JOURNAL OF BACTERIOLOGY, Dec. 1983, p. 1332–1337
0021-9193/83/121332-06$02.00/0

Vol. 156, No. 3

Ultrastructure and Extreme Heat Resistance of Spores from Thermophilic *Clostridium* Species

H. H. HYUN,[1] J. G. ZEIKUS,[2]†* R. LONGIN,[2] J. MILLET,[2] AND A. RYTER[2]

Department de Biologie Moleculaire, Institut Pasteur, Paris, France,[2] and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706[1]

Received 10 May 1983/Accepted 19 September 1983

The heat resistance and ultrastructural features of spore suspensions prepared from *Clostridium thermocellum* LQRI, *Clostridium thermosulfurogenes* 4B, and *Clostridium thermohydrosulfuricum* 39E were compared as a function of decimal reduction time. The decimal reduction times at 121°C for strains LQRI, 4B, and 39E were 0.5, 2.5, and 11 min. The higher degree of spore heat resistance was associated with a spore architecture displaying a thicker cortex layer. Heat resistance of these spores was proportional to the ratio of spore cortex volume to cytoplasmic volume. These ratios for spores of strains LQRI, 4B, and 39E were 1.4, 1.6, and 6.6, respectively. The extreme heat resistance and autoclavable nature of *C. thermohydrosulfuricum* spores under routine sterilization procedures is suggested as a common cause of laboratory contamination with pure cultures of thermophilic, saccharide-fermenting anaerobes.

The cellular basis for the extreme heat resistance of bacterial endospores remains an active research topic, principally in the genus *Bacillus*. Previous studies by Murrell and Warth (13) on the mechanism of spore heat resistance reported that *Bacillus* spores which developed in the presence of cycloserine had normal dipicolinic acid contents but lacked cortex development and were not thermally stable. Millet and Ryter (11) showed that *Bacillus* sporulation mutants blocked in cortex formation were not heat stable. Gould and Dring (5) proposed that the cortex of an endospore functioned as an osmoregulatory organelle and conferred heat resistance by prevention of cytoplasmic dehydration. Recently, Beaman et. al. (2) examined five types of *Bacillus* spores and reported that an exponential increase in the heat resistance correlated directly with the wet density and inversely with the water content and with the cytoplasm/cytoplasm plus cortex volume ratio.

Less attention has been directed toward understanding the heat resistance of endospores formed by thermophilic bacteria. Previously, Warth (18) demonstrated that spores from thermophilic bacteria were more heat resistant than those from mesophilic or psychrophilic species. Problems associated with extreme heat resistance of spores from thermophilic anaerobes (e.g., *Clostridium thermosaccharolyticum*) have been reported in the canned food industry (22). Recently, Donelly and Basta (4) showed that *Desulfotomaculum nigrificans*, a thermophilic, sulfate-reducing bacterium, produced spores of unusually high heat resistance with a decimal reduction time (*D*) at 121°C of 5.6 min. This value was higher than that indicated for the aerobic sporeforming thermophile, *Bacillus stearothermophilus* (1, 4).

During the past 8 years, our laboratory has been examining the metabolic properties of different thermophilic bacterial species that transform polysaccharides to ethanol (14, 25). In the past 4 years, our laboratory cultures have been routinely contaminated despite cautious use of rigorous asceptic cultural techniques. Unknowingly, we have sent to other investigators *Clostridium thermocellum* cultures that were contaminated by pentose-fermenting species. Also, upon receipt of nonsporeforming, thermophilic, ethanol-producing bacteria (3, 20, 24) from other investigators or major culture collections, we have routinely noted the presence of contaminating spores even after modification of our medium autoclaving procedures. These problems led us to examine whether spores of thermophilic anaerobes could withstand normal autoclaving procedures. In view of these facts, the present research report was directed toward comparing the heat resistance and ultrastructural features of three thermophilic ethanologenic species: *C. thermocellum* LQRI (9), *Clostridium thermohydrosulfuricum* 39E (23), and *Clostridium thermosulfurogenes* 4B (16a). The data show

† Present address: Department of Bacteriology, University of Wisconsin, Madison, WI 53706.

that spore suspensions of *C. thermohydrosulfuricum* can easily withstand normal 15 to 20 min autoclaving and that the extreme thermal resistance of thermophilic anaerobic spores correlates with the ratio of spore cortex volume to spore cytoplasmic volume.

