Lipids in the inner membrane of dormant spores of *Bacillus* species are largely immobile

Ann E. Cowan*†, Elizabeth M. Olivastro*, Dennis E. Koppel**, Charles A. Loshon*, Barbara Setlow*, and Peter Setlow*†

*Department of Molecular, Microbial, and Structural Biology and †Center for Biomedical Imaging Technology, University of Connecticut Health Center, Farmington, CT 06032

Edited by Richard M. Losick, Harvard University, Cambridge, MA, and approved April 5, 2004 (received for review October 23, 2003)

Bacterial spores of various *Bacillus* species are impermeable or exhibit low permeability to many compounds that readily penetrate germinated spores, including methylamine. We now show that a lipid probe in the inner membrane of dormant spores of *Bacillus megaterium* and *Bacillus subtilis* is largely immobile, as measured by fluorescence redistribution after photobleaching, but becomes free to diffuse laterally upon spore germination. The lipid immobility in and the slow permeation of methylamine through the inner membrane of dormant spores may be due to a significant (1.3- to 1.6-fold) apparent reduction of the membrane surface area in the dormant spore relative to that in the germinated spore, but is not due to the dormant spore’s high levels of dipicolinic acid and divalent cations.

Dormant bacterial spores of various *Bacillus* species are extremely resistant to chemical agents that readily kill germinated spores or growing cells (1, 2). One factor important in dormant spore resistance to such chemicals appears to be their slow permeation into the spore core or protoplast. A variety of data indicate that it is the spore’s inner membrane that is the major permeability barrier restricting the passage of small molecules into the spore core (3–7), although the lipid composition of the inner membrane exhibits no anomalies that might explain its unusual properties (8–12). In addition, the dormant spore’s inner membrane has the potential to expand significantly, because electron microscopy indicates that the volume this membrane encompasses (the spore core): (i) decreases as much as 2-fold late in sporulation (13); and (ii) increases up to 2-fold in the first minute of spore germination, when the spore’s large peptidoglycan cortex is degraded and the germ cell wall expands (13–15). This increase in core volume during spore germination takes place without new membrane synthesis, because ATP production is not required, and restores relatively normal permeability to the germinated spore’s plasma membrane (4, 5, 16, 17).

Whereas dyes that stain growing cells, including fluorescent lipid probes, do not stain dormant spores (B.S. and P.S., unpublished results) as expected (15, 18), lipid probes can be incorporated into the developing spore’s membranes during sporulation (19). Given the latter finding, we decided to add fluorescent lipid probes during sporulation to prepare spores with labeled membranes, and then use the technique of fluorescence redistribution after photobleaching (FRAP) to directly analyze the fluidity of the spore’s inner membrane, because this technique has been used successfully in analyzing the fluidity and organization of molecules in a number of other systems, including spores of *Bacillus* species (20–25).

Methods

**Strains and Spore and Cell Preparation.** The isogenic *Bacillus subtilis* strains used in this work are derivatives of strain 168 and were: PS832, a prototrophic derivative of strain 168; PS3207, *cwlD::cat* strain in which the *cwlD* gene responsible for production of muramic acid-6-lactam in the spore cortex (14, 26) has been deleted and replaced with a chloramphenicol resistance marker; and PS3328 (27), *cotE::tet* in which the *cotE* gene responsible for proper assembly of much of the spore coat as well as outer spore membrane integrity (28) has been deleted and replaced with a tetacycline resistance marker. The fluorescent probes used were pyridinium, 4-[6-[(diethylamino)phenyl]-1,3,5-hexatrienyl]-1-[3-(triethylammonio) propyl], dibromide (FM-4-64), and pyridinium, 4-[2-(6-(dibutylamino)-2-naphthalenyl) ethenyl]-1-(3-sulfopropyl)-, hydroxide, and salt (di-4-ANEPPS), both from Molecular Probes. These probes have been used to label membranes in living cells, including bacteria (19, 29–31). Spores of *B. subtilis* strains were prepared at 37°C in 2× Schaeffer’s glucose medium without antibiotics and containing fluorescent probes (2 μg/ml FM-4-64 or 4–7.7 μM di-4-ANEPPS) added at hr 1–2 of sporulation, and spores were cleaned and stored as described (32, 33). Spores of *Bacillus megaterium* QMB1551 (originally obtained from H. S. Levinson, U.S. Army Natick Laboratories, Natick, MA) were prepared at 30°C in supplemented nutrient broth medium, labeled with fluorescent lipid probes as described above, and cleaned and stored as described (34). All dormant spore preparations were 98% free of growing or sporulating cells, germinated spores, or cell debris.

