were not remarkably heat resistant. Their *D*-values ranged from <1 min at 90°C to >200 min at 70°C (Table 1). All three strains had *D*₇₀ values of >100 min in milk, which is consistent with the reported failure of milk pasteurization to kill spores of the surrogate *B. anthracis* Sterne (23, 24). In contrast, canning procedures should completely inactivate *B. anthracis* spores. The standard process of 2.4 min at 121°C for low-acid canned foods should result in a 68-log reduction in *B. anthracis*

TABLE 1. *D-values for Bacillus species^a*

Strain	Buffer, pH 7.0			Milk			Buffer, pH 4.5			Orange juice		
	70°C	80°C	90°C	70°C	80°C	90°C	70°C	80°C	90°C	70°C	80°C	90°C
<i>B. anthracis</i>												
Sterne	153.9	10.5	2.9	138.0	19.1	1.5	70.3	5.2	0.80	40.3	8.5	1.05
Pasteur	116.5	8.5	0.86	205.1	15.7	1.0	9.2	1.9	0.85	9.3	3.0	0.68
Vollum	226.7	29.9	4.9	198.1	24.3	6.7	99.6	8.0	1.6	81.7	7.6	2.0
<i>B. cereus</i>												
ATCC 9818	1,405.5	83.7	22.0	654.2	46.5	12.8	430.2	86.8	8.6	320.4	41.4	19.8
ATCC 4342	224.0	27.0	4.8	257.0	28.6	3.3	61.8	12.6	3.1	60.0	12.4	4.2
ATCC 7004	77.9	14.1	0.74	119.7	6.0	1.1	56.0	4.6	0.9	54.2	5.1	0.95
<i>B. subtilis</i> ATCC 6633	905.1	51.0	6.0	346.0	49.3	4.2	162.7	19.5	12.3	120.4	26.4	5.4
<i>B. thuringiensis</i>												
ATCC 13366	123.4	12.9	1.4	85.9	22.2	0.55	46.0	4.5	0.50	60.1	7.5	1.1
ATCC 10792	283.0	16.8	1.7	221.2	23.8	1.7	76.3	7.2	1.0	43.7	7.0	1.2
<i>B. mycoides</i> ATCC 21929	269.8	35.9	3.6	131.0	52.3	2.5	112.5	7.9	2.4	63.1	11.5	1.6

^a *D*-values are in minutes.

viability at $D_{121} = 0.035$ min, calculated from the *D*- and *z*-values (Table 2) for the virulent *B. anthracis* Vollum in milk. The D_{90} values for *B. anthracis* in pH 7.0 buffer (Table 1) are consistent with those of the 17 strains examined by Murray (21). The most resistant strain in that study had a D_{90} value approximately 50% greater than that for *B. anthracis* Vollum. Given the differences in sporulation procedures, recovery media, and experimental methodology, this difference is of little practical importance. *D*-values for *B. anthracis* Pasteur were significantly less than those for *B. anthracis* Vollum and lower than those for any of the strains examined by Murray (21). The thermal resistance characteristics reported here cannot be compared with those published by Novak et al. (23) for *B. anthracis* Sterne and *B. cereus* ATCC 9818 because those authors considered their *D*-values "invalid because of the low r^2 values obtained for the linear representations of collected data points" (23).

The thermal resistance of the three *B. anthracis* strains

fell within the range of the other *Bacillus* species examined (Fig. 1). The thermal resistance of the surrogate *B. anthracis* Sterne was not significantly different from the virulent *B. anthracis* Pasteur and Vollum, but there were significant differences ($P < 0.001$) in heat resistance among the individual *Bacillus* strains examined. The three *B. anthracis* strains had similar *z*-values in milk and buffer at pH 7.0, but the *z*-value for *B. anthracis* Pasteur increased twofold in acidic media (Table 2).

The vaccine strain *B. anthracis* Sterne lacks the pXO2 plasmid, which codes for the poly- γ -D-glutamic acid capsule that makes *B. anthracis* so virulent (19). However, *B. anthracis* Sterne contains the pXO1 plasmid encoding the lethal and edema toxins and cannot be considered completely nonpathogenic (16). Based on the *D*-values in Figure 1, *B. anthracis* Sterne appears to be an appropriate thermal surrogate for more virulent *B. anthracis* strains if class IIa biosafety laboratory practices are used.