MATERIALS AND METHODS

Organisms and growth conditions. *C. thermocellum* (9), *C. thermosulfurogenes* (16a), and *C. thermohydrosulfuricum* (23) were routinely cultured using anaerobic procedures described previously (23) but with a medium autoclaving time of 45 min at 121°C. Cultures were maintained in Bellco anaerobic pressure tubes (18 by 142 mm; Bellco Glass, Inc., Vineland, N.J.) that contained 10 ml of medium and an N_2-CO_2 (95:5) gas headspace and which were closed with a pressure bung and metal serum crimp seal.

For preparation of spore suspensions with either *C. thermohydrosulfuricum* or *C. thermosulfurogenes*, the organisms were grown on a low-phosphate-buffered basal medium (LPBB medium [24]) containing 0.05% yeast extract, 0.2% xylose, and 1.0% 4-morpholinepropanesulfonic acid (MOPS) for 3 days, and the final culture broth served as the spore suspension. *C. thermocellum* was grown on CM3-3 medium with 0.3% yeast extract and 0.5% cellobiose. CM3-3 medium was modified from that reported by Petre et. al. (15) and contained (per liter): KH_2PO_4, 1.5 g; $K_2HPO_4 \cdot 3H_2O$, 2.2 g; $(NH_4)_2SO_4$, 1.3 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.0025 g; 0.2% resazurin, 1 ml, 10% $NaHCO_3$, 50 ml; 2.5% cysteine · HCl, 20 ml; and vitamin solution, 20 ml. $NaHCO_3$ and cysteine · HCl solutions were added separately after sterilization, and vitamin solution was added after filter sterilization. The vitamin solution contained (per liter of distilled water): thiamine · HCl, 25 mg; pyridoxine · HCL, 25 mg; pyridoxamine · HCl, 25 mg; pyridoxal · HCl, 25 mg; calcium panthotenate, 25 mg; riboflavin, 25 mg; nicotinic acid, 25 mg; lipoic acid, 25 mg; *p*-aminobenzoate, 1 mg; folic acid, 1 mg; biotin, 0.1 mg; vitamin B_{12}, 0.1 mg.

Electron microscopy. The 3-day-old sporulating cultures were fixed with osmium tetroxide according to the method of Ryter and Kellenberger (16). After prefixation, the bacterial suspensions were centrifuged, concentrated in agar (19), and then fixed overnight in osmium tetroxide. The cells were postfixed with uranyl acetate and then dehydrated in acetone and embedded in Epon. Thin sections were stained for 3 min with lead citrate and examined in a Siemens electron microscope.

Determination of spore cortex to cytoplasmic volume. Both spore cortex and cytoplasm volumes were measured from representative electron micrographs of mature spores. The dimensions of the individual mature spores examined did not vary more than ±5% for growth in four separate thin sections of each species.

The volumes of spore cortex plus cytoplasm and cytoplasm were calculated by the equations $4/3\ \pi\gamma^3$ for the spherical spores (i.e., strains 4B and 39E) and $4/3\ \pi\ a^2b$ for the oval spores, where γ is the radius, a is the radius of the short axis, and b is the radius of the long axis. Therefore, the cortex volume was calculated by subtracting the volume of cytoplasm from the volume of spore cortex plus cytoplasm.

