APPLIED MICROBIOLOGY, Oct. 1973, p. 614-616

Vol. 26, No. 4
Printed in U.S.A.

NOTES

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

WALTER W. BOND, MARTIN S. FAVERO, AND MARILYN R. KORBER

The Environmental Microbiology Section, Phoenix Laboratories, Ecological Investigations Program, Center for Disease Control, Phoenix, Arizona 85014

Received for publication 5 June 1973

An unusual mesophilic *Bacillus* sp. was isolated from heated soil, and a cleaned spore preparation showed extraordinary resistance to dry heat (D_{125C} = 139 h) and relative sensitivity to moist heat (D_{80C} = 61 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_{125C} 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medium and heating system used, have shown D_{125C} values ranging from 8 to 50 min (W. W. Bond and M. S. Favero, unpublished data); values of subcultured environmental isolates from spacecraft, assembly areas, and soils have ranged from 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5 to 100 min (3; unpublished data).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 μm (U.S. series equivalent 325; W. S. Tyler Co., Mentor, Ohio). The resultant spore and soil concentrations were 2.7×10^6/ml and 0.03 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_{125C} value of 24 h was calculated from a best-fit regression line of the mean data points (ignoring N_0, 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-

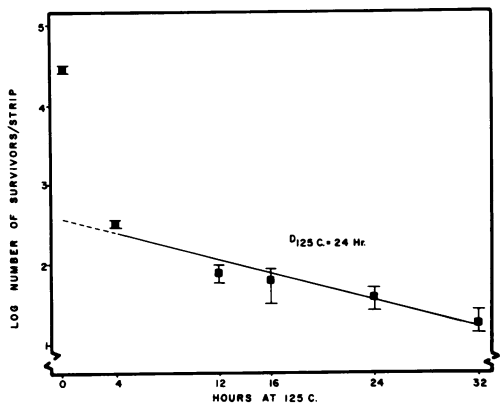

FIG. 1. *Survival of naturally occurring spores in Cape Kennedy soil at 125 C dry heat.*

ical to slightly oval, central to subterminal spores having rough, stainable walls. After leaving the swollen sporangium, the spores appeared to increase in size from 1.2 μm to approximately 1.7 μm while assuming a more oval 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 wall 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 acetylmethylcarbinol 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. The

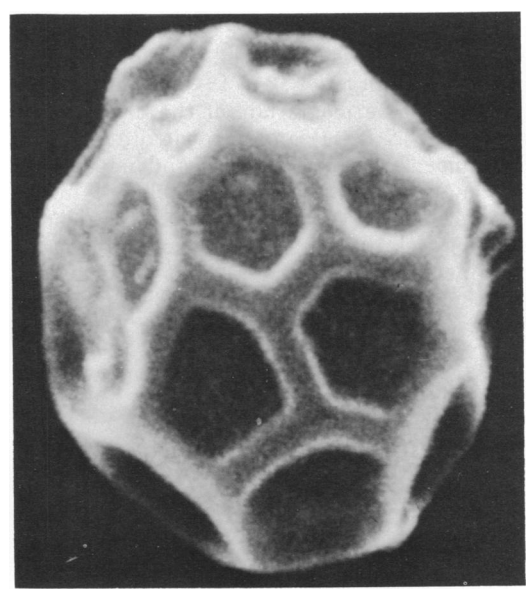

FIG. 2. *Scanning electron micrograph of a spore of Bacillus sp. ATCC 27380. ×30,000. Courtesy of D. J. Gould, Div. 2515, Sandia Laboratories, Albuquerque, N.M.*

