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The Brownian diffusion of dormant and germinating spores of *Bacillus megaterium*

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Light scattering techniques provide a non-destructive probe into structural aspects concerning the dormancy, heat resistance and germination of bacterial spores. Quasi-elastic light scattering techniques are applied to a study of the diffusion and scaling properties of dormant and germinating *Bacillus megaterium* spores (strain KM). A translational coefficient of $(5{\cdot}01 \pm 0{\cdot}10) \times 10^{-9}$ cm^2/s is obtained for the dormant spore, with little apparent change during the early stages of germination. Dormant and germinated spores, however, give different scaling characteristics. The significance of these observations in terms of theories concerning the dormancy and heat resistance of spores is discussed.

In an earlier communication (Harding & Johnson 1984) the usefulness of quasi-elastic light scattering (QLS) techniques in the investigation of bacterial spores and their germination was described. Because the structural integrity of the bacterial spore is an essential factor in the development of heat resistance, the use of non-destructive probes such as QLS is capable of providing new and hitherto unobtainable data on the morphological and molecular determinants of resistance.

It seems likely that the factors responsible for the heat resistance of spores of a given species, for example *Bacillus subtilis*, are also responsible for the heat resistance of spores of other species. There is little morphological variation in spore structure as visualized by optical and electron microscopy, although variations in spore cortex and coat thickness, in protoplast size, extent of cortex cross-linking and protoplast dehydration occur. The principal differences are that some species of spore possess an additional thin outermost layer (the 'exosporium') and that the lag period between the addition of germinant and the onset of germination can vary from less than 1 min to 20 min within a population of spores even, under optimum environmental conditions. This can be explained as differences of diffusion times of the germinant to the receptor sites or differences in the number of receptor sites for the germinant—whether or not these sites be enzymatic (see, for example, Racine *et al.* 1981).

In an attempt to test the generality of our conclusions on the theories of dormancy and heat resistance of spores of *B. subtilis*, we have now extended our measurements to spores of *Bacillus megaterium*. In this paper we demonstrate that the germination characteristics, as visualized by QLS, are very similar.

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Materials and Methods

BACTERIAL SPORES

Spores of *Bacillus megaterium* (strain KM) were chosen because they are spheroidal, of low

asymmetry and because they lack exosporia: these properties greatly simplify their dynamic behaviour in solution. The spores were supplied in freeze-dried form by Dr D.J. Ellar, and had previously been prepared and purified using the methods described by Scott & Ellar (1978). The same buffer was used to resuspend the spores as had been used by these workers, viz 50 mmol/l-K_2HPO_4 (pH 7·5). A stock solution of 1·0 mol/l L-alanine in the same solvent was diluted to 40 mmol/l to initiate germination. As with the earlier experiments with *B. subtilis*, adequate attention was given to the rapid mixing of germinant using either a pipette or a plunger device (Harding & Johnson 1984).

All the solvents used had been degassed to reduce the tendency of the spores to aggregate. In some instances samples were ultrasonicated using a 500 W sonicator (Dawe Instruments, UK) with a 38·73 kHz transducer to assist in their dispersion—this was particularly useful since it reduced the time of standing required to achieve a reasonably monodisperse supernatant.

Unlike *B. subtilis*, however, *B. megaterium* had been stored (<0°C) in freeze-dried form (as opposed to a frozen dispersion form for *B. subtilis*) and satisfactory dispersion of the freeze-dried material was obtained only after standing in solvent (at 4°C) for 3 d or somewhat less after ultrasonics. A value for the 'polydispersity factor', PF (Pusey 1974; Harding & Johnson 1984) for the dispersed material of 0·05 ± 0·03 was obtained, similar to that for *B. subtilis*.

LIGHT SCATTERING

Turbidity measurements, used to monitor the progress of germination, were performed at a wavelength, λ of 580·0 nm in a double beam Pye-Unicam SP800b spectrophotometer.

