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Analysis of dye binding by and membrane potential in spores of *Bacillus* species

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Summary

Aims—To determine roles of coats in staining *Bacillus subtilis* spores, and whether spores have membrane potential.

Methods and results—Staining by four dyes and autofluorescence of *B. subtilis* spores that lack some (*cotE*, *gerE*) or most (*cotE gerE*) coat protein was measured. Wild-type, *cotE* and *gerE* spores autofluoresced and bound dyes, but *cotE gerE* spores did not autofluoresce and were stained only by two dyes. A membrane potential-sensitive dye DiOC₆(3) bound to dormant *B. megaterium* and *B. subtilis* spores. While this binding was abolished by the protonophore FCCP, DiOC₆(3) bound to heat-killed spores, but not to dormant *B. subtilis cotE gerE* spores. However, DiOC₆(3) bound well to all germinated spores.

Conclusions—The autofluorescence of dormant *B. subtilis* spores and the binding of some dyes are due to the coat. There is no membrane potential in dormant *Bacillus* spores, although membrane potential is generated when spores germinate.

Significance of the Study—The elimination of the autofluorescence of *B. subtilis* spores may allow assessment of the location of low abundance spore proteins using fluorescent reporter technology. The dormant spore's lack of membrane potential may allow tests of spore viability by assessing membrane potential in germinating spores.

Keywords

Bacillus; spores; spore staining; membrane potential; spore coat

Introduction

There are a variety of situations in which it is essential to rapidly detect spores of *Bacillus* species, since spores of these species are extremely resistant to many harsh treatments and the growing cells can often cause food spoilage and disease (Setlow 2006; Setlow and Johnson 2007). While detection of spores as organisms that can give rise to colonies after heat treatments that are lethal for growing cells is extremely reliable, this analysis is not especially rapid. Consequently, there is significant interest in more rapid methods for spore detection. One approach that has been suggested is the use of spore fluorescence, either autofluorescence or

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fluorescence due to the binding of an appropriate dye with detection of the fluorescent spores by flow cytometry (Laflamme *et al.*, 2004, 2005b, 2006; Cronin and Wilkinson 2007).

In general, spores stain poorly with many reagents that readily stain growing bacteria, in particular nucleic acid stains, as these stains cannot readily gain access to the spore core, the site of all nucleic acids (Gould 1969; Setlow *et al.*, 2002). However, spores can often be stained weakly by such reagents, and in some cases this weak staining has been visualized by confocal fluorescence microscopy as a ring outside the spore core (Melly *et al.*, 2002; Setlow *et al.*, 2002). The material that is binding these reagents and giving rise to spore fluorescence is not known and could be either the outer proteinaceous spore coat or the thick peptidoglycan cortex that is between the coat and core (Melly *et al.*, 2002; Setlow *et al.*, 2002). In addition to the coat and cortex, the spore also has two membranes, an outer one between the cortex and coat, and an inner membrane that surrounds the spore core. These membranes are additional sites where stains could bind. Indeed, spore formation in the presence of at least several membrane-targeted lipid probes results in significant incorporation of these probes into the spore's inner membrane (Cowan *et al.*, 2004). In addition to fluorescence due to binding of various stains, spores also commonly exhibit significant autofluorescence (Laflamme *et al.*, 2005b, 2006).

There appear to be a number of reasons to determine the causes of both the commonly observed weak staining of layers outside the core of dormant spores, as well as the spore's autofluorescence. First, knowledge of the spore structures and components important in this staining could be important in the use of specific stains to detect both total and viable spores. Second, learning the basis for this weak staining might suggest ways to eliminate it. This might be useful in applications such as determination of spore membrane potential, in which non-specific staining by a lipophilic probe could make interpretation of results difficult. Finally, knowledge of the structures and components giving rise to spore autofluorescence might allow elimination of this autofluorescence. This might then facilitate use of fluorescent reporter technology for applications such as determining the localization and/or movement of very low abundance proteins in spores. Because of the potential benefits in learning the basis for spore autofluorescence and the weak but significant binding of a number of commonly used stains, we have undertaken to learn the reason for these spore properties, focusing primarily on the large proteinaceous spore coat and using spores of *B. subtilis*. This work has provided strong evidence that the spore's autofluorescence is due largely if not exclusively to the spore coat, as is the binding of some dyes, while at least two other dyes may bind largely to the cortex in the intact dormant spore. We have further used this knowledge to demonstrate that dormant spores of several *Bacillus* species appear to have no detectable membrane potential, in contrast to the situation in outgrowing spores.