TABLE 2. *z-values of Bacillus species^a*

Strain	Buffer, pH 7.0	Milk	Buffer, pH 4.5	Orange juice
<i>B. anthracis</i>				
Sterne	11.6	10.2	10.3	12.6
Pasteur	9.4	8.7	19.3	17.6
Vollum	12.0	13.6	11.1	12.4
<i>B. cereus</i>				
ATCC 9818	10.5	11.7	11.8	16.6
ATCC 4342	12.0	10.6	15.5	17.3
ATCC 7004	9.9	9.8	11.1	11.4
<i>Bacillus thuringiensis</i>				
ATCC 13366	10.3	9.1	10.2	11.6
ATCC 10792	9.0	9.5	10.7	13.0
<i>B. mycoides</i> ATCC 21929	10.3	11.6	12.0	12.5
<i>B. subtilis</i> ATCC 6633	9.2	10.5	17.8	14.9
Mean \pm SD	10.4 \pm 1.1 A	10.5 \pm 1.9A	13.0 \pm 3.1 B	14.0 \pm 2.3 B

^a *z*-values are in degrees C. Means with the same letter are not statistically different ($P > 0.05$).

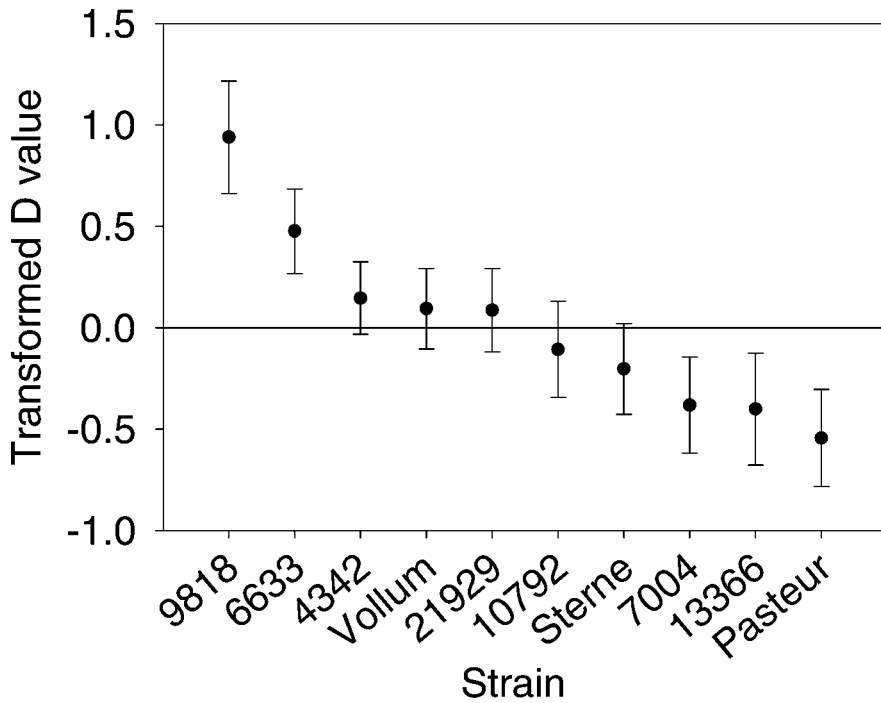


FIGURE 1. Transformed D-values and 95% confidence intervals for strains of *Bacillus* spp.

B. cereus strains ATCC 9818, ATCC 4342, and ATCC 7004 were used in this study because they represent strains with high, medium, and low heat resistance (9, 18), a designation we confirmed. *B. cereus* ATCC 9818 was significantly more heat resistant than were the other strains in this study (Fig. 1) and would make a conservative (fail-safe) surrogate.

B. subtilis ATCC 6633 was examined because it has been validated against *B. anthracis* Sterne as a surrogate

for UV resistance (22). *B. anthracis* surrogates validated against multiple stressors would be very useful. The *D*-values reported in Table 1 indicate that *B. subtilis* ATCC 6633 would be a fail-safe nonpathogenic surrogate for thermal resistance studies. The *D*- and *z*-values (Table 2) of *B. thuringiensis* ATCC 13366 and ATCC 10792 were similar and otherwise unremarkable compared with those of the other *Bacillus* strains. The *D*-values of *B. mycoides* ATCC 21929 were not significantly different from those of many other *Bacillus* strains tested (Table 1 and Fig. 1).