The quasi-elastic light scattering apparatus was essentially the same as described earlier (Harding & Johnson 1984), except that, in addition to the Spectra-Physics 15 mW He-Ne laser (λ = 632·8 nm), an air-cooled argon ion laser (Model 82, Point Source Lasers, UK) was used. This latter laser has an output adjustable from 5 mW to 60 mW at a wavelength of 488·0 nm, and an intensity feedback mechanism so that the intensity was constant to within 2·5% over long time periods. Fluorimeter cuvettes (1 cm square) were used, filled to a minimum level of 0·7 mm, to minimize convection effects in the cell.

The QLS apparatus was used to evaluate the apparent translational (z-average) diffusion coefficient $D_{z,\,APP}$ for both dormant and germinating spores, and hence the radii (r) of the equivalent Stokes spheres using the relation

$$r = \frac{kT}{6\pi\eta D_{z,\,APP}} \qquad (1)$$

where η is the solvent viscosity, k is the Boltzmann constant and T the temperature (K). The spores scattered so strongly that ultrafiltration of the spore suspensions was unnecessary and a particle concentration of $\approx 6 \times 10^7$ per ml gave sufficient photon counts in about 50 s to allow an accurate $D_{z,\,APP}$ to be evaluated.

Bacillus megaterium spores approximate prolate ellipsoids of revolution of dimensions 1·2 × 0·7 μm (from Beaman *et al.* 1984) so that rotational as well as translational motion would be expected to contribute to the correlation function. Chen *et al.* (1977) have demonstrated that for particles of this size range rotational motion is so slow in comparison with translational motion as to be negligible, except at low scattering angles ($\lesssim 20°$). Accordingly a scattering angle of 90° was chosen for the diffusion measurements. High scattering angles were also desirable so as to minimize the effects of any possible traces of dust (Godfrey *et al.* 1982). $D_{z,\,APP}$ values were obtained from a plot of $\ln[g^{(2)}(t) - 1]$ *vs* channel number (b), where $g^{(2)}(t)$ is the normalized intensity correlation function and b is related to time, t, by $b\tau$, τ being the chosen sample time. The basic equation may be written (Pusey 1974)

$$\ln[g^{(2)}(t) - 1]^{1/2} \equiv \ln[g^{(1)}(t)] = -D_{z,\,APP}\,q^2 t \qquad (2)$$

where the Bragg wave vector q is given by

$$q = [4\pi n/\lambda] \sin\left(\frac{\theta}{2}\right) \qquad (3)$$

n being the refractive index of the solution, λ the wavelength (*in vacuo*), θ the scattering angle and $g^{(1)}(t)$ is the field autocorrelation function.

Autocorrelation 'scaling' plots (Chen *et al.* 1977) for dormant and germinated spores were obtained for angles ranging from 90° down to 8°.

Results and Discussion

As for *B. subtilis*, linear plots of the logarithm of the normalized autocorrelation function *vs* time were obtained at 90°, for both dormant and germinating spores (Fig. 1). The *z*-averaged diffusion coefficient for the dormant spore at 35°C in the K_2HPO_4 buffer was $(5{\cdot}01 \pm 0{\cdot}10) \times 10^{-9}$ cm^2/s (corrected to water at 25°C this is $4{\cdot}10 \times 10^{-9}$ cm^2/s). The corresponding Stokes radius is 0·60 μm; this compares with a value of 0·59 μm for *B. subtilis* (Harding & Johnson 1984).

Diffusion coefficient measurements were also determined on the incompletely dispersed spores, and were as low as $4{\cdot}0 \times 10^{-9}$ cm^2/s (at 34·9°C); as expected, higher values for the polydispersity factor were obtained (0·15 ± 0·10). Ultrasonics produced no observable effects on the diffusion properties of 'dispersed' spores; viz those with a $D_{z,APP}$ of $5{\cdot}0 \times 10^{-9}$ cm^2/s (Table 1).