Materials and methods

Strains used, spore preparation and purification

The *B. megaterium* strain used was QMB1551, originally obtained from H.S. Levinson, U.S. Army Laboratories, Natick, MA, USA. The *B. subtilis* strains are isogenic and are derivatives of strain PS832, a prototrophic laboratory derivative of strain 168. PS533, the wild-type *B. subtilis* strain, also carries plasmid pUB110 encoding resistance to kanamycin (10 mg l⁻¹) (Setlow and Setlow 1996). PS3328 carries a tetracycline resistance (Tc^r; 10 mg l⁻¹) cassette replacing the majority of the coding sequence of the *cotE* gene (Paidhungat *et al.*, 2001). Strain PS4149 carries a spectinomycin resistance (Sp^r) cassette replacing the majority of the *gerE* gene's coding sequence, and strain PS4150 carries the *cotE* and *gerE* mutations from strains PS3328 and PS4149, respectively (Ghosh *et al.*, 2008). Spores of *B. subtilis* strains were prepared on 2xSG medium agar plates at 37°C, and the spores were harvested and purified as described (Nicholson and Setlow 1990; Paidhungat *et al.*, 2000). *B. megaterium* spores were prepared on SNB medium (Goldrick and Setlow 1983) agar plates at 30°C, and were purified

as described above for *B. subtilis* spores. All spores used in this work were free (> 98%) of growing or sporulating cells or germinated spores. In a few cases spores were chemically decoated and washed as described (Bagyan *et al.*, 1998).

Fluorescence microscopy of dormant spores

Dormant spores at an optical density at 600 nm (O.D.₆₀₀) of 30-40 in phosphate-buffered saline (25 mmol l⁻¹ KPO₄ buffer (pH 7.4) – 100 mmol l⁻¹ NaCl) were stained for 30 min to 2 hr at 23°C with 1 or 50 mg l⁻¹ 4',6'-diamino-2-phenylindole (DAPI) (Molecular Probes, Eugene OR, USA), 10 mg l⁻¹ acridine orange (Sigma Chemical Company, St. Louis, MO, USA), 10 mg l⁻¹ 10-N-nonyl acridine orange (Molecular Probes, Eugene, OR, USA), or 5 µmol l⁻¹ pyridinium, 4-(2-(6-(dibutylamino)-2 naphthalenyl)-1-(3-sulfopropyl)-, hydroxide, inner salt (di-4-ANEPPS) (Molecular Probes, Eugene, OR, USA). Aliquots (~ 5 µl) of stained or unstained spores were applied to agarose coated slides, a coverslip applied and the edges of the coverslip were sealed with clear nail polish. Fluorescence images of spores were obtained using a 63×1.4 NA lens on a Zeiss LSM510 laser scanning confocal microscope with the confocal pinhole fully opened to collect fluorescence from the entire depth of the individual spores. Conditions for examining DAPI fluorescence were: excitation at 364 nm; dichroic mirror UV/488; and emission at ≥ 385 nm. Conditions for examining ANEPPS, acridine orange and 10-N-nonyl acridine orange fluorescence were: excitation at 488 nm; dichroic mirror UV/488/543/633; and emission at ≥ 560 nm. Conditions for examining autofluorescence were: excitation at 488 nm; dichroic mirror UV/488; and emission at ≥ 505 nm.

Spore germination and preparation of growing cells

Spores were germinated following a heat shock (30 min at 75°C for *B. subtilis* spores; 15 min at 60°C for *B. megaterium* spores) of spores at an O.D.₆₀₀ of 1-2 in water. After cooling to room temperature, *B. subtilis* PS533 spores were germinated at an O.D.₆₀₀ of 0.1 (~10⁷ spores/ml) at 37°C in 25 mmol l⁻¹ Tris-HCl buffer (pH 8) plus 100 µmol l⁻¹ L-alanine. *B. megaterium* spores at an O.D.₆₀₀ of 0.1 (~2×10⁶ spores ml⁻¹) were germinated at 37°C in 20 mmol l⁻¹ KPO₄ buffer (pH 7.5) and 50 mmol l⁻¹ glucose. Greater than 90% of the spores of both species germinated in 60 min under these conditions.