For germination, spores of *B. megaterium* and *B. subtilis* in water at an OD600 of ~10 were heat-shocked for 30 min at 60°C or 70°C, respectively. After cooling on ice, spores were germinated at 37°C and an OD600 of 1 in 50 mM Tris-HCl (pH 8.0) containing 5 mM L-alanine (*B. subtilis*) or 5 mM D-glucose (*B. megaterium*). In some cases, KCN was added to 10 mM to block ATP production (17). Spores were germinated for 7 min (*B. megaterium*) or 45 min (*B. subtilis*) and germination was ≥95% complete as determined by phase contrast microscopy. Germinated *cwlD* spores cannot be reliably identified by phase contrast microscopy. Consequently, for microscopy and FRAP analyses the germinated *cwlD* spores were isolated by centrifugation, run on a Nycodenz density gradient, and the band of lower-density-germinated *cwlD* spores was isolated and Nycodenz was removed by centrifugation (14). However, for measurement of the rate of methylamine permeation into the core of *cwlD* spores, spores germinated for 60 min were used without density gradient purification.

Sporules of *B. megaterium* were decoted by treatment with 1% SDS-0.1 N NaOH for 30 min at 37°C, and the treated spores were washed by repeated centrifugation. *B. subtilis* spores were decoated and washed as described (35). This latter decoating procedure removes not only the coats but also the outer membrane from *B. subtilis* spores (36).

Vegetative cells of *B. megaterium* and *B. subtilis* were prepared by growth in LB medium (32) at 30°C and 37°C, respectively. The di-4-ANEPPS dye was added to 4 μM at an OD600 of ~0.2, cells were grown for an additional hour and were then harvested by
centrifugation and resuspended in dye-free medium before microscopy.

Measurement of Methylamine Permeation into Spores. The rate of methylamine permeation into B. subtilis spores was measured essentially as described (4, 7, 37). Spores (~15 mg of dry weight per ml) were incubated at 4°C or 22°C in 200 mM Tris-HCl (pH 8.8) with 5 μM [14C]methylamine and H2O (both from New England Nuclear). At various times aliquots were centrifuged, the supernatant fluid made 5% in trichloroacetic acid and the pellet was suspended in 5% trichloroacetic acid, samples incubated overnight at 22°C and then assayed for radioactivity. The spore core volume was determined as described above, but substituting 5 μM [14C]sorbitol for methylamine. Water has been reported to penetrate the entire spore, whereas sorbitol does not (4–7). The amount of methylamine in the spore core and the core pH were calculated as described (4, 7).

Microscopy and FRAP Analysis. For microscopy, 10 μl of spores were placed on an agarose coated slide (18, 20) and a coverslip was applied and sealed with clear nail polish. Measurements of the areas of spores were obtained from epifluorescence images collected by using a 100×, 1.4 N.A. planapochromat objective on a Zeiss Axioplan2 microscope equipped with a Photometrics (Roper Scientific, Trenton, NJ) PXL-EV37 cooled charge-coupled device camera. Area measurements were performed by using METAMORPH software (Universal Imaging, Downingtown, PA). FRAP experiments were performed on a Zeiss LSM510 confocal microscope by using a 100×, 1.4 N.A. planapochromat objective. The 488-nm line from an argon laser was used for excitation, and the pinhole was fully opened so that all fluorescence from the cell was collected. The scan axis was oriented such that the x direction was perpendicular to one axis of the cell. To minimize the time for image collection, the scan area was limited to a box slightly larger than the spore. By using the time series function in the Zeiss AIM software, 5 prebleach images of the spore were collected, and a square region overlying one-half of the spore was rapidly photobleached by using unattenuated laser light. Immediately (4–6 ms) after photobleaching, 45 successive images were collected to monitor the fluorescence redistribution after the photobleaching. In most cases there was no delay between collection of images, and the total time between successive images (collection time plus blank time before the next image was collected) ranged from 66 to 600 ms.