Heat resistance. The spore suspensions (i.e., cultural fluid) of each organism were adjusted to pH 7.0 with 1 N NaOH, and 2-ml samples were distributed into sterile, N_2-gassed, anaerobic pressure tubes. The tubes were then heated at the indicated times and temperatures in an oil bath. Control tubes with an inserted thermometer were used for lag time corrections. After being heated at the desired temperature, tubes were withdrawn at given time intervals and cooled on ice, and samples were diluted into LPBB medium. For cell survivor counts, the diluted samples were plated in an anaerobic chamber (Coy Products, East Lansing, Mich.) onto either LPBB medium containing 0.1% yeast extract, 0.5% xylose, and 2.5% agar for *C. thermohydrosulfuricum* and *C. thermosulfurogenes* or onto CM3-3 medium containing 0.1% yeast extract, 0.5% cellobiose, and 2.5% agar for *C. thermocellum*. The number of survivors was determined by the average of duplicate plate count experiments.

The death rate constant (k) and D were calculated by the methods of Aiba and Humphrey (1), with $\ln N/N_0 = -kt$ and $D = 2.303/k$, where N is the number of survivors, N_0 is the number of survivors at zero time, and t is time. Activation energy (E) was obtained from the logarithmic plot of the Arrhenius equation, $K = \alpha' e^{-E/RT}$, where α' is the empirical constant, R is the gas constant, and T is the absolute temperature.

RESULTS

Figure 1 illustrates the representative ultrastructural features of *C. thermocellum* spores. Developing sporangia were distinctly swollen. Mature spores were oval shaped and contained a well-formed, electron-transparent cortex and electron-dense wall coat layers. Less than 20% of the cells examined contained spores, and numerous abortive spores were detected which displayed architectural features distinct from mature spores (e.g., lack of a cortex or defined spore coat layers). The average ratio of spore cortex volume to cytoplasmic volume was 1.4.

Figure 2 shows the ultrastructural features of *C. thermosulfurogenes* spores. Less than 10% of the cells examined contained spores, and both abortive and mature spores were observed. Developing sporangia were distinctly swollen. Mature spores were spherical and contained a well-defined cortex and a large cytoplasm. The average ratio of spore cortex volume to spore cytoplasm volume was 1.6.

Figure 3 shows the representative ultrastructural features of *C. thermohydrosulfuricum* spores. Less than 20% of the cell culture examined contained mature and abortive spores. Developing sporangia were not distinctively swollen. Mature spores were spherical and contained a very thick cortex but a small cytoplasm. The average ratio of spore cortex volume to cytoplasm volume was 6.6.

