Some Physical Properties of Coat Material from *Bacillus stearothermophilus* Spores

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**Abstract.** Coat material from *Bacillus stearothermophilus* spores has been examined for the following properties: X-ray diffraction pattern, infrared absorption spectrum, mechanical strength, and melting temperatures of the crystalline regions. The X-ray diffraction pattern of the coat material is different from that of both α- and β-keratin. The high melting temperature of the crystalline material indicates that its bonding is more stable than that of α- or β-keratin. The mechanical strength of the coat material $= 10^9$ N/m$^2$ is shown to be high enough to allow the coat to support the internal pressure in bacterial spores. This pressure has been postulated to produce a partial dehydration, which increases the ability of bacterial spores to withstand high temperatures in water.

Bacterial spores of all species have the same general morphology, consisting of an inner core (protoplast) surrounded by a germ cell wall, cortex, coat, and in some species a thin, loosely fitting envelope, the exosporium. The peptidoglycan composition of the cortex is reasonably well understood [11], although its physical structure is less well known. The coat is composed of an entirely different material, being proteinaceous and partly crystalline. The crystalline material in the coat of *Bacillus subtilis* is reported [9] to be in both the α helical and β pleated sheet forms of keratin, the major component of wool, horn, and feathers. Amino acid analysis of the coat from *B. subtilis* reveals a composition not greatly different from that of keratin, although there is less cysteine [9].

It has been suggested that the coat is critically involved in the basic mechanism of the heat resistance of bacterial spores [1]. This theory is based on the premise that the interior of the mature spore is held in a partially dehydrated state through pressure applied by the cortex, which is supported by the coat. The mechanical strength of the coat must be known before one can decide whether or not the theory is reasonable. This paper contains the results of the first experimental determinations of the mechanical strength. Some other physical properties of coat material, including X-ray diffraction pattern, infrared absorption spectrum, and the melting temperature of the crystalline regions when dry, are reported in this paper.

The whole of the coat material has been examined, even though it is well known [2, 8] that the coat of bacterial spores may consist of several layers with differing degrees of organization as seen with an electron microscope. The most important point of this study is the determination of the mechanical strength of the whole coat; the other physical studies are concerned with attempts to understand the basis for that strength. Coat material from *Bacillus stearothermophilus* spores was used throughout the study, since its spores are among the most thermostable [11].

**Materials and Methods**

Spores of *B. stearothermophilus* ATCC 7953 were grown, washed, and cleaned as previously described [11] and then freeze-dried.

**Preparation of coat material.** Ten-ml lots of freeze-dried spores (10 mg/ml), suspended in 0.05 M ammonium acetate at pH 7.2, were shaken at 0°C in bottles with 20-g glass beads (grade 12-13) at 950 reciprocations/min for 15-45 min until over 99.9% of the spores were disrupted. The disrupted spore suspension and beads were washed rapidly onto a grade 0 sintered glass funnel, and the beads were washed twice by shaking in a measuring cylinder with five times their volume of ice-cold distilled water. The suspension and washings were then centrifuged for 20 min at 10,000 g and 1°C to give a deposit of coat and cortex. This was washed five times with ice-cold glass-distilled water. The coat and cortex fraction was resuspended in 0.05 M ammonium

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acetate, pH 7.2, containing lysozyme (0.2 mg/ml final) and 2:1 toluene–CHCl₃ (1 ml/100 ml) and incubated with shaking at 37°C for 2 h. The coats, after digestion of the cortex by the lysozyme, were sedimented at 10,000 g for 20 min at 0°C, washed six times with ice-cold glass-distilled water, recentrifuged, and freeze dried.

**X-ray diffraction.** X-ray diffraction powder photographs were obtained by irradiating freeze-dried coat material in the form of a 1-cm disc which was produced by pressing the powdered coat with a pressure of 700 x 10⁵ N/m² in a hydraulic press used for making discs from powders for infrared spectroscopy. The equipment used was a Phillips PW 1011 generator and a Phillips vertical goniometer. Copper Kα radiation with an average wavelength of 1.54178 angstroms was used. The X-ray generator power was 50 kV, 28 mA. Slits for divergence and receiving were 1°, and the slit for scatter was 4°. A proportional counter was employed for the detection with a graphite filter. Scanning rate was 1/2° of the Bragg angle (2θ) per minute, and the full-scale count was 1000 counts per second.

**Mechanical strength.** A small piece (1 mm x 1 mm x 200 μm) of a disc similar to that used in the X-ray diffraction experiments was placed between the anvils of a modified [7] Instron tensile or compression testing machine. The compressive strength of the sample of coat material was determined at room conditions, 60% RH, 20°C.

**Scanning electron micrographs.** Discs were made from *B. stearothermophilus* spores in the same manner as for the X-ray diffraction experiments, but the pressure applied to the spores was 600 x 10⁵ N/m² or 1200 x 10⁵ N/m². Pieces of the discs were gold shadowed and examined in a Cambridge scanning electron microscope.

**Infrared spectrum.** A disc of coat material as used for X-ray diffraction was analyzed with a Perkin Elmer infrared spectrophotometer, and the transmittance was determined between wavelengths of 2.5 and 8.5 microns.

**Melting of the crystalline portion of coat material with a differential scanning calorimeter.** The melting of a sample of coat material was examined with a Perkin Elmer DSC2, which had been calibrated by use of Indium. For the experiments, the coat material was kept dry by being sealed into a volatile-sample holder. The temperature was raised at 5°C/min.

**Results and Discussion**

**Crystalline structure.** The X-ray diffraction pattern of coat material (Fig. 1) reveals a strong crystalline powder pattern with reflections corresponding to crystalline spacings of 3.34 Å and 9.9 Å and an amorphous ring at 4.3 Å. This can be compared with an X-ray diffraction pattern of a sample of powdered rhinoceros horn (Fig. 1) taken in the same way. The crystalline material in rhinoceros horn has the α helical structure of α-keratin. The notable difference is the absence of the 3.34 Å spacing in the diffraction pattern of rhinoceros horn. The amorphous ring is the same; 4.3 Å for the horn, and 4.3 Å for the coat, while the other spacings are similar; 10.3 Å for the horn, 9.9 Å for the coat. The 5.1 Å arc characteristic of α-keratin cannot be differentiated from the amorphous ring in the power diffraction pattern of rhinoceros horn. There is a large difference in the amplitudes of the ≈9.9 Å spacing reflections, with the amplitude for the coat material being at least five times larger than that of the horn. The ≈9.9 Å spacing reflection in the β-keratin of feathers is also much smaller than that of the coat. Differential scanning calorimetry was used to determine the melting temperatures of the α-keratin of rhinoceros horn and for the coat material. In the dry state the crystalline material of the coat melted at ≈260°C, and there was no sign of a melting at ≈215°C, the melting temperature found for α-keratin [3].