Image processing was performed with METAMORPH software. An average background value determined from a cell-free area was first subtracted for each image in a time series, and average intensity values were calculated perpendicular to a line drawn down the long axis of the spore. The analysis of the fluorescence redistribution was performed with the software system MLAB, constructed by Civilized Software (Bethesda). The diffusion coefficient (D) and mobile fraction (R) were calculated based on a normal-mode analysis (38), as described below.

FRAP Theory and Methods. The FRAP analysis used is the normal-mode analysis described previously for membrane components (38). The normal-mode analysis is an appropriate method in cases where the sample geometry is not infinitely large compared with the bleached area, but rather, is a bounded region on the same scale as the bleached region and has a relatively symmetric geometry. In our case, the membrane of a spore is very much like the surface of a sphere.

The problem is first reduced to a one-dimensional problem by determining average fluorescence along one dimension of the two-dimensional image. For an azimuthally symmetric concentration distribution characterized by a single lateral diffusion coefficient D and a mobile fraction R, on a spherical surface of radius r, the diffusion equation has the general solution (38):

\[
c(x,t) = \sum_{n=0}^{\infty} A_n P_n(x) [Re^{-\lambda_n r} + (1 - R)],
\]

where \( \Gamma = 2D/r^2 \) is the fundamental relaxation rate, x is the cosine of equatorial angle \( \Theta \) (see Fig. 1), and \( P_n(x) \) is the Legendre polynomial of order n. Coefficients \( A_n \) are determined by the particular initial concentration distribution, \( c(x,0) \).

We define a normalized “first moment” of the distribution:

\[
\mu_1(t) = \int_{-1}^{1} \frac{P_1(x) c(x,t) dx}{\int_{-1}^{1} P_0(x) c(x,t) dx},
\]

where \( P_0(x) = 1 \), and \( P_1(x) = x \). Combining Eqs. 1 and 2, and applying the orthogonality relation for the Legendre polynomials, it follows directly that \( \mu_1(t) \) selects the first normal mode of the distribution, and that:

\[
\mu_1(t) = A \left[ Re^{-\Gamma t} + (1 - R) \right],
\]

with \( A = A_1/3A_0 \).

If we assume a uniform prebleach concentration distribution, possible corrections for the effects of cell geometry can be carried out to a good approximation in the calculation of \( c(x,t) \) with point-by-point normalizations of each postbleach fluorescence scan, \( F(x,t) \), by \( F(x,-) \), a prebleach scan. We can thus take

\[
\hat{\mu}_1(t) = \hat{M}_1(t)/\hat{M}_0(t),
\]

where

\[
\hat{M}_1(t) = \frac{2}{N} \sum_{i=1}^{N} F(x_i,t)/F(x_i,-)
\]

and

\[
\hat{M}_0(t) = \frac{2}{N} \sum_{i=1}^{N} F(x_i,t)/F(x_i,-)
\]

as an experimental estimate of \( \mu_1(t) \). In Eqs. 5a and 5b, we take for \( i = 1, N: \)

\[
F(x_i,-) \equiv \frac{1}{2} F_{\text{max}}
\]

and

\[
F(x_i,t)
\]

Fig. 1. Confocal fluorescence micrographs of dormant (A) or germinated (B) B. megaterium spores that had incorporated di-4-ANEPPS into the spore’s inner membrane during sporulation. Spores germinated for 7 min with glucose and KCN, and images of dormant and germinated spores were obtained as described in Methods. (Bars, 10 μm.)
where $F_{\text{max}}$ is the maximum value of $F(x, t)$ to approximate the range of $F(x, t)$ from $-r$ to $+r$.