Table 1. Effect of ultrasonics on a dispersion of *Bacillus megaterium*

Period of ultrasonication (s)	$D_{z,APP}$ ($\times 10^9$ cm^2/s)	PF
30	5·01 ± 0·09	0·044 ± 0·021
60	4·97 ± 0·08	0·062 ± 0·040
90	5·03 ± 0·11	0·061 ± 0·040
120	5·03 ± 0·10	0·042 ± 0·026

O.D. = 0·47; K_2HPO_4 buffer (pH = 7·5); temperature = 34·9°C; 500 W sonicator bath with 38·73 kHz transducer.

PF, Polydispersity factor (Pusey 1974).

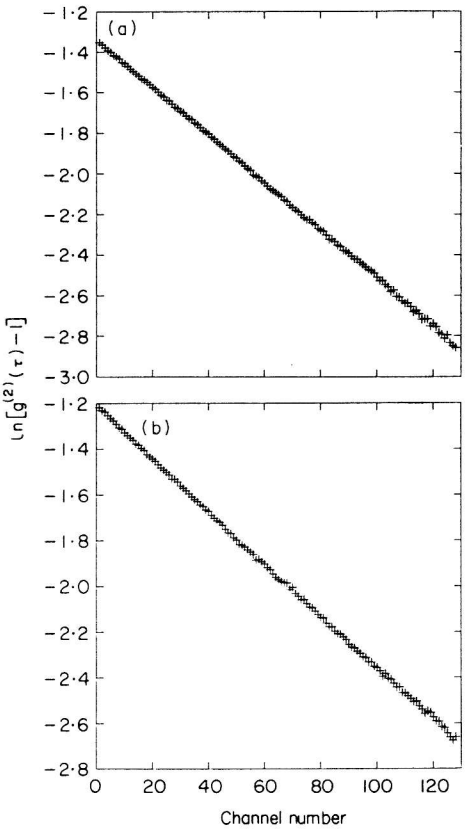

Fig. 1. Plot of $\ln[g^{(2)}(t) - 1]$, where $g^{(2)}(t)$ is the normalized autocorrelation function, *vs* channel number *b* for *Bacillus megaterium* spores: (a) dormant spores and (b) germinating spores, 16·5 min after the addition of germinant. Scattering angle = 90°, temperature = 34·9°C, spore concentration = 6×10^7 particles/ml, He-Ne laser (λ = 632·8 nm, 15 mW), sample time τ = 20 μs.

Control experiments on the mixing procedures (Harding & Johnson 1984) were performed, as for *B. subtilis*. After the addition of germinant little change in the apparent translational diffusion coefficient was manifested during the next 90 min (Fig. 2), by which time optical density measurements performed in parallel with the QLS confirmed that the majority of the spores had germinated: after a short lag period (≈5 min) a continuous fall in absorbance from 0·57 to 0·24 was observed. At the end of the absorbance measurements the cuvette containing the spore suspension was inverted and replaced to test for a possible decrease in absorbance due to sedimentation; only a very small change in absorbance was observed (<0·02). The sensitivity of the QLS measurements was such that over short time intervals (≈1 min) a volume contraction of the cortex ≥6% (equivalent to a 2% increase in $D_{z,APP}$) could be excluded. These results are therefore consistent with our earlier observations for *B. subtilis*, but not with the packed cell volume measurements of Izard and Wills (cited by Murrell 1980).

