Low heat resistance of *Bacillus sphaericus* spores correlated with high protoplast water content

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1. SUMMARY

The low heat resistance ($D_{100} = 0.554$ min, $z = 13.4^\circ$C) of dormant lysozyme-sensitized spores of *Bacillus sphaericus* 9602 was correlated with a low protoplast wet density (1.305 g/ml) equivalent to a high protoplast water content (61.0%, wet weight basis). These values for these unusual spores were consistent with those correlated previously in 28 spore types of seven other species.

2. INTRODUCTION

Dormant spores of *Bacillus sphaericus* differ from those of commonly studied aerobic species in having a nearly spherical-shaped body, a complex structured coat, and an elongated loose-fitting exosporium [1]. These spores are also distinctive in containing within the exosporium a mosquitocidal protein crystal [2,3] and having an incoherent 16S rRNA phylogeny [4].

The unusual characteristics of *B. sphaericus* spores and their apparent usefulness as models for light scattering studies [5,6] led us to compare them with other species of bacterial spores in which the moist-heat resistance over a wide range is correlated with the protoplast water content. Water distributed unequally within the fully hydrated dormant spore, with the protoplast relatively dehydrated and the integument more hydrated, was quantified for the first time by Beaman et al. [7] using three lysozyme-sensitive morphotype spores of *Bacillus megaterium* and a differential permeability technique. These findings were extended by Nakashio and Gerhardt [8] to four other spore species, which cluster differently from *B. megaterium* in their correlation between heat resistance and protoplast water content. Further study by Beaman and Gerhardt [9] using 28 types of lysozyme-sensitive spores among seven *Bacillus* species with a buoyant density technique [10] revealed limits of about 57% and 28% in protoplast water content, where mineralization and thermal adaptation then independently control heat resistance.

The relative dehydration in a central region has, from first observation, been indicated by the light-microscopic image of the bacterial spore with a refractile core and a nonrefractile periphery. This qualitative image has been refined by measuring the refractive index of spores with microscopic techniques [11,12], photometric immersion...
refractometry [13], and laser diffractometry [5,6]. The limitation in these uses of refractive index measurements to obtain water content of the core in situ is the inability to obtain an experimentally determined value of the refractive index increment of the protoplast itself, which therefore must be assumed or estimated in the converting calculation.

We here report measurements of protoplast buoyant wet density on lysozyme-sensitized spores of *B. sphaericus* 9602, correlation of their high protoplast water content with their low moist-heat resistance, and comparison of the results with previous findings in other species.

### 3. MATERIALS AND METHODS

Spores of *Bacillus sphaericus* 9602, obtained from Ulanowski et al. [5], were produced in about 90% of the cells by use of a sporulation medium containing the following: 0.23% Bacto-peptone (Difco); 0.41% Bacto-soytone (Difco); 0.14% beef extract (Difco); 0.06% yeast extract (Difco); 0.04% glucose; 0.15% NaCl; and 0.16% K2HPO4. Sterile salt solutions were added to obtain final concentrations of 0.013% CaCl2 x 2H2O; 0.01% MgCl2 x 6H2O; 0.003% MnSO4 x H2O; 0.0007% ZnSO4 x 7H2O; 0.00018% FeSO4 x H2O; and 0.00016% CuSO4 x 5H2O. Also added were filter-sterilized solutions to obtain final concentrations of 2.5 mg/l thiamine; 100 mg/l lysine, and 0.50 mg/l biotin [14]. Two-liter flasks containing 400 ml of the sporulation medium were inoculated (5%) with a 6-h culture and shaken at 150 RPM at 30°C for about 42 h. The spores were harvested by centrifugation at 3000 x g for 10 min, washed twice with 0.05 M TRIS and 0.05 M EDTA at pH 7, and treated with 200 µg per ml lysozyme to remove vegetative cell debris. The clean spores were stored in water at 4°C.