To obtain growing cells of *B. subtilis* strain PS533, cells grown overnight at 37°C on an LB medium agar plate (Paidhungat and Setlow 2000) containing kanamycin were inoculated into 4 ml of LB medium, grown at 37°C to an O.D.₆₀₀ of ~ 1, diluted 30-fold into prewarmed (37°C) Spizizen's minimal medium plus 0.1% Casamino acids (Spizizen 1958) and aliquots grown for ~ 50 min with or without various additions and/or other treatments prior to flow cytometry.

Flow cytometry

Dormant spores at an O.D.₆₀₀ of 0.1 were stained for flow cytometry in 10 mmol l⁻¹ Na₂HPO₄-1.8 mmol l⁻¹ KH₂PO₄-140 mmol l⁻¹ NaCl-2.7 mmol l⁻¹ KCl (adjusted to pH 7.4 with HCl) and with 6.3 nmol l⁻¹ 3,3'-dihexyloxacarbocyanine iodide [DiOC₆(3)] (Shapiro *et al.*, 1979; Cabrini and Verkman 1986; Rotenberg and Wu 1998; Laflamme *et al.*, 2005a) for at least 30 min prior to flow cytometry. Germinated spores were labeled with the same dye concentration in germination medium, with the dye added at the start of spore germination. To stain growing cells, the dye was added to 6.3 nmol l⁻¹ at the time of dilution into Spizizen's medium. To assess the effects of an agent that collapses membrane potential, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (Sigma Chemical Company, St. Louis, MO, USA), a proton ionophore dissolved in ethanol, was added to 100 µmol l⁻¹ 10-30 min prior to flow cytometry giving a final concentration of 2.3% ethanol. Control experiments showed that the ethanol alone did not cause the effects of FCCP addition.

Flow cytometry was carried out on a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) with excitation at 488 nm. Fluorescence signals were collected through a 530 nm band pass filter, and at least 10^4 spores were analyzed in each run.

Results

Staining and autofluorescence of *B. subtilis* spores containing various amounts of coat protein

As found previously (Melly *et al.*, 2002; Setlow *et al.*, 2002; Cowan *et al.*, 2004), wild-type *B. subtilis* spores stained noticeably with several dyes, including two (DAPI (50 mg l^{-1}) and acridine orange) that are primarily nucleic acid-specific, as well as a membrane probe, di-4-ANEPPS, and a dye that binds to anionic phospholipids, 10-N-nonyl acridine orange (Petit *et al.*, 1992) (Fig. 1a, 2a, 3a, 4a). Staining of these spores with a lower concentration of DAPI (1 mg l^{-1}) was also evident, but was much less intense than with the higher DAPI concentration (data not shown). In addition, the wild-type spores exhibited significant autofluorescence at wavelengths above 505 nm when excitation was at 488 nm (Fig. 5a). The latter wavelengths were used since they are those commonly used to detect fluorescence from green fluorescent protein (GFP), a reporter protein commonly used to detect the location of proteins in bacteria.

The layers in intact dormant spores that are the logical candidates for the binding of these stains are the coat and cortex, as these layers are large and permeable to molecules $\geq 1 \text{ kDa}$ in size (Gerhardt *et al.*, 1972; Driks 1999), although it is possible that spore membranes could also bind lipophilic dyes. However, the spore's inner membrane is almost certainly not permeable to any of these dyes (Gerhardt *et al.*, 1972). The spore coat has many hydrophobic protein components, and may also be the source of the spore's autofluorescence, since coats are reported to contain dityrosine cross-links between coat proteins, as well as a one or more pigments (Driks 1999, 2002; Henriques and Moran 2007). To assess whether the spore coats or cortex are indeed responsible for the spore's autofluorescence and peripheral staining, we examined these properties in spores of two strains with single mutations that interfere with coat assembly and structure, *cotE* and *gerE* (Zhang *et al.*, 1992; Zhang *et al.*, 1994; Driks 1999, 2002; Henriques and Moran 2007), but neither mutation abolished or even lessened the spore's autofluorescence or its peripheral staining (Fig. 1c,d; Fig. 2c,d; Fig. 3b,c; Fig. 4c,d, Fig. 5b,c), including with 1 mg l^{-1} of DAPI (data not shown). Indeed, the staining intensity with DAPI and acridine orange actually increased significantly in *cotE* and *gerE* spores.