Figures 4, 5, and 6 compare the thermal

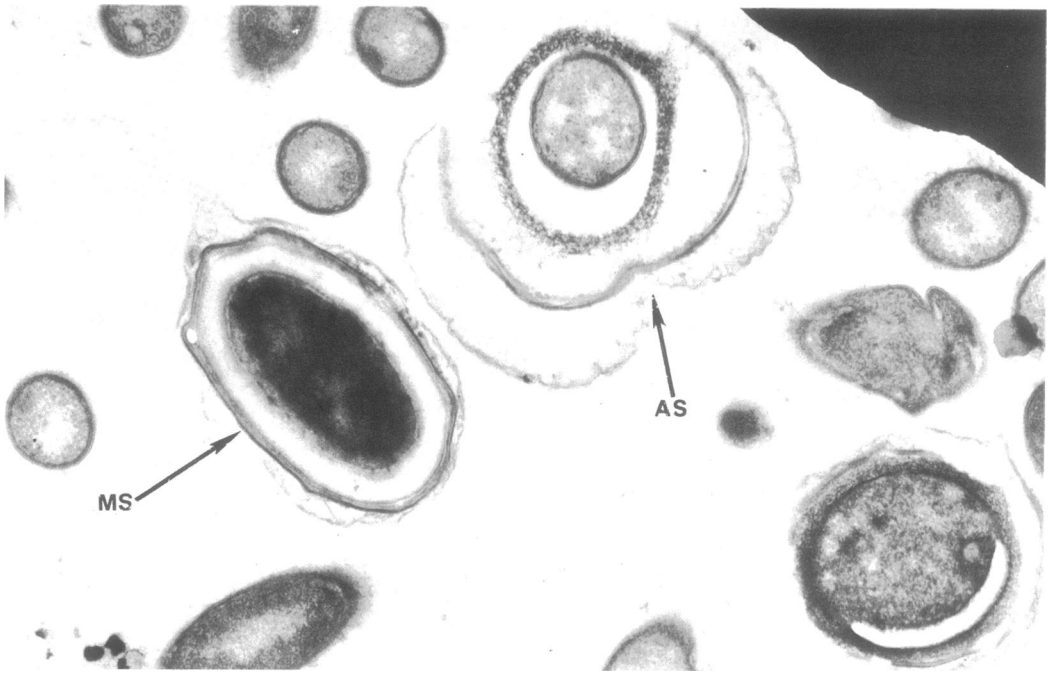

FIG. 1. Electron micrograph of thin sections of *C. thermocellum* prepared from a sporulated culture. Both mature spores (MS) and abortive spores (AS) are shown by arrows. Magnification: 40,000×.

survivor curves and the thermal death rate plots of spore suspensions from *C. thermohydrosulfuricum*, *C. thermosulfurogenes*, and *C. thermocellum*. The thermal survivor curves were biphasic at lower temperatures for all spores, indicating the presence of abortive, non-heat-resistant spores which were viable in the total spore population. *k* and *D* values were obtained from the latter linear phases. Vegetative cells of *C. thermohydrosulfuricum* were too heat sensi-

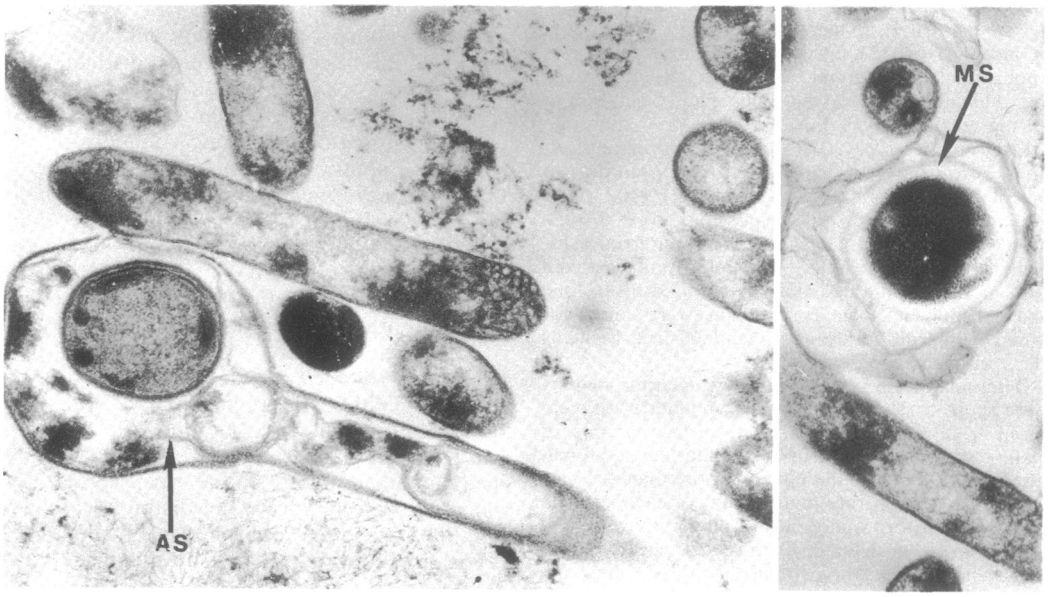

FIG. 2. Electron photomicrographs of thin sections of *C. thermosulfurogenes* prepared from a sporulated culture. Both mature spores (MS) and abortive spores (AS) are shown by arrows. Magnification: 38,000×.