**Results and Discussion**

As expected based on previous work (19), we were able to incorporate the lipid probes FM-4-64 and di-4-ANEPPS into spores by sporulation in the presence of these compounds. As found previously with FM-4-64, addition of these molecules did not alter the sporulation process (ref. 19 and data not shown). These probes could have been incorporated into either the spore’s inner or outer membrane, or both membranes. However, the level of probe in the cleaned spores was not reduced significantly by treatments that removed the spore’s outer membrane and much of the spore coats (data not shown). This finding suggests that most if not all of the probe molecules in the cleaned spores are in the inner membrane. This is not to say that probe is not incorporated into the outer membrane during sporulation; indeed, previous work has indicated that this does take place (19). However, any probe molecules in the spore’s outer membrane must be lost during the ∼2-week period of incubation and treatment needed for cleaning the spores.

Examination of decoated *B. megaterium* spores stained with either di-4-ANEPPS (Fig. 1A) or FM-4-64 (data not shown) by confocal microscopy showed that dormant spores appeared as brightly staining rings with a dark center, as expected if the inner membrane was the site of the probe. When labeled spores were germinated in the presence of KCN to block ATP production (17), the size of the stained ring increased significantly (Fig. 1B), as expected, because the spore core volume increases early in spore germination in an energy-independent process. Measurements of the radii of the di-4-ANEPPS- and FM-4-64-stained rings in dormant and germinated decoated spores indicated that this volume increased ∼1.6-fold upon germination (data not shown), and thus that the membrane area would have to expand on the order of 1.3-fold to allow this volume expansion. Similar results were obtained with decoated and intact spores of *B. subtilis*, where the volume was found to increase ∼2-fold, requiring an increase in surface area on the order of 1.6-fold. These values are close to the ∼2-fold expansion of the volume of the spore core calculated from analyses of electron micrographs of dormant and early germinated *B. subtilis* spores (14, 15, 39), and is further evidence that the fluorescent probes are located in the dormant spore’s inner membrane. Note, however, that the latter electron micrographs generally overestimate the energy-independent expansion of spore core volume early in germination, because germination is generally carried out under conditions allowing energy metabolism as well as protein synthesis.

FRAP analysis of di-4-ANEPPS-labeled decoated dormant spores of *B. megaterium* yielded a mobile fraction of only 0.38, indicating that more than half of the lipid probe is immobile within the time scale of the FRAP experiment (Fig. 2A and Table 1). In contrast, the mobile fraction of di-4-ANEPPS in spore membranes more than doubled after germination, and exhibited a 5-fold increase in diffusion coefficient (D; Fig. 2B and Table 1). Assuming that the probe di-4-ANEPPS is distributed equally in the bulk of the lipid bilayer, this result indicates that most of the lipid molecules within the inner membrane are immobile in dormant spores, but the great majority of lipid molecules become mobile early in spore germination. The mobile fraction of di-4-ANEPPS in germinated *B. megaterium* spores was similar to that obtained in vegetative cells, although there was an additional 2-fold increase in D in vegetative cells (Table 1). Generally similar results were obtained with *B. subtilis* spores, as almost 70% of the di-4-ANEPPS in decoated dormant spores was immobile on the time scale of the FRAP experiments.

After germination, the mobile fraction increased to >0.75, although in this case there was no significant increase in D (Table 1). Vegetative cells of *B. subtilis* showed a similar high mobile fraction but a 15-fold increase in D to a value similar to that obtained for *B. megaterium* (Table 1). The significance of the <100% mobile fraction of the lipid probe in germinated spores and vegetative cells of *B. megaterium* and *B. subtilis* is not clear, but it is possible that some or all of the residual immobile fraction may reflect an inability to completely account for photobleaching during monitoring in the analysis.