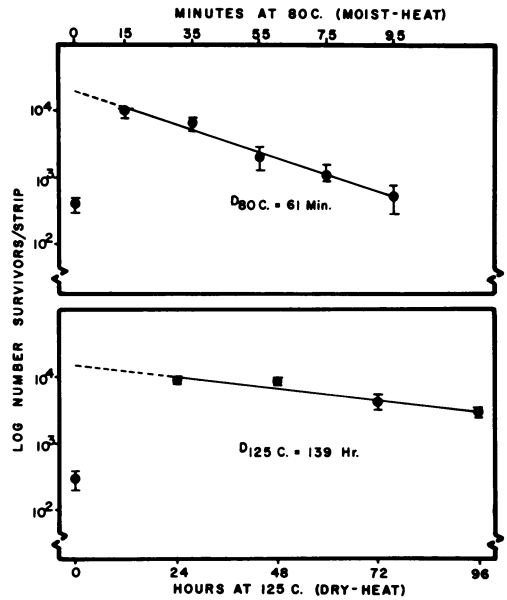

FIG. 3. *Survival of spores of Bacillus sp. ATCC 27380 exposed to 80 C moist heat or 125 C dry heat.*

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.

Services were provided in support of the planetary quarantine requirements of the National Aeronautics and Space Administration under Contract W-13,062.

We are indebted to Ruth Gordon of Rutgers University for her helpful comments and assistance with identification schemes for *Bacillus* spp. Sincere appreciation is also expressed to H. Orin Halvorson and to personnel of the Applied Science Division (Div. 5252), Sandia Laboratories, Albuquerque, New Mexico, for their cooperation.

LITERATURE CITED

1. American Public Health Association, Inc., American Water Works Association, and Water Pollution Control Federation. 1965. Standard methods for the examination of water and waste water including bottom sediments and sludges, 12th ed. American Public Health Association, Inc., New York.
2. Bond, W. W., M. S. Favero, N. J. Petersen, and J. H. Marshall. 1970. Dry-heat inactivation kinetics of naturally occurring spore populations. Appl. Microbiol. **20**:573-578.
3. Bond, W. W., M. S. Favero, N. J. Petersen, and J. H. Marshall. 1971. Relative frequency distribution of $D_{125\,C}$ values for spore isolates from the Mariner-Mars 1969 spacecraft. Appl. Microbiol. **21**:832-836.
4. Craven, C. W., J. A. Stern, and G. F. Ervin. 1968. Planetary quarantine and space vehicle sterilization. Astronaut. Aeronaut. **6**:18-48.
5. Ernst, R. R. 1968. Sterilization by heat, p. 703-740. *In* C. A. Lawrence and S. S. Block (ed.), Sterilization, disinfection, and preservation. Lea and Febiger, Philadelphia.
6. Holt, S. C., and E. R. Leadbetter. 1969. Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. Bacteriol. Rev. **33**:346-378.
7. Macek, T. J. 1973. Biological indicators and the effectiveness of sterilization procedures, p. 19-34. *In* G. Briggs Phillips and William S. Miller (ed.), Industrial sterilization. Duke University Press, Durham, N.C.
8. Pflug, I. J. 1973. Heat sterilization, p. 239-282. *In* G. Briggs Phillips and William S. Miller (ed.), Industrial sterilization. Duke University Press, Durham, N.C.
9. Pflug, I. J., and C. F. Schmidt. 1968. Thermal destruction of microorganisms, p. 63-105. *In* C. A. Lawrence and S. S. Block (ed.), Sterilization, disinfection, and preservation. Lea and Febiger, Philadelphia.
10. Robinow, C. F. 1960. Morphology of bacterial spores, their development and germination, p. 207-248. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 1. Academic Press Inc., New York.
11. Sivinski, H. D., D. M. Garst, M. C. Reynolds, C. A. Trauth, Jr., R. E. Trujillo, and W. J. Whitfield. 1973. The synergistic inactivation of biological systems by thermoradiation, p. 305-335. *In* G. Briggs Phillips and William S. Miller (ed.), Industrial sterilization. Duke University Press, Durham, N.C.
12. Smith, N. R., R. E. Gordon, and F. E. Clark. 1952. Aerobic spore-forming bacteria. Agricultural monograph no. 16, U.S. Department of Agriculture, U.S. Government Printing Office, Washington, D.C.