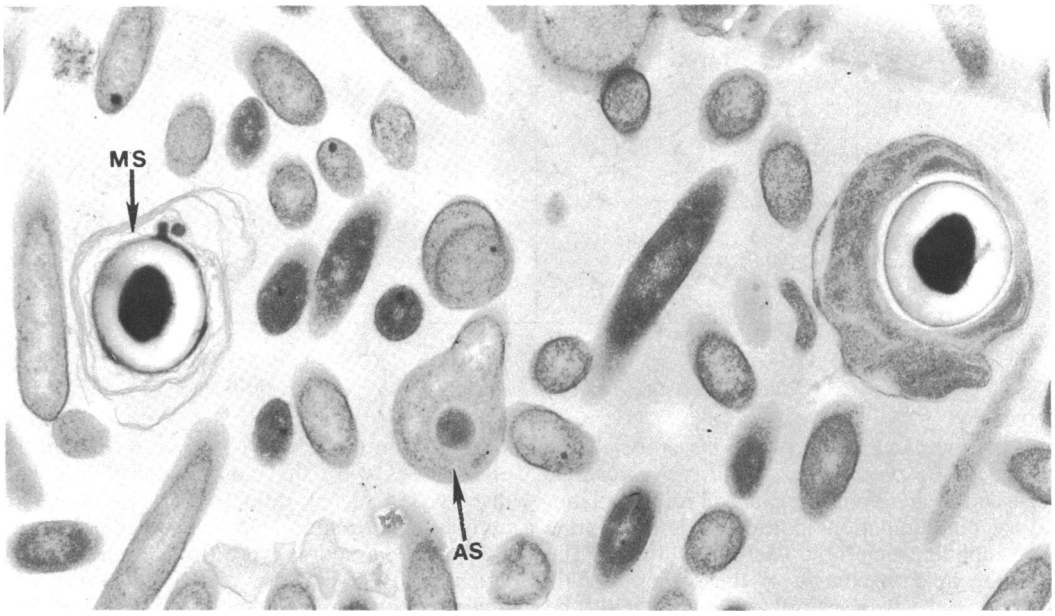

FIG. 3. Electron micrograph of thin sections of *C. thermohydrosulfuricum* prepared from a sporulated culture. Both mature spores (MS) and abortive spores (AS) are shown by arrows. Magnification: 30,000×.

tive to measure death rates at temperatures higher than 85°C. Vegetative cells of *C. thermosulfurogenes* and *C. thermocellum* were even more heat sensitive, and cells were all (~10^7 cells per ml) killed by a 3-min heat treatment at 75°C.

Spores of *C. thermohydrosulfuricum* survived heating at 132 and 121°C for short periods and were extremely heat resistant below 121°C (Fig. 4). The calculated thermal activation energy was ca. 213.4 kJ/g mol. Spores of *C. thermosulfurogenes* were considerably less heat resistant at 115 and 110°C (Fig. 5). Their calculated thermal activation energy was ca. 324.3 kJ/g mol. Spores of *C. thermocellum* were the most readily inactivated by high temperature (Fig. 6) and displayed a thermal activation energy of ca. 364.4 kJ/g mol.

DISCUSSION

Table 1 compares published values for spore heat resistance as a function of *D* with those from the thermophilic, anaerobic species examined here. It is quite remarkable that spores of *C. thermohydrosulfuricum* displayed a D value at 121°C of 11 min, as compared with the value of 3 min for *B. stearothermophilus* spores, which are commonly used as the standard to judge autoclaving procedures for materials used