One factor that may contribute to the immobility of lipids in the dormant spore’s inner membrane is that removal of much of...
the forespore core water during sporulation, with the attendant decrease in core volume (39), may be accompanied by significant changes in the membrane leading to a high proportion of gel phase lipid. An additional factor that may affect the mobility of lipids in the inner membrane is the spore core’s enormous depot (≥15% of spore core dry weight) of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)] (15). DPA is accumulated late in spore formation and is excreted in the first minute of spore germination (3, 15). DPA in the spore core is likely in a 1–1 chelate with divalent cations, predominantly Ca²⁺, and this chelate is likely present as a relatively dehydrated crystalline lattice (40–42). Because the divalent cations chelated with DPA are free to form further interactions, it is possible that a meshwork of DPA, Ca²⁺, and phospholipid head groups may form at the inner surface of the spore’s inner membrane, thus immobilizing the phospholipids in the inner leaflet of this membrane. If this is the case, then lipid probes might become mobile in spores that have lost DPA but whose core has not swollen because of a block in cortex degradation.

To test this latter prediction, we used spores of B. subtilis carrying a mutation in the cwlD gene (26). Spores of this strain have an altered cortex that does not contain muramic acid–lactam and is thus not attacked by spore cortex-lytic enzymes (3, 14). Consequently, whereas DPA is released during nutrient-triggered germination of cwlD spores, the cortex is not degraded and the spore core does not swell appreciably, if at all (3, 14, 16, 26). Indeed, fluorescence microscopy of di-4-ANEPPS-stained dormant and germinated cwlD spores indicated that the spore core did not swell during spore germination (data not shown), as shown previously by electron microscopy (14). There was no increase in the D or the mobile fraction of the di-4-ANEPPS in the inner membrane of germinated cwlD spores compared with values in dormant cwlD or wild-type spores (Fig. 3 and Table 1). This result indicates that release of DPA and its associated divalent cations is not sufficient to relieve the constraints to lateral diffusion of lipids in the spore’s inner membrane.

The relative immobility of a lipid probe in germinated cwlD spores leads to the prediction that the rate of permeation of small molecules into the core of germinated cwlD spores should be similar to the slow permeation of small molecules into the dormant spore’s core. To test this prediction, we measured the rate of uptake of methylamine into dormant and germinated wild-type and cwlD spores. The pH in the core of dormant spores of Bacillus species is somewhat low at 6.3–6.5, compared with values in germinated spores or growing cells of 7.5–7.8, and furthermore, the low pH in dormant spores is maintained for long periods even upon incubation at a high external pH (4, 7). Consequently, when incubated at a high external pH, dormant spores take up a large amount of methylamine into the core, because protonated methylamine does not cross the inner membrane. As found previously with dormant B. megaterium spores (4, 7), the rate of methylamine uptake by dormant wild-type B. subtilis spores was extremely slow at 4°C, taking ≥16 h to reach maximal uptake, and even taking 4 h at 22°C (Fig. 4). The maximum value for methylamine uptake into the spore core at 4°C was used as described (4, 37) to calculate a pH in the dormant spore core of ≥6.5. The rate of methylamine uptake by dormant cwlD spores was similar to that of dormant wild-type spores at both 4°C and 22°C (Fig. 4), and this rate was also similar in dormant cotE spores (Fig. 4A). Because the outer membrane is not an effective permeability barrier in cotE spores (28), this latter result indicates that it is the inner membrane that is likely the rate-limiting barrier to passage of methylamine into the spore core.

In contrast to the slow methylamine penetration into the dormant spore core, methylamine uptake by germinated wild-type spores at 4°C was too fast to measure (Fig. 4A), although the amount of methylamine uptake was much lower than into dormant spores because the germinated core pH was calculated

![Fig. 3. FRAP analysis of di-4-ANEPPS in dormant and germinated cwlD spores of B. subtilis. (A) and germinated cwlD spores of B. subtilis. (A) Fluorescence intensity (F(t, τ)) averaged across the spore along a line drawn down the longest axis of the spore (x, t) before and after photobleaching. , average of five prebleach scans; , first postbleach scan; , last postbleach scan (corrected for photobleaching that occurs during continuous monitoring; see Methods). The dashed lines depict the actual values for the last postbleach scan before correcting for some photobleaching during monitoring. The vertical bars show the regions defined as the beginning and end of the spore for the determination of the diffusion coefficient. (B and D) Fits to Eq. 5a in Methods, from which D and R are derived. (A and B) Data from a representative dormant cwlD spore in which half the spore was photobleached. (C and D) Results from a representative germinated cwlD spore in which half the spore was photobleached.]

