Inactivation of *Bacillus* Spores in Dry Systems at Low and High Temperatures

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A plot of the thermal resistance of *Bacillus subtilis* var. *niger* spores (log *D* value) against temperature was linear between 37 and 190 °C ($z = 23$ °C), provided that the relative humidity of the spore environment was kept below a certain critical level. The corresponding plot for *Bacillus stearothermophilus* spores was linear in the range 150 to 180 °C ($z = 29$ °C) but departed from linearity at lower temperatures (decreasing z value). However, the z value of 29 °C was decreased to 23 °C if spores were dried before heat treatment. The straight line corresponding to this new z value was consistent with the inactivation rate at a lower temperature (60 °C). The data indicate that bacterial spores which are treated in dry heat at an environmental relative humidity near zero are inactivated mainly by a drying process. By extrapolation of the thermal resistance plot obtained under these conditions for *B. subtilis* var. *niger* spores, the *D* value at 0 °C would be about 4 years.

INTRODUCTION

The resistance of bacterial spores to dry heat differs considerably from the resistance to moist heat in both inactivation rate (*D* value) and temperature coefficient (z value), indicating that the denaturation follows different pathways in the two systems. Dry-heat inactivation has generally been considered to be primarily an oxidation process (Rahn, 1945; Sykes, 1965; Ernst, 1968). However, this is contradicted by the findings of Pheil *et al.* (1967), for example, who showed that the dry-heat resistance of spores is higher in oxygen than in air. Considering the strong influence of water activity on the dry-heat resistance of spores (see, for example, Murrell & Scott, 1966), a possible explanation for dry-heat inactivation could be the removal of bound water critical for maintaining the helical structure of proteins. This is supported by results showing that a certain level of water is necessary for the maintenance of heat stability in spores (Rowe & Silverman, 1970; Soper & Davies, 1971). Further, Marshall, Murrell & Scott (1963) found that spores stored for long periods at very low water activity were inactivated at room temperature.

Inactivation kinetics of bacterial spores are usually described by the thermal resistance plot (the logarithm of the inactivation rate plotted against temperature). When this is linear, the slope gives the z value. The model is empirical (Bigelow, 1921) and has been widely used for calculations of spore inactivation. However, Rahn (1945) has pointed out that the linear relationship obtained is only a valid approximation in narrow temperature ranges. Deviations from the straight line thermal resistance plot have been demonstrated in several studies (Amaha, 1953; Levine, 1956; Esselen & Pflug, 1956; Licciardello & Nickerson, 1963; Wang, Scharer & Humphrey, 1964; Edwards, Busta & Speck, 1965). In all of these, moist heat was used. Reported deviations from linearity for dry-heat inactivation are few and may (for example, Oag, 1940) be due to experimental errors. The aim of the present work was to study the dry-heat inactivation kinetics of

Bacillus spores over a wide temperature range in order to get information concerning the mechanisms of dry-heat inactivation of bacterial spores.

METHODS

Spore material. Spores of *Bacillus subtilis* var. *niger* ATCC9372 and *B. stearothermophilus* NCTC10339 were produced and harvested as described by Molin & Östlund (1976). The incubation temperature for spore production was 37 °C for *B. subtilis* var. *niger* and 55 °C for *B. stearothermophilus*. Spores were stored in 95 % (v/v) ethanol at +4 °C.

Samples for the inactivation studies were prepared by distributing 0.1 ml of spore suspension over a glass cover-slip (0.1 × 18 × 18 mm). After the diluent had evaporated, the spores were left evenly distributed over the glass surface (Molin & Östlund, 1975). Samples contained 6×10^7 *B. subtilis* var. *niger* spores or 1×10^7 *B. stearothermophilus* spores.

Before heat treatment the samples were usually stored for 24 h over silica gel followed by 5 to 15 days at 33 % relative humidity at room temperature (20 to 24 °C). In one series of experiments, however, the spores of *B. stearothermophilus* were stored over silica gel or over KOH (under vacuum) for 12 days before the heat treatment.

Inactivation experiments. Spore samples (spores applied to the cover-slip) were usually inactivated by heat treatment in a resistometer as described by Molin & Östlund (1975). The experimental conditions could be described as an open system allowing free gas exchange with the environment [as opposed to a closed one as defined by Pflug & Schmidt (1968), for example]. The humidity of the surrounding air was continuously recorded using a hair hygrometer. Values of between 6 and 10 g water (m air)⁻³ were recorded. Spore samples were heated in the resistometer at fixed temperatures in the range 90 to 190 °C. Samples were also heated in a vacuum desiccator over dry KOH in a hot-air oven (Horo, model 043; A. R. Horwell, London) at 37, 60 and 100 °C.