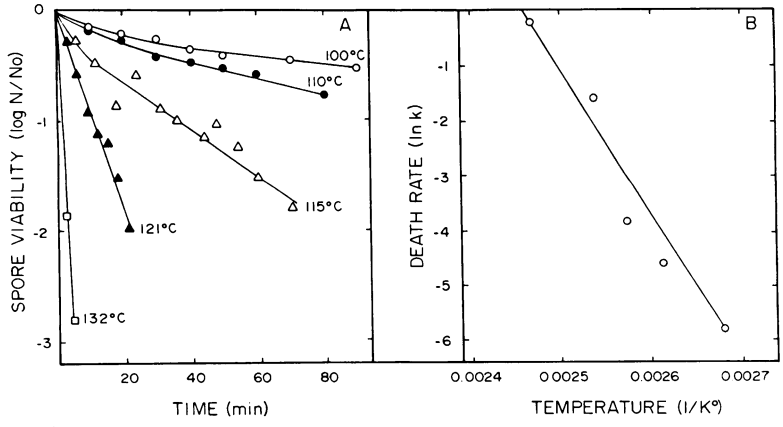

FIG. 4. Thermal resistance of *C. thermohydrosulfuricum spore suspensions.*

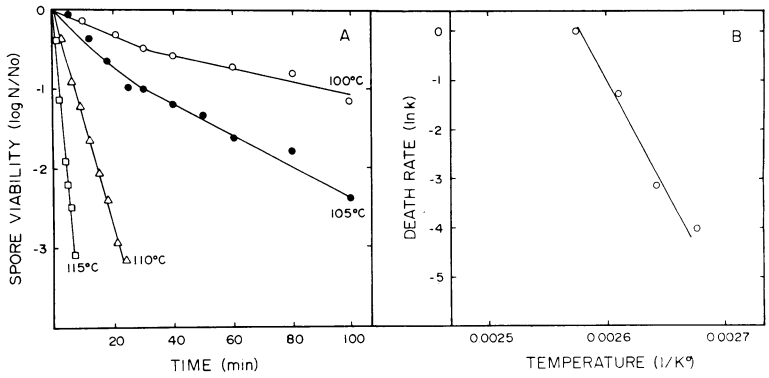

FIG. 5. Thermal resistance of *C. thermosulfurogenes* spore suspensions.

in microbial cultural work. The autoclavable nature of *C. thermohydrosulfuricum* spores is regarded as an important cause of frequent laboratory contamination in saccharide-fermenting thermophilic, anaerobic bacterial cultures. The extreme heat resistance of these thermophilic spores suggests that investigators should be more cautious in preparation of cultural materials to maintain pure cultures of thermophilic bacteria. In addition to the thermophilic heterotrophic sporeformers examined here, lithotrophic species that ferment H_2-CO_2 have also been described (7, 21). Thus, one should also be wary of contamination by sporeformers during autotrophic cultivation of thermophilic species such as *Methanobacterium thermoautotrophicum* (26). A minimum autoclaving time of 30 min at 121°C is suggested for preparation of cultural materials to grow thermophilic bacteria.

The ultrastructural and heat-resistance data presented here support the current functional role for the spore cortex as the major determining factor in the heat stability of endospores (5, 13). The differences in heat stability of spores from thermophilic anaerobes were directly related to the thickness of the cortex and inversely related to the cytoplasm/cytoplasm plus cortex volume ratio (2). Thus, *C. thermohydrosulfuricum* spores were the most heat resistant and displayed a higher ratio of cortex volume to cytoplasmic volume. On the other hand, *C. thermocellum* spores were the least heat resistant and displayed a lower ratio of cortex volume to cytoplasmic volume. Other investigators (5, 13) suggested that the contraction of the cortex is important in the maintenance of spore heat resistance. Thus, spores with a very thick cortex layer, such as those produced by *C. thermohydrosulfuricum*, would contract considerably and may be less prone to dehydration by heat. Other components in these spores, such as Ca^{2+} or diaminopimelic acid, may also be of importance in the transformation of heat-labile vegetative cells into heat-stable spores by facilitating contraction of the cortex via reduction in electrostatic repulsion of ionic groups in the cortex (5). Therefore, spores with a very thick cortex may require more Ca^{2+} or diaminopimelic acid in contraction of the cortex, resulting in less water activity of the cytoplasm and higher heat resistance. This feature awaits further analysis in the extremely heat-resistant spores of *C. thermohydrosulfuricum*.

