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Heat Resistance Correlated with DNA Content in *Bacillus megaterium* Spores

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Two subpopulations of *Bacillus megaterium* spores (1.360 and 1.355 g/ml) were obtained by density gradient centrifugation. The heavier spores had a higher thermoresistance (e.g., D_{80} = 186 versus 81 min) and a higher DNA content (1.25×10^{-14} versus 0.65×10^{-14} g per spore, apparently corresponding to digenomic versus monogenomic spores). No appreciable differences were found in the mineral and dipicolinic acid contents or in the inactivation kinetics of the two subpopulations. The implications of the findings are discussed with regard to mechanisms of heat resistance and of inactivation.

The DNA content of bacterial spores, expressed on a per-spore basis, varies from species to species as much as fourfold, with *Bacillus megaterium* spores containing twice the minimum amount (7). Spore species with higher DNA contents show "multiple-hit" curvilinear inactivation kinetics during exposure to ionizing radiation (18), suggestive of "multiple chromosome sets" (7), "polyploidy" (10), or, in the currently used term, multiple genomes. Multigenomic spore populations can be separated into subpopulations with a narrowed range of DNA content by density gradient centrifugation; the higher the density, the higher is the DNA content of the subpopulation (12).

The heat resistance of bacterial spores is attributable to at least three basic determinants that affect the vital protoplast, as reviewed recently (8). Dehydration is the only determinant necessary and sufficient in itself for the elevated level of thermoresistance characteristic of spores, but mineralization and thermal adaptation also can contribute significantly.

The question arose whether an increased content of DNA in spores can affect their heat resistance and their heat inactivation kinetics. Consequently, we undertook to isolate spore subpopulations from a multigenomic species, *B. megaterium*, by means of density gradient centrifugation. We then examined the inactivation kinetics of the subpopulations and compared their heat resistances with their DNA contents and with their dipicolinic acid (DPA) and mineral contents, which also can affect buoyant density (6) and thus heat resistance (1).

Spores of *B. megaterium* ATCC 33729, an exosporiumless variant of strain QM-B1551, were produced and cleaned as described previously (13). The cleaned spores were separated into two nonoverlapping bands by centrifugal buoyant-density sedimentation in incremental discontinuous gradients (1.40 to 1.30 g/ml) of Nycodenz (Nyegaard and Co., Oslo, Norway), as described previously (3, 14). Nycodenz does not adversely affect spore dormancy or viability during the separation process (14). Spore counts were determined in a Petroff-Hauser counting chamber by using a phase-contrast microscope.

The heat resistance of each of the two spore types was determined at 80, 85, and 90°C and expressed as a *D* value (minutes required for a decimal reduction in CFU), as described previously (1, 2). The *D* value at each temperature was obtained from a plot of log survivors versus time by least-squares computing of the slope of the rectilinear portion, which extended over 1 to 4 decades of decline, depending on temperature. The rectilinear regressions extended essentially from the origin, with a lag apparently representing heat activation of spore germination sometimes occurring in the first few minutes.

DNA was determined by the diphenylamine assay of Burton (5), with the following modifications. A 0.2-ml suspension of about 5×10^9 spores was added to 2 ml of chilled 0.5 N perchloric acid and held on ice for 60 min. The suspension was then sonicated briefly and held in a 90°C water bath for 10 min. Samples were then centrifuged at $1,700 \times g$ for 10 min, and the supernatant fractions were collected. This process was repeated twice to achieve maximum yields (9). The supernatant fractions of each spore suspension were pooled and analyzed with salmon sperm DNA as a standard (5). Determinations were expressed as grams of DNA per spore.

DPA was assayed as described by Janssen et al. (11). Minerals were extracted by the method of Bender and Marquis (4) and analyzed by plasma emission spectrophotometry (Spectra Span VB; Applied Research Laboratories, Dearborn, Mich.). The results were expressed as micromoles of DPA or mineral per spore and as mole ratios.

Two close but distinct subpopulations of the dormant spores were obtained after centrifugation in the Nycodenz gradients, one in a band with buoyant wet density of 1.355 g/ml and another in a band of 1.360 g/ml.