While the *cotE* and *gerE* mutations disrupt spore coat assembly and structure, a significant amount of the spore coat remains on dormant *cotE* and *gerE* spores (Driks 1999; Klobutcher *et al.*, 2006; Ghosh *et al.*, 2008). Therefore, we also examined the autofluorescence and staining of spores of a *cotE gerE* mutant strain, as these spores have recently been shown to lack almost all coat layers, except for a very thin layer of insoluble and most likely highly cross-linked protein (Ghosh *et al.*, 2008). As noted above, spore staining with either acridine orange or a low or high DAPI concentration was unaffected by the double *cotE gerE* mutation (Fig. 1e; Fig. 2e; and data not shown). In contrast, spore autofluorescence as well as spore staining by di-4-ANEPPS were almost absent in *cotE gerE* spores (Fig. 3d, 5d). The staining of the *cotE gerE* spores with 10-N-nonyl acridine orange was also greatly reduced, with only a few spores showing any notable staining (Fig. 4e). One possible explanation for this latter result is that some of the *cotE gerE* spores retain a small amount of coat protein, and this is what is binding the 10-N-nonyl acridine orange. To test this possibility we chemically decoated wild-type and *cotE gerE* spores and then examined the decoated spores after staining with DAPI and 10-N-nonyl acridine orange, and also measured the decoated spore's autofluorescence. The chemical decoating had no effect on either the autofluorescence of wild-type or *cotE gerE* spores (data not shown), the 10-N-nonyl acridine orange staining of wild-type spores (Fig. 4a,b) or the DAPI staining of *cotE gerE* spores (Fig. 1e,f), but actually increased the DAPI and acridine

orange staining of wild-type spores (Fig. 1a,b; Fig. 2a,b). The chemical decoating also eliminated the staining of the small percentage of *cotE gerE* spores with 10-N-nonyl acridine orange (Fig. 4e,f), although had no significant effect on the acridine orange staining of *cotE gerE* spores (Fig. 2e,f).

Analysis of membrane potential in spores

The finding that much of the staining of dormant *B. subtilis* spores with the lipophilic stains di-4-ANEPPS and 10-N-nonyl acridine orange was likely due to binding to spore coat proteins prompted us to re-examine the use of the binding of a membrane potential-sensitive dye to assess the membrane potential in dormant and germinated spores of several *Bacillus* species (Laflamme *et al.*, 2005a). It is not surprising that fully germinated spores would have a membrane potential, since metabolism begins in the outgrowing spore soon after completion of germination (Paidhungat and Setlow 2002; Setlow 2003). However, significant binding of a membrane potential-sensitive dye to dormant spores has also been reported, suggesting that dormant spores may have a significant potential difference across at least one of the spore membranes (Laflamme *et al.*, 2005a). This result is unexpected, since dormant spores lack major high-energy small molecules such as ATP and reduced pyridine nucleotides, although oxidized pyridine nucleotides, AMP and some ADP are present (Paidhungat and Setlow 2002; Setlow 2003). However, the binding of the membrane potential—sensitive dye to dormant spores appears to be specific, since it was eliminated by addition of a proton ionophore that can dissipate membrane potential (Laflamme *et al.*, 2005a).