The biphasic survivor curves observed for

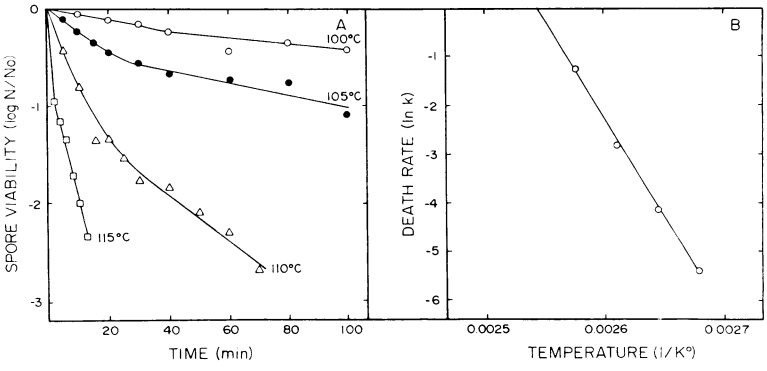

FIG. 6. Thermal resistance of *C. thermocellum* spore suspensions.

TABLE 1. Heat resistance of spores as a function of *D*

Organism	*D* value (min)		
	100°C	110°C	121°C
C. thermocellum LQRI	200	8	0.5
C. thermosulfurogenes 4B	520	33	2.5
C. thermohydrosulfuricum 39E	770	123	11
D. nigrificans[a]			5.6
Bacillus subtilis[b]			0.9
B. stearothermophilus FS1518[b]			3.0
B. stearothermophilus FS617[b]			0.8
Clostridium sporogenes PA3679[b]			1.3

[a] Data obtained from reference 4.
[b] Data obtained from reference 1.

spores from thermophilic anaerobes at lower thermal inactivation temperatures can be explained in terms of a heterogeneous population of spores with differing heat stability. The presence of abortive spores in which the cortex was either lacking or incomplete was observed when spore suspensions were examined by electron microscopy. Thus, the heterogeneous population of abortive and mature spores would result in biphasic survivor curves at a low temperature, whereas at a high temperature, fast inactivation of abortive spores during the heating process would result in only mature spores at zero times and hence linear survivor curves. Previously, investigators have explained the appearance of nonlinear survivor curves by either the multiple critical site theory (10, 12), experimental artifacts (17), heterogeneity of spore heat resistance in populations before heating (6), or heterogeneity which is developed during heating as a result of spore heat adaptation (8).

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, by a research fellowship to J.G.Z. from Institut Pasteur, and by grants from Solvay and Cie to Institut Pasteur and the University of Wisconsin.