Chen *et al.* (1977) described a method of 'scaling' whereby the normalized correlation function $g^{(2)}(t)$ for several angles is plotted as a function of q^2t, where t is the time in seconds, and q is the Bragg wave vector defined by Eq (3). These curves should superimpose (i.e. 'scale')

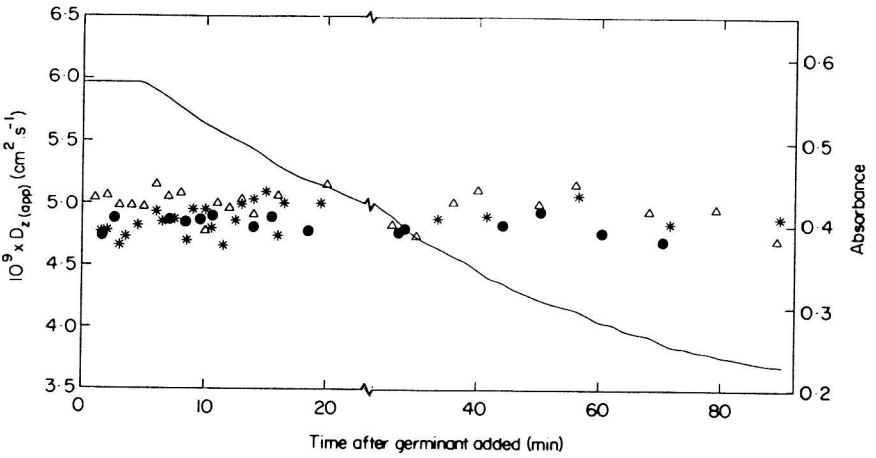

Fig. 2. Plot of the *z*-average diffusion coefficient, $D_{z,APP}$ measured at a scattering angle of 90° as a function of time after the addition of germinant (L-alanine diluted to 40 mmol/l). △, Run 1; He-Ne laser (15 mW) Temperature = 34·9°C, mixing by pipette, spore concentration = 6×10^7 particles/ml; ●, Run 2; He-Ne laser (15 mW) Temperature = 34·7°C, mixing by plunger, 4×10^7 particles/ml; *, Run 3; Ar-ion laser (25 mW) Temperature = 34·9°C, mixing by pipette, 6×10^7 particles/ml. The continuous line represents a plot of absorbance at 580 nm against time, obtained simultaneously with Run 3 in a Pye-Unicam recording spectrophotometer at 34·9°C.

for spherical isotropic scattering particles (Chen *et al.* 1977) even if their dimensions are comparable with the wavelength of the incident radiation (i.e. for generalized Mie as well as Rayleigh-Gans-Debye scatterers). It was thought initially that these curves for spheroidal particles of low asymmetry such as bacterial spores of *B. subtilis* and *B. megaterium* would also superimpose. As with spores of *B. subtilis* however, such curves for *B. megaterium* clearly do not superimpose (Fig. 3), and exhibit an oscillatory behaviour with change in angle. This demonstrates the sensitivity of the scaling property to even modest degrees of asymmetry (although the effects of polydispersity and possible aggregates cannot be ruled out). Furthermore, *germinated* spores of *B. megaterium* reveal a similar, though less marked non-scaling property (Fig. 3b); viz, the curves appear to scale at the higher angles. This difference in scaling behaviour is probably ascribable to changes in internal and/or external structure upon germination; quantification of these changes in terms of changes in the oscillatory behaviour is, however, a non-trivial problem (see e.g. Chen *et al.* 1977). The better scaling of the germinated spores is possibly a reflection of the decrease in internal refractive index with a corresponding change from Mie to Rayleigh-Gans-Debye scatterers, or more probably a result of the general observation (Gould 1969) that bacterial spores become slightly more spherical upon germination.

Our observations on *B. megaterium* and our earlier results for *B. subtilis* exclude the occurrence of significant volume changes during the early stages of germination. Any theory concerning the mechanisms responsible for the dormancy and heat resistance of spores (and loss thereof upon germination) which involves such changes is not supported by the experiments described. Our results are consistent, however, with the theory of Ellar (1978): viz that dehydration by osmosis from the spore to the mother cell occurs throughout sporulation and the rigid cortex acts merely to maintain the dehydrated state, and it is neither a *result* of an expanded cortex (Gould & Dring 1975), nor a contracted cortex (Lewis *et al.* 1965).

At very late stages of germination some degradation of the cortex would be expected to become appreciable, and further work at longer times is anticipated. In addition, further studies on the structure of spores at different stages in their development in the mother cell are now called for.