Recovery of surviving spores. Spores were removed from the glass cover-slips by ultrasonic treatment for 3 min in distilled water. The efficiency of the washing was enhanced by crushing the glass with a pipette before sonication. The spores were recovered by incubation on Tryptone Glucose Extract Agar (Difco) for 48 h at 37 °C for *B. subtilis* var. *niger* and at 55 °C for *B. stearothermophilus*.

Analysis of data. The thermal inactivation rate could, for both the organisms tested and at all temperatures, be represented quite accurately by a straight line. The data were analysed by linear regression from which the *D* values with 95 % confidence limits were calculated. Each *D* value was calculated on the basis of at least 15 heat-treated samples. The reported *D* values are based on a reduction of 4 to 5 logs.

Because of small irregularities in the inactivation rate at the beginning of the heat treatment, the spore count at zero time was excluded from the calculations. The extent of deviation from linearity is shown by the ratio of the *y*-intercept of the regression line (*y*₀) to the logarithmic value of the initial spore number (log *N*₀), i.e. no deviation from linearity gives *y*₀/log *N*₀ = 1.0 whereas, for example, a shoulder in the curve gives a value greater than 1.0.

The *z* value for *B. subtilis* var. *niger* spores was calculated by the method of least squares from six different *D* values in the temperature range 140 to 190 °C. The *z* values for *B. stearothermophilus* were calculated from three to four *D* values in the range 140 to 180 °C.

RESULTS

The resistance of spores to dry heat, given by the *D* value, was higher for *B. subtilis* var. *niger* than for *B. stearothermophilus* within the temperature range investigated (Table 1). The thermal resistance plot of *B. subtilis* var. *niger* spores inactivated in the resistometer was linear (*z* = 23 °C) in the higher temperature range, but curved upwards (decreasing *z* value) at lower temperatures. However, the inactivation data for spores heated in a desiccator over KOH in the temperature range 37 to 100 °C fitted the extrapolation of the straight part of the thermal resistance plot (Fig. 1).

The thermal resistance plot for spores of *B. stearothermophilus* was similar to that for *B. subtilis* var. *niger*, being linear in the higher temperature range (*z* = 29 °C) and curving upwards at lower temperatures (Fig. 2). However, the deviation from linearity was much more pronounced for *B. stearothermophilus*. Furthermore, the inactivation rate of spores heated at a lower temperature in a desiccator over KOH did not correspond to the straight part of the thermal resistance plot.

Table 1. Dry-heat inactivation of *B. subtilis* var. *niger* and *B. stearothermophilus* spores

Temp. (°C)	<i>B. subtilis</i> var. <i>niger</i>		<i>B. stearothermophilus</i>	
	<i>D</i> value with 95 % confidence limits*	$y_0/\log N_0$	<i>D</i> value with 95 % confidence limits*	$y_0/\log N_0$
190	1.0 (0.92-1.2) s	1.0	—	—
180	2.7 (2.5-3.1) s	1.0	1.8 (1.3-2.8) s	0.86
170	7.8 (7.1-8.6) s	1.0	4.2 (3.3-5.7) s	0.87
160	18 (16-22) s	1.1	9.8 (8.6-11) s	0.94
150	58 (53-65) s	1.0	19 (17-21) s	0.98
140	2.7 (2.5-2.8) min	1.1	—	—
120	30 (27-35) min	1.1	—	—
100	8.8 (8.6-9.1) h	1.1	2.8 (2.6-3.1) h	0.99
100	4.5 (3.7-5.8) h†	1.1	—	—
90	—	—	12 (10-14) h	1.0
60	3.9 (3.4-4.2) days†	1.0	2.3 (1.8-3.1) days†	0.97
37	44 (41-48) days†	1.0	—	—

* The *z* values of *B. subtilis* var. *niger* spores (23 °C) and *B. stearothermophilus* spores (29 °C) were calculated from *D* values in the temperature ranges 140 to 190 °C and 150 to 180 °C, respectively.

† Spores heated over solid KOH.

Fig. 1

Fig. 2

Fig. 1. Thermal resistance of *B. subtilis* var. *niger* spores in a dry environment (*D* values measured in s). ●, Spores heated in a resistometer; ○, spores heated in a vacuum desiccator over KOH. The dashed line is the extrapolated curve calculated from the *D* values in the range 140 to 190 °C.