Table 1. Diffusion coefficient (D) and mobile fraction (R) for di-4-ANEPPS in B. megaterium and B. subtilis spores

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th></th>
<th></th>
<th>cwlD</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D/(10⁻⁵ cm²s⁻¹)</td>
<td>R</td>
<td>n</td>
<td>D/(10⁻⁵ cm²s⁻¹)</td>
<td>R</td>
<td>n</td>
</tr>
<tr>
<td>B. megaterium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dormant</td>
<td>1.31 ± 0.40</td>
<td>0.38 ± 0.07</td>
<td>6</td>
<td>0.82 ± 0.40</td>
<td>0.24 ± 0.07</td>
<td>10</td>
</tr>
<tr>
<td>Germinated</td>
<td>7.34 ± 1.62</td>
<td>0.85 ± 0.04</td>
<td>5</td>
<td>0.25 ± 0.25</td>
<td>0.29 ± 0.12</td>
<td>8</td>
</tr>
<tr>
<td>Vegetative</td>
<td>18.24 ± 6.63</td>
<td>0.71 ± 0.12</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. subtilis</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dormant</td>
<td>1.10 ± 0.98</td>
<td>0.31 ± 0.16</td>
<td>9</td>
<td>0.82 ± 0.40</td>
<td>0.24 ± 0.07</td>
<td>10</td>
</tr>
<tr>
<td>Germinated</td>
<td>1.85 ± 1.24</td>
<td>0.75 ± 0.07</td>
<td>10</td>
<td>0.25 ± 0.25</td>
<td>0.29 ± 0.12</td>
<td>8</td>
</tr>
<tr>
<td>Vegetative</td>
<td>16.87 ± 0.50</td>
<td>0.75 ± 0.01</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values (presented as mean ± SD) were measured and calculated as described in Methods. All measurements were made on decoated spores. Essentially identical results were obtained by using coated spores.
as \( \approx 7.6 \) at time 0. The germinated \( cwlD \) spores also had a core pH of 7.6 at time 0 of measurement, and again took up less methylamine than did dormant spores. However, in contrast to germinated wild-type spores, germinated \( cwlD \) spores took up methylamine slowly, both at 4°C and 22°C (Fig. 4). Thus, the inner membrane of germinated \( cwlD \) spores retains the low permeability properties of the dormant spore’s inner membrane, which is consistent with the relative immobility of lipids in the inner membrane of germinated \( cwlD \) spores.

The data in this communication strongly indicate that the inner membrane of dormant spores of \( B. subtilis \) species has some novel properties; in particular that lipids in the inner membrane are largely immobile. Several previous studies (43–45) used electron spin resonance spectroscopy to assess the mobility of spin labeled lipid probes in spore membranes, and this work also suggested that lipids in the spore’s inner membrane were largely immobile and became mobile early in spore germination. Unfortunately, in this early work it appeared likely that much of the spin label was associated with protein, in particular spore coat protein, with perhaps only a small fraction associated with the spore’s inner membrane (43). This result is clearly not the case in the current work, because FRAP analysis with both intact and decoted spores gave very similar results.