The ability to separate the spores into two density bands enabled a direct comparison of their heat inactivation kinetics and heat resistances. Both populations of spores showed rectilinear regressions (data not presented). The *D* values, obtained directly at 80, 85, and 90°C and by extrapolation at 100°C (Table 1), showed that the heavier spores were significantly more resistant to heat than the lighter spores. For example, the difference in *D* values was 130% at 80°C, which is usually considered the lethal temperature for this relatively susceptible spore species. The *D* values were then plotted versus temperature (Fig. 1) to generate *z* values (the temperature changes needed to reduce the *D* values by a factor of 10). The *z* values for the two spore types were similar (Table 1).

The analyses for DNA contents (Table 1) revealed that the lighter spores contained 0.65×10^{-14} g of DNA per spore and the heavier spores contained 1.25×10^{-14} g of DNA per

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TABLE 1. Characteristics of lighter and heavier spores of *B. megaterium* ATCC 33729

Characteristic[a]	Value for the following buoyant density band:	
	1.355 g/ml	1.360 g/ml
Heat resistance		
D value (min)		
D_{80}	80.9	186
D_{85}	11.5	23.7
D_{90}	2.02	3.78
D_{100}[b]	0.049	0.073
z value (°C)[c]	6.2	5.9
DNA content (g, 10^{-14})	0.65	1.25
Mineral content (μmol, 10^{-10})		
Ca	7.38	7.68
K	3.44	3.12
Mg	0.938	1.02
Mn	0.271	0.298
Na	0.266	0.312
Mg/Ca mole ratio	0.127	0.133
DPA content (μmol, 10^{-10})	6.82	7.08
Ca/DPA mole ratio	1.08	1.08

[a] The data for each characteristic represent the averages of at least triplicate determinations on pooled bands from a single spore crop.
[b] Computed by least-squares extrapolation from the measurements obtained at 80, 85, and 90°C.
[c] Calculated from the thermal destruction curves shown in Fig. 1.

spore, apparently corresponding to monogenomic and digenomic spores, respectively. Both values were within the range of DNA contents reported for this species (10). Two plasmids (ca. 10 and 30 MDa) were identified in both the heavier and lighter spores and so were believed not to contribute appreciably to the difference in DNA contents.

Of the five main minerals found in spores, only Na varied more than 10% between the lighter and heavier spores (Table 1), and Na is not considered to be a main determinant of spore heat resistance (4) or of buoyant density (6). The DPA content and the Mg/Ca and Ca/DPA mole ratios, all considered to contribute to spore heat resistance (17), differed little or not at all between the two spore types. Thus, these characteristics apparently did not contribute to the differences in density and heat resistance between the two spore types.

Dehydration of the protoplast is the primary determinant of buoyant wet density and heat resistance of spores (1, 2, 8). However, it is highly unlikely that the increased *D* value for digenomic spores can be attributed to decreased water content because the protoplast water content in various spore species, including *B. megaterium* spores, is constant at a minimum limit of about 28%. At this limit, determinants other than protoplast dehydration (here, the DNA content) independently cause changes in heat resistance.

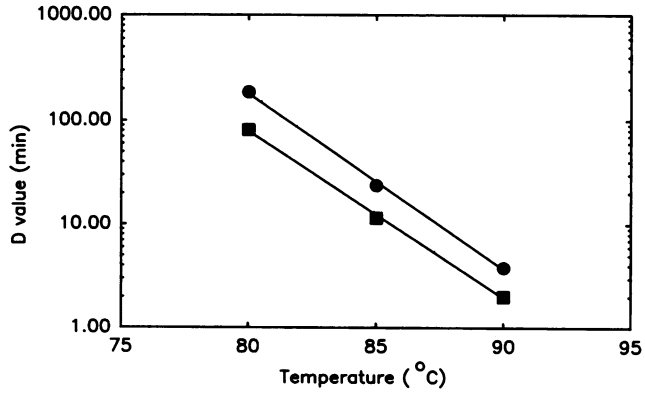

FIG. 1. Thermal destruction curves for lighter (■) and heavier (●) spores of *B. megaterium* ATCC 33729; the *z* values for lighter and heavier spores were 6.2 and 5.9°C, as calculated by least-squares analysis.

The positive correlation found between the heat resistance (as characterized by *D* and *z* values) and DNA content of *B. megaterium* spores might suggest that DNA is another general determinant of spore thermoresistance. Unlike dehydration, mineralization, and thermal adaptation, however, an increased DNA content occurs in only a fraction of rather than throughout a spore population. Furthermore, selection of multigenomic spore species to enhance survival does not seem to have occurred in nature, as some monogenomic species reportedly are more resistant than multigenomic species (15). Even within this one species, nature has not selected for only multigenomic spores to confer greater resistance. We are left with the conclusion that the increased DNA content occurring in a fraction of the population can indeed increase heat resistance in spores but that this factor does not seem likely to determine thermoresistance in general as do dehydration, mineralization, and thermal adaptation.