LITERATURE CITED

1. **Aiba, S., and A. E. Humphrey.** 1978. Biochemical engineering, p. 240–244. Tokyo University Press, Tokyo.
2. **Beaman, T. C., J. T. Greenamyre, T. R. Corner, H. S. Pankratz, 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. **Ben-Bassat, A., and J. G. Zeikus.** 1981. *Thermobacteroides acetoethylicus* gen. nov. sp., a new chemoorganotrophic, anaerobic bacterium. Arch. Microbiol. **128**:365–371.
4. **Donnelly, L. S., and F. F. Busta.** 1980. Heat resistance of *Desulfotomaculum nigrificans* spores in soy protein infant formula preparations. Appl. Environ. Microbiol. **40**:721–725.
5. **Gould, G. W., and G. J. Dring.** 1975. Heat resistance of bacterial endospores and the concept of an expanded osmoregulating cortex. Nature (London) **258**:402–405.
6. **Han, Y. W.** 1975. Death rates of bacterial spores: nonlinear survivor curves. Can. J. Microbiol. **21**:1464–1467.
7. **Kerby, R., and J. G. Zeikus.** 1983. Growth of *Clostridium thermoaceticum* on H_2-CO_2 or CO as energy source. Curr. Microbiol. **8**:27–30.
8. **Komemushi, S., K. Okubo, and G. Terui.** 1968. Kinetics of the thermal deaths of microorganisms. IV. On the change of death rate constant of bacterial spores in the course of heat sterilization. J. Ferment. Technol. **46**:249–256.
9. **Lamed, R. J., and J. G. Zeikus.** 1980. Ethanol production by thermophilic bacteria: relationship between fermentation product yields of and catabolic enzyme activities in *Clostridium thermocellum* and *Thermoanaerobium brockii*. J. Bacteriol. **144**:569–578.
10. **Meynell, G. G., and E. Meynell.** 1970. Theory and practice in experimental bacteriology. Cambridge University Press, London.
11. **Millet, J., and A. Ryter.** 1972. Mutants de *Bacillus subtilis* marburg bloques tardivement dans leur sporulation. Ann. Inst. Pasteur (Paris) **122**:395–406.
12. **Moats, W. A.** 1971. Kinetics of thermal death of bacteria. J. Bacteriol. **105**:165–171.
13. **Murrell, W. G., and A. D. Warth.** 1967. Composition and heat resistance of bacterial spores, p. 1–24. *In* L. L. Campbell and H. D. Halvorson (ed.), Spores III. American Society for Microbiology, Ann Arbor, Mich.
14. **Ng, T. K., P. J. Weimer, and J. G. Zeikus.** 1977. Cellulolytic and physiological properties of *Clostridium thermocellum*. Arch. Microbiol. **114**:1–7.
15. **Petre, J., R. Longin, and J. Millet.** 1981. Purification and properties of an endo beta-1,4 glucanase from *Clostridium thermocellum*. Biochimie **63**:629–639.
16. **Ryter, A., and E. Kellenberger.** 1958. Etude au microscope electronique de plasmas contenant de l'acide desoxyribonucleique. I. Les nucleoides des bacteries en croissance. Z. Naturforsch. Sect. C. **13**:597–605.
16a. **Schink, B., and J. G. Zeikus.** 1983. *Clostridium thermosulfurogenes* sp. nov., a new thermophile that produces elemental sulfur from thiosulfate. J. Gen. Microbiol. **129**:1149–1158.
17. **Stumbo, C. R.** 1965. Thermobacteriology in Food Processing. Academic Press, Inc., New York.
18. **Warth, A. D.** 1978. Relationship between the heat resistance of spores and the optimum and maximum growth temperatures of *Bacillus* species. J. Bacteriol. **134**:699–705.
19. **Whitehouse, R., I. C. Benichau, and A. Ryter.** 1977. Procedure for the longitudinal orientation of rod-shaped bacteria and the production of a high cell density of procaryotic and eucaryotic cells in thin sections of electron microscopy. Biol. Cell **30**:155–158.
20. **Wiegel, J., and L. G. Ljungdahl.** 1981. *Thermoanaerobacter ethanolicus* gen. nov. sp. nov., a new extreme thermophilic anaerobic bacterium. Arch. Microbiol. **128**:343–350.
21. **Wiegel, J., M. Braun, and G. Gottschalk.** 1981. *Clostridium thermoautotrophicum* species novum, a thermophile producing acetate from molecular hydrogen and carbon dioxide. Curr. Microbiol. **5**:255–260.
22. **Xezones, H., T. L. Seymiller, and I. J. Hutchings.** 1965. Processing requirements for a heat tolerant anaerobe. Food Technol. **20**:111–112.
23. **Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge.** 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. **143**:432–440.
24. **Zeikus, J. G., P. W. Hegge, and M. A. Anderson.** 1979. *Thermoanaerobium brockii* gen. nov. spec. nov., a new caldoactive anaerobic bacterium. Arch. Microbiol. **122**:41–47.
25. **Zeikus, J. G., and T. K. Ng.** 1981. Thermophilic saccharide fermentations, p. 263–290. *In* G. Tsao (ed.), Annual reports on fermentation processes, vol. 5. Academic Press, Inc., New York.
26. **Zeikus, J. G., and R. S. Wolfe.** 1972. *Methanobacterium thermoautotrophicus* sp. n., an anaerobic, autotrophic, extreme thermophile. J. Bacteriol. **109**:707–713.