Although the majority of lipid in the inner membrane of dormant spores is immobile, a significant fraction of this lipid remains mobile, albeit with a lower \( D \) value than in growing cells. The obvious question that arises is what is the organization of mobile and immobile lipid in the inner membrane? We can envisage two general answers to this question. First, perhaps the lipid in the two leaflets of the inner membrane have very different mobility, slow in one and immobile in the other. It has been shown in model membranes that regions of gel phase lipids can exist in individual monolayers (46, 47). However, another feature of dormant spores that might contribute to differential lipid mobility in the two leaflets of the spore’s inner membrane is the low level of water in the core, compared with the much higher level in the germ cell wall/cortex area (2). Perhaps this occurrence results in much lower mobility of lipids in the inner leaflet of the inner membrane. Because the core water level rises appreciably upon normal spore germination (but much less so upon germination of \( cwlD \) spores; refs. 2, 14, and 16), this finding might restore mobility to the lipids in the inner leaflet of the inner membrane. Using quenching agents in the external solution may allow one to differentiate the mobility of fluorescence probes in the two leaflets. The second explanation is that there are distinct domains in the inner membrane, one in which lipid is immobile and one where lipid remains mobile, albeit with a reduced \( D \) value. There is at least one report that the spore’s inner membrane has a significantly higher protein content than the growing cell membrane (48), and a high level of protein in the spore’s inner membrane might serve to organize large areas of ordered domains under the special conditions of dehydration of the spore core. Evidence consistent with the existence of distinct domains in the membrane of growing cells of \( B. subtilis \) has been presented (49). It was also reported that the inner membrane of \( B. megaterium \) spores could be resolved into approximately equivalent amounts of two fractions of similar lipid composition, but markedly different protein content and composition (50). In contrast, the inner membrane of germinated spores gave rise to only a single fraction that had a protein content and composition intermediate between the two fractions from the dormant spore’s inner membrane. Whereas this observation has never been pursued to determine whether it is indeed a true reflection of the structure of the inner membrane of spores of \( B. subtilis \) species, it is certainly provocative in view of our findings on lipid mobility in this membrane and the plethora of evidence for different domains in other biological membranes (51, 52). Fluorescence probes with different solubilities for lipid phases may provide tools for exploring the possible structure of lipid domains in the spore membrane.

Whatever the explanation for the fraction of lipid in the dormant spore’s inner membrane that remains mobile in FRAP analysis, the majority of lipid in this membrane is immobile and even the mobile lipid has a much lower \( D \) value than lipid in growing cell membranes. The presence of a high fraction of immobile lipid in the dorman spore inner membrane is independent of the spore’s large depot of DPA and divalent cations, but the majority of this lipid becomes mobile during germination if the spore’s cortex is degraded, although the \( D \) value for lipids in the germinated spore membrane remains low, in particular in \( B. subtilis \) spores.

Spore cortex hydrolysis in the early minutes of germination leads to a rapid and significant expansion of the inner membrane surface area as calculated from the volume encompassed by the inner membrane. Consequently, it is tempting to suggest that the increased lipid mobility in the germinated spore’s inner membrane reflects the mechanisms responsible for the expansion of the membrane. The increase in membrane surface area does not require ATP production and there is no evidence for internal membrane stores in spores or for significant invaginations of the dormant spore’s inner membrane that could supply additional membrane. However, work on both pure lipid bilayers and biological membranes indicate that the area per lipid molecule can increase only by 2–4% without loss of membrane integrity (53–55). One possibility is that the cortex acts to stabilize the spore membrane in a compressed state during sporulation. Degradation of the cortex during germination may allow relaxation of the membrane concomitant with rehydration of the spore core. In addition, given the high protein content of the inner membrane in dormant spores, changes in membrane protein structure as the spore is rehydrated could also account for an expansion in membrane surface area. Finally, it is also possible that free lipids complexed with proteins could provide a source of additional material for membrane expansion.

The significantly restricted mobility of lipid probes in the inner membrane of dormant spores suggests the presence of a substantial amount of gel phase lipid that would be expected to greatly decrease the passive permeability of this membrane, as has been observed in several studies (56–58) and in this work. The low permeability of the spore’s inner membrane would also be important in the spore’s retention of its pool of small molecules for long periods of time when spores are suspended in water. Finally, there are a number of proteins present in the dormant spore’s inner membrane, including receptors for nutrient germinants and perhaps channels for DPA, that operate in the environment of this membrane to allow spore germination.
(3, 59, 60). The activities of some enzymes present in the spore’s inner membrane have also been shown to change markedly in the first minute of spore germination in the absence of protein synthesis (48). Because the activities of several membrane proteins are altered by lateral compression on the membrane (61), how the properties of spore membrane proteins are altered by the novel characteristics of the membrane in which they reside is a key question, the answers to which may provide insight into the mechanism of both spore dormancy and spore germination.

This work was supported by a grant from the Army Research Office (to P.S.) and National Institutes of Health Grant GM19698 (to P.S.). The Center for Biomedical Imaging Technology is also supported by National Institutes of Health Grant RR13186.