NOTES

Bacillus sp. ATCC 27380: a Spore with Extreme Resistance to Dry Heat

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An unusual mesophilic Bacillus sp. was isolated from heated soil, and a cleaned spore preparation showed extraordinary resistance to dry heat ($D_{125\text{C}} = 139 \text{ h}$) and relative sensitivity to moist heat ($D_{65\text{C}} = 61 \text{ min}$). Biochemical tests and morphology fit no described species.

For the past several years, we have been involved in the study of dry-heat inactivation kinetics of bacterial spores as related to terminal sterilization of spacecraft destined to detect extraterrestrial life. These investigations have included spores in their naturally occurring environments (soils and dusts) as well as environmental isolates cultured with various types of media (2, 3).

Prior to 1960, relatively little was known about inactivation by dry heat at temperatures below 160 C, and workers initially involved in the problem of sterilizing a spacecraft at approximately 125 C began using spores of Bacillus subtilis var. niger to study changes in dry-heat resistance levels due to pressure, gases, moisture, and combinations of these and other physical parameters. The spores of this organism, now commonly used as a biological indicator for dry-heat cycles, came to be considered as a representative of "average" resistance (4).

In earlier studies of naturally occurring spore populations in 25 soil samples collected in various parts of the United States, we observed $D_{125\text{C}}$ values (length of time necessary to effect 90% kill at 125 C) ranging from 16 to 126 min (2). Pure spore crops of B. subtilis var. niger, depending upon the type of sporulation medium and heating system used, have shown $D_{125\text{C}}$ values ranging from 8 to 50 min (W. W. Bond and M. S. Favero, unpublished data); values of subcultured environmental isolates from spacecraft, assembly areas, and soils have ranged from <5 to 100 min (3; unpublished data).

During a study of naturally occurring spores, an unusually dry-heat-resistant population was encountered when a suspension of soil from Cape Kennedy was tested. The raw soil sample had been washed with 95% ethyl alcohol through a graded series of stainless steel sieves, the smallest opening being 43 $\mu$m (U.S. series equivalent 325; W. S. Tyler Co., Mentor, Ohio). The resultant spore and soil concentrations were $2.7 \times 10^9/\text{ml}$ and $0.03 \text{ g/ml}$, respectively. Portions (0.1 ml) of the suspension were applied to stainless steel strips (0.5 inch by 0.5 inch), and the dry heat resistance level was determined by a method reported earlier (2) using Trypticase soy agar (TSA; BBL) supplemented with soluble starch and yeast extract (3) as the recovery medium. Figure 1 shows the survivor curve when four strips were heated at each interval and the $D_{125\text{C}}$ value of 24 h was calculated from a best-fit regression line of the mean data points (ignoring the unheated controls) by using a least squares method.

During an end-point determination with this naturally occurring spore population, in which survivors at the 48-h interval were recovered in broth (3), an unusual, slow-growing sporeformer was isolated after 1 month of incubation at 32 C. Positive broth tubes were recognized only when vigorously agitated, thereby showing a clear, compact, mucoid sediment with no turbidity in the supernatant broth. When subcultured on supplemented TSA at 32 C, growth and sporulation were also slow, requiring 10 to 14 days to achieve detectable levels of sporulation.

Young cultures of the isolate exhibited spher-
spores approximately oval in shape. Vegetative cells became pleomorphic in older cultures, and the highly sculptured spore surfaces were discernible using light microscopy; the less dense forms showing surface texture were most likely spore cell debris left after germination. Figure 2 is a scanning electron photomicrograph of a single spore showing the surface morphology in detail with suggestion of exosporium remnants. The honeycomb pattern of polygonal depressions surrounded by straight ridges is similar to that seen in electron photomicrographs of "B. megaterium 350" described by Robinow (10) and the freeze-etched preparations of B. polymyxa and B. fastidiosus shown by Holt and Leadbetter (6).

Attempts at identification of this isolate by standard biochemical methods (12; R. E. Gordon, W. C. Haynes and C. H-N. Pang, The Genus Bacillus, U.S.D.A., Agricultural Handbook no. 427, in press) have shown that it fits no described pattern. Positive tests were obtained with (i) growth in 2, 4, and 10% NaCl broth, and (ii) production of catalase. Negative tests were (i) motility, (ii) utilization of citrate, (iii) hydrolysis of starch, gelatin, or casein, (iv) production of acetyl methylcarbinol or indole, (v) reduction of nitrate or methylene blue, (vi) growth at 45°C (growth was poor at 37°C), and (vii) utilization of carbohydrates (arabinose, glucose, lactose, sorbitol, rhamnose, mannitol, or xylose). Growth on supplemented TSA in a Brewer Anaerobic Jar (BBL) was negative in 14 days at 32°C. The Bacillus sp. isolate was submitted to the American Type Culture Collection and was given an accession number of 27380.

An actively growing culture was streaked on thickly poured 100- by 15-mm plates of AK #2 sporulation agar (Difco) supplemented with 20 μg of magnesium sulfate per ml and 80 μg of calcium chloride per ml. After incubation for 20 days at 32°C, growth was harvested and cleaned by a method described previously (2), and spores were resuspended in phosphate-buffered distilled water (BDW; reference 1) held at 4°C. Survival of the spores when exposed to dry heat at 125°C was determined by the stainless steel strip assay method (3) modified by insonating for 60 s with a Biosonic III Ultrasonic Probe (2) at 60% maximum intensity prior to plating the rinse solution from each strip. The moist heat D value was determined by placing inoculated stainless steel strips in tubes containing 10 ml of BDW and heating the tubes at 80°C for appropriate intervals in an oil bath (Blue M Electric Co., Blue Island, Ill., Mod. MW1115A). Assay subsequent to cooling of the tubes was identical to the dry-heat determination. All heating and assay manipulations were conducted in a vertical laminar flow clean room (3). Plates were incubated at 32°C and counted at 2-day intervals for 2 weeks, and Fig. 3 shows the results when five test units were heated at each interval.

Fig. 1. Survival of naturally occurring spores in Cape Kennedy soil at 125°C dry heat.

Fig. 2. Scanning electron micrograph of a spore of Bacillus sp. ATCC 27380. ×30,000. Courtesy of D. J. Gould, Div. 2515, Sandia Laboratories, Albuquerque, N.M.
Fig. 3. Survival of spores of Bacillus sp. ATCC 27380 exposed to 80 °C moist heat or 125 °C dry heat.

concave downward (8) shapes of both survivor curves indicate that Bacillus sp. ATCC 27380 spores require either dry- or moist-heat activation (5); it is emphasized that the maximum level of germination in the dry-heat system was reached only after 24 h at 125 °C. The gentle slope of the dry-heat curve (D = 139 h) indicates that 125 °C is barely the threshold temperature of lethality.

The extreme magnitude of the dry-heat resistance (5, 7-9, 11) and also the difference in resistance levels between dry and moist heat may make this organism a valuable tool in the elucidation of spore germination mechanisms, the biophysical nature of spores, mechanisms of heat inactivation, and possibly as a more stringent biological indicator for dry-heat sterilization cycles.

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