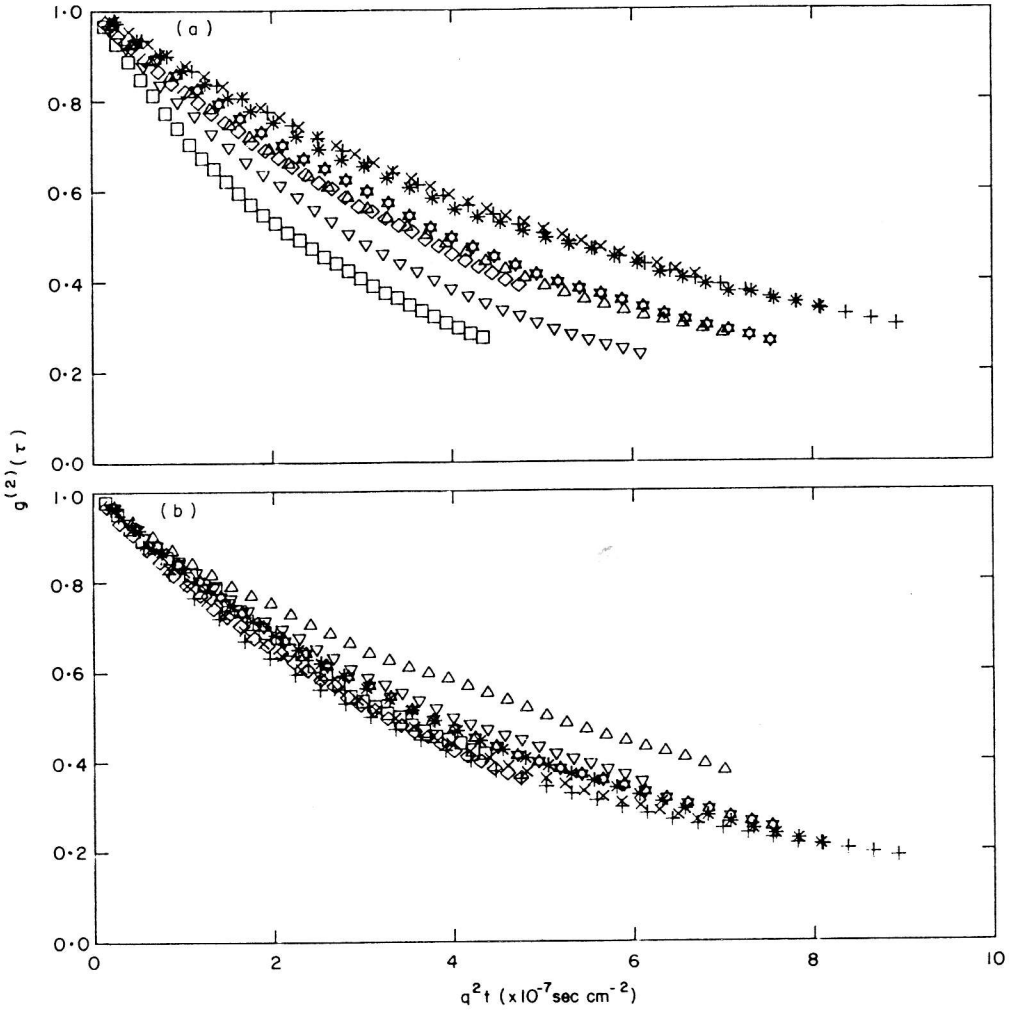

Fig. 3. Scaling experiments: plot of $g^{(2)}(t)$ against q^2t for different scattering angles, where q is the Bragg wave vector. Spore concentration was 6×10^7/ml and the temperature was 34·9°C. (a) Dormant spores, (b) germinated spores. □ 8°; ◇ 10°; ▽ 15°; △ 20°; ◁ 25°; * 35°; × 60°; + 90°.

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