Fig. 2. Thermal resistance of *B. stearothermophilus* spores in a dry environment (*D* values measured in s). ●, Spores heated in a resistometer; ○, spores heated in a vacuum desiccator over KOH; △, spores dried over KOH (under vacuum) before heat treatment in a resistometer. The dashed line is the extrapolated curve calculated from the *D* values represented by △.

The *z* value for *B. stearothermophilus* spores changed from 29 to 26 °C when the spores were dried over silica gel before heat treatment, and from 29 to 23 °C when the spores were dried over KOH (under vacuum). The *D* values (with 95% confidence limits) for spores dried over silica gel were 19 (16-24) s, 7.9 (6.7-9.5) s and 3.4 (2.7-4.5) s, when heated at 150, 160 and 170 °C, respectively. The *D* values for spores dried over KOH are indicated in Fig. 2. The *D*₆₀ value obtained for spores heated in a desiccator over KOH was consistent with the extrapolated thermal resistance plot (*z* = 23) for spores dried over KOH before heating (Fig. 2).

DISCUSSION

The inactivation data for *B. subtilis* var. *niger* spores heat-treated in the resistometer was consistent with the thermal resistance model of Bigelow (1921) in the higher temperature range, but deviated from this model at lower temperatures (Fig. 1). However, because the spores were inactivated in an open system which allowed free gas exchange with the environment, the relative humidity of the spore environment was higher at lower temperatures and this may have influenced the inactivation rate. The critical influence of the relative humidity on spore resistance is well known (see, for example, Murrell & Scott, 1966; Angelotti *et al.*, 1968; Brannen & Garst, 1972).

The inactivation data for spores heated at lower temperatures but at very low relative humidity (in a desiccator over KOH) corresponded to the straight part of the thermal resistance plot (Fig. 1). This strongly supports the suggestion that the deviation in z value is caused by a change in the relative humidity of the heating system.

The thermal resistance plot of *B. stearothermophilus* spores differed from that of *B. subtilis* var. *niger* spores in three ways: (i) the z value in the temperature range 150 to 180 °C was 6 °C higher for *B. stearothermophilus*; (ii) the plot curved upwards more steeply at lower temperatures indicating a greater decrease in the z value; and (iii) spores heated over KOH at low temperatures were not inactivated in accordance with the extrapolation of the straight part of the thermal resistance plot.

The difference in inactivation kinetics between the two organisms may be explained by a difference in the water relations of the spores. It has been shown by Fox & Pflug (1968), for example, that the inactivation kinetics of spores subjected to dry heat is closely related to the loss of water during treatment. Thus, the z value increased when the spores were heated in systems with increasing dehydration rates. The same pattern may hold if spores of different water content or water-holding capacity are exposed to dry heat. Also the water location within the spore and the number of critical attachments for water may influence the inactivation kinetics.

The critical influence of a small amount of water in the spores on the apparent z value is demonstrated by the fact that the z value of *B. stearothermophilus* was decreased by decreasing the water content of the spores before heat treatment. Thus, by drying the spores over KOH before heating, the z value was decreased by 6 °C. It is striking that the thermal resistance plot for pre-dried *B. stearothermophilus* spores ($z = 23$ °C) corresponded to the D_{60} value obtained at very low relative humidity, and that the z value obtained is the same as that for *B. subtilis* var. *niger* spores. These results suggest that the irregularities in the thermal resistance plot for *B. stearothermophilus* resulted from a water-related factor in the spore (for example, an 'excess' of water). When the effect of this factor is neutralized, the thermal resistance plots for *B. stearothermophilus* and *B. subtilis* var. *niger* spores correspond over the entire temperature range.

Considering the z value of *B. subtilis* var. *niger* spores to be constant in the range 37 to 190 °C and assuming that the inactivation mechanism is actually a drying process (the spores were inactivated at the growth temperature), one might extrapolate the thermal resistance plot of *B. subtilis* var. *niger* spores to still lower temperatures. Thus, the D value at 0 °C, would be about 4 years. Brannen (1970) has suggested the D_0 value for *B. subtilis* spores stored in a vacuum (10^{-8} Torr = 1.33×10^{-6} Pa) would be between 140 and 2500 years. However, his calculations were based on inactivation data from a relatively narrow temperature range. The data were also extrapolated in accordance with the Arrhenius equations, i.e. the inactivation rate was plotted versus $1/T$; this gave a thermal resistance plot curving upwards (the z value decreased with a decreasing temperature). Nevertheless, if one assumes a constant z value over the entire temperature range and extrapolates the inactivation data of the present investigation, the D value of *B. subtilis* var. *niger* spores near 0 K would be about 10^{12} years. This suggests that the

bacterial spore will theoretically keep its viability for a long time when stored in a cool environment, for example, in space.

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