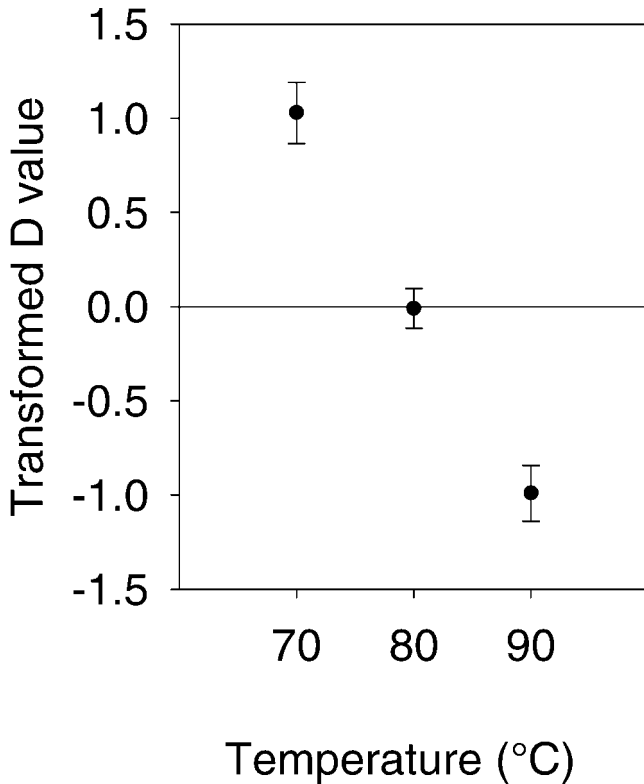


FIGURE 2. The effect of temperature on transformed D-values for *Bacillus* spp. Bars indicate 95% confidence intervals.

Influence of heating temperature. The influence of temperature on *D*-values was highly significant ($P < 0.001$; Fig. 2). Because Figures 1 through 3 present statistically transformed *D*-values, Figure 2 also serves as a useful benchmark for illustrating the relative importance of strain-to-strain differences (Fig. 1) and heating medium differences (Fig. 3) compared with the more familiar temperature effect (Fig. 2).

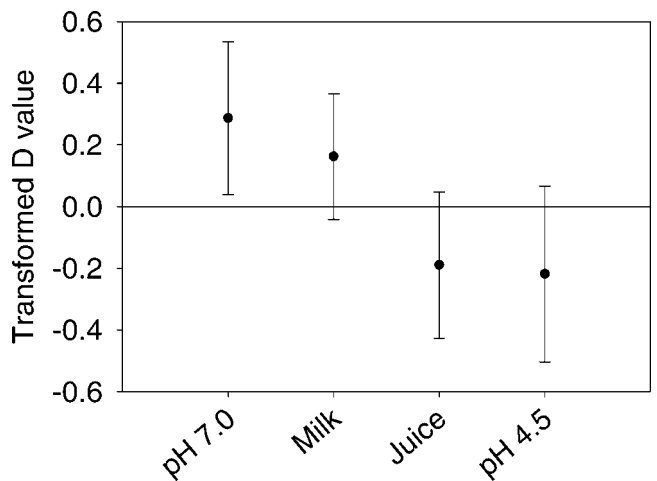


FIGURE 3. The effect of substrate on transformed D-values for *Bacillus* spp. Bars indicate 95% confidence intervals.

Influence of heating medium. There were significant differences ($P = 0.008$) in z -values for pH 7.0 buffer and milk compared with pH 4.5 buffer and orange juice (Fig. 3). This finding is consistent with Murray's observation (21) that *B. anthracis* thermal death times obtained at pH 4.0 were shorter than those obtained at pH 7.0. Decreases in thermal resistance of *Bacillus* spores with decreasing pH have been widely reported (4, 7, 18, 20, 25) and attributed to spore demineralization at low pH values (1). Heating of acidic media also increased the z -values of some strains.

z -values. The z -values in pH 7.0 buffer and milk were approximately 10.5°C and did not differ significantly among the strains (Table 2). Previously reported *B. cereus* z -values of 7.6°C (9) and 8.3°C (17) are slightly lower than the 10.7°C reported here. In their survey of *B. cereus* strains, Rajkowski and Mikolajcik (25) reported z -values of 7.4 to 14.5°C. All of these published values are much lower than the z -values of >20°C reported by Novak et al. (23) for *B. anthracis* Sterne and *B. cereus* ATCC 9818, which were "dependent on D -values having rather low r^2 values." The average z -values obtained in pH 4.5 buffer and orange juice (Table 2) were significantly higher than those obtained in pH 7.0 buffer and milk, but the significance of this increase was driven by large differences in a few strains. *B. anthracis* Sterne, *B. cereus* ATCC 4342, and *B. subtilis* ATCC 6633 had markedly higher z -values in both acidic media compared with the neutral media. The z -value of *B. cereus* ATCC 9818 was markedly higher only in orange juice. Bender and Marquis (1) observed that although D -values decreased with decreasing pH, z -values increased for *Bacillus megaterium*, *B. subtilis*, and *Bacillus stearothermophilus*.

We determined D -values and z -values for virulent *B. anthracis* spores and potential surrogates in multiple heating media, including two foods. These data are consistent with previously reported thermal death times (21, 23) and confirm that *B. anthracis* spores are not unusually heat resistant. These spores can be expected to survive pasteurization but not canning. Based on D - and z -values, spores from *B. anthracis* Sterne, *B. cereus* ATCC 4342, *B. cereus* ATCC 9819, and *B. subtilis* ATCC 6633 are valid surrogates for virulent *B. anthracis* spores in thermal resistance studies.

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