The observed correlation between thermoresistance and DNA might also suggest that DNA is a critical target in the inactivation of spores by heat, as it is in their inactivation by radiation (18). One might reason that a spore with multiple genomes should be more resistant to heat than a spore with a single genome because only one undamaged copy may be necessary for survival from heat treatment. However, the "multi-hit" curvilinear kinetics seen with radiation inactivation (18) were not found here with thermal inactivation of the multigenomic spores. Instead, "single-hit" rectilinear kinetics occurred. This finding tends to support other kinetic evidence indicating that the denaturation of DNA is not limiting in the thermal inactivation of bacterial spores (16).

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LITERATURE CITED

1. **Beaman, T. C., and P. Gerhardt.** 1986. Heat resistance of bacterial spores correlated with protoplast dehydration, mineralization, and thermal adaptation. Appl. Environ. Microbiol. **52**:1242–1246.
2. **Beaman, T. C., J. T. Greenamyre, T. R. Corner, and P. Gerhardt.** 1982. Bacterial spore heat resistance correlated with water content, wet density, and protoplast/sporoplast volume ratio. J. Bacteriol. **150**:870–877.
3. **Beaman, T. C., H. S. Pankratz, and P. Gerhardt.** 1988. Heat shock affects permeability and resistance of *Bacillus stearothermophilus* spores. Appl. Environ. Microbiol. **54**:2515–2520.
4. **Bender, G. R., and R. E. Marquis.** 1985. Spore heat resistance and specific mineralization. Appl. Environ. Microbiol. **50**:1414–1421.
5. **Burton, K.** 1968. Determination of DNA concentration with diphenylamine. Methods Enzymol. **12B**:163–166.
6. **Dean, D. H., and H. A. Douthit.** 1974. Buoyant density heterogeneity in spores of *Bacillus subtilis*: biochemical and physiological basis. J. Bacteriol. **117**:601–610.
7. **Fitz-James, P. C., and I. E. Young.** 1959. Comparison of species and varieties of the genus *Bacillus*. Structure and nucleic acid content of spores. J. Bacteriol. **78**:743–754.
8. **Gerhardt, P., and R. E. Marquis.** 1989. Spore thermoresistance

mechanisms, p. 17–37. *In* I. Smith, R. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.
9. **Hanson, R. S., and J. A. Phillips.** 1981. Chemical composition, p. 328–364. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
10. **Hitchins, A. D.** 1978. Polarity and topology of DNA segregation and septation in cells and sporangia of the bacilli. Can. J. Microbiol. **24**:1103–1134.
11. **Janssen, F. W., A. J. Lund, and L. E. Anderson.** 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26–27.
12. **Johnston, G. C., and I. E. Young.** 1972. Variability of DNA content in individual cells of *Bacillus*. Nature (London) New Biol. **238**:164–166.
13. **Koshikawa, T., T. C. Beaman, H. S. Pankratz, S. Nakashio, T. R. Corner, and P. Gerhardt.** 1984. Resistance, germination, and permeability correlates of *Bacillus megaterium* spores successively divested of integument layers. J. Bacteriol. **159**:624–632.
14. **Lindsay, J. A., T. C. Beaman, and P. Gerhardt.** 1985. Protoplast water content of bacterial spores determined by buoyant density sedimentation. J. Bacteriol. **163**:735–737.
15. **Lindsay, J. A., and W. G. Murrell.** 1985. Changes in density of DNA after interaction with dipicolinic acid and its possible role in spore heat resistance. Curr. Microbiol. **12**:329–334.
16. **Munblit, V. Y., and V. I. Trofimov.** 1985. Compensation effect in the processes of thermal-inactivation of microorganisms. Biophysics **30**:103–109.
17. **Murrell, W. G., and A. D. Warth.** 1965. Composition and heat resistance of bacterial spores, p. 1–24. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Washington, D.C.
18. **Woese, C. R.** 1958. Comparison of the X-ray sensitivity of bacterial spores. J. Bacteriol. **75**:5–8.