The native spores were made lysozyme-sensitive by disrupting the coats and the outer pericortex membrane by treatment first with 8 M urea and then with 8 M urea, 1% mercaptoethanol and 1.5% sodium dodecyl sulfate [15]. The lysozyme-sensitized spores were purified by layering on a 58% sucrose solution (density = 1.275 g/ml) and centrifuging twice at 6000 x g for 3 h. The lysozyme-sensitive spores came to the bottom whereas the lysozyme-resistant and germinated spores remained on top.

Wet density was determined with Nycodenz gradients as described previously [9,10].

Heat resistance, expressed as a D value (decimal reduction time at a given temperature) was measured as described previously [9]. A z value refers to the number of degrees Celsius to bring about a 10-fold reduction in D value.

Electron microscopy methods, with HXSA/VCD used as the embedding resin, were as described previously [16].

### 4. RESULTS

Spores of *B. sphaericus* 9602 were found to be only partially susceptible to the chemical agents (thioglycolic acid, or sodium dodecyl sulfate plus dithiothreitol) that produce lysozyme sensitization in other spores [9]. Treatment with urea, sodium dodecyl sulfate and mercaptoethanol [15], however, produced spores that were almost all lysozyme susceptible. The lysozyme-sensitized spores became permeable to glucose and Nycodenz at the outer membrane.

Comparison of electron micrographs of thin-sectioned, stained, native and lysozyme-sensitized spores (Fig. 1) showed that the inner coat-outer membrane complex was removed by the treatment [15], accounting for the conversion to lysozyme sensitivity. The treatment also removed most of the exosporium, inclusion crystal, and capsular material which were seen outside the coat of the native spore.

The moist heat resistance of *B. sphaericus* 9602 spores was found to be very low. Intact, gradient-purified, native *B. sphaericus* spores had a D90 value of 22.7 min, a D95 value of 7.95 min, and a D100 value of 2.88 min (z value of 11.2°C). The lysozyme-sensitized spores had a D90 value of 16.6 min, a D95 value of 7.96 min, a D100 value of 2.97 min (z value of 13.4°C), and an extrapolated D100 value of 0.554 min.

As determined by use of a Nycodenz gradient, the buoyant wet density of the protoplasm of lyso-
zyme-sensitized *B. sphaericus* 9602 spores was 1.305 g/ml. By use of an experimental correlation equation [10], this value was converted to a protoplast water content value of 61.0 g of water per 100 g of wet protoplast (which is equivalent to 156 g of water per 100 g of dry protoplast and to 79.6 g of water per 100 ml of wet protoplast).

For native lysozyme-resistant spores, the buoyant wet density (of the sporoplast) was 1.275 g/ml for a major gradient band and 1.250 g/ml for a minor band. The correlation equation for conversion to protoplast water content of lysozyme-sensitive spores [10] is not applicable to lysozyme-resistant spores.

5. DISCUSSION

The low heat resistance of *B. sphaericus* 9602 spores ($D_{100} = 2.88$ min for native spores and 0.554 min for lysozyme-sensitized spores) was similar to that of the psychrophilic *B. macquariensis* spores [9]. The correlation of low heat resistance with low protoplast wet density (1.305 g/ml) and high protoplast water content (61.0%) for lysozyme-sensitive *B. sphaericus* spores was consistent with the general correlation established with 28 spore types of seven other *Bacillus* species [9].

Our findings were also reasonably similar to the value for heat resistance ($D_{90} = 6.20$ min for native *B. sphaericus* 9602 spores) recently obtained by L.A. De Pieri (personal communication) and the protoplast wet density (1.31 g/ml for the native spores) recently reported by Ulanowski et al. [5] using laser diffractometry. Our findings differ, however, from their value for protoplast water content (29%, wet weight basis). Their value was calculated using a specific refraction increment (refractive index increment) that was estimated from the chemical composition of spores, apparently of various entire spores and not of the *B. sphaericus* spore protoplast. This limitation and
the complex peripheral structure of *B. sphaericus* spores complicate the use of various refractive index measurements [5,6,11–13], which otherwise seem so promising.

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REFERENCES