To re-investigate the possible membrane potential in dormant and germinating spores, we used both *B. subtilis* and *B. megaterium* spores, and measured the uptake of the membrane potential-sensitive dye DiOC₆(3) by flow cytometry as described (Laflamme *et al.*, 2005a). As reported previously, dormant spores of both *Bacillus* species took up a significant amount of this dye, and the amount of dye adsorbed was greatly reduced in the presence of the protonophore FCCP (Fig. 6a-f; Table 1). Chemical decoating of *B. subtilis* or *B. megaterium* spores did not reduce the spore fluorescence upon addition of this dye (Table 1). This observation indicated that membrane potential-sensitive dye binding was not due to a potential across the spore's outer membrane, as this membrane is largely removed during chemical coat removal (Buchanan and Neyman 1986). However, it was most surprising that treatment of spores of both species for 1 hr at 95°C, a treatment that killed > 99.9% of the spores and released > 95% of all small molecules (data not shown), had no effect on the binding of DiOC₆(3) by these spores (Table 1). This suggested that perhaps the binding of this dye to dormant spores was due to non-specific adsorption to spore coat material, much as was the binding of di-4-ANEPPS and 10-N-nonyl acridine orange as noted above. Consequently, we also examined the binding of DiOC₆(3) to dormant *cotE gerE* (PS4150) spores that lack the great majority of coat proteins. As found with di-4-ANEPPS and 10-N-nonyl acridine orange, there was minimal if any binding of this dye to these spores (Table 1), suggesting that the binding of this dye to wild-type spores is again due to adsorption to coat proteins.

Membrane potential in germinated spores and growing cells

While it appears likely that there is no significant membrane potential in dormant spores, it has been reported that DiOC₆(3) binding can detect membrane potential in germinated/outgrowing spores (Laflamme *et al.* 2005a). Therefore we also examined outgrowing spores as well as growing cells for membrane potential using flow cytometry to assess DiOC₆(3) binding to cells in these stages of growth. Similar to what was found previously (Laflamme *et al.*, 2005a), DiOC₆(3) was bound significantly to outgrowing *B. subtilis* and *B. megaterium* spores, and this binding was largely eliminated by FCCP (Tables 1, 2). That at least some of the DiOC₆(3) binding to germinated wild-type spores and all of the binding to the growing wild-type cells was due to a potential across the plasma membrane of the germinated spores and

growing cells was further suggested by the elimination of most dye binding by heat treatment of the outgrowing spores and growing cells (Tables 1,2). The binding of DiOC₆(3) to outgrowing *cotE gerE* (PS4150) spores that lack most spore coat layers was also largely sensitive to both FCCP and heat treatment (Table 1), strongly suggesting that this binding is again due to dye uptake in response to membrane potential.

Discussion

The work in this communication leads to a number of new conclusions. The first is that the great majority of the peripheral staining of dormant spores of at least *B. subtilis*, and perhaps spores of all related species, by lipophilic stains such as di-4-ANEPPS, 10-N-nonyl acridine orange and DiOC₆(3) is due to the binding of these reagents to the spore coats. This is true even in spores that have been chemically decoated, a procedure that removes much coat protein, although leaving an insoluble and very difficult to dissolve shell that has been termed a “rind” (Klobutcher *et al.*, 2006). The precise structure and makeup of this rind is not known, but in *B. subtilis* spores this structure is resistant not only to detergent extraction, but also to digestion by proteases *in vitro* and during predation on spores by eukaryotic microbes (Klobutcher *et al.*, 2006). Even the rind remaining after lysis of *cotE* or *gerE* spores is resistant to hydrolysis by proteases and dissolution by detergents (Ghosh *et al.*, 2008). While it is not clear what spore coat components are most important in adsorption of the stains listed above, since their binding was not reduced appreciably in *cotE* or *gerE* spores or by chemical decoating, it may be the insoluble rind material that is primarily responsible for the non-specific adsorption of these stains. Whatever the precise coat components that adsorb these stains, this presumably non-specific adsorption must clearly be kept in mind when interpreting results obtained with spores stained with lipophilic agents.

A second notable conclusion is that the peripheral staining of dormant spores by DAPI and acridine orange appears not to be due to binding of this dye to spore coats. Indeed, spores with mutations interfering with coat structure or assembly or decoated wild-type spores were stained better by these agents than were intact wild-type spores, and loss of almost all coat protein in *cotE gerE* spores also did not reduce spore staining with DAPI and acridine orange. Thus it is possible that it is the spore cortex that is stained by these two agents in dormant spores, as suggested previously (Setlow *et al.*, 2002). In contrast, the staining of wild-type spores incubated with di-4-ANEPPS is almost certainly due to adsorption of this dye to the outer spore layers, most likely the spore coat, as the staining with di-4-ANEPPS was almost completely abolished in *cotE gerE* spores, but not in either *cotE* or *gerE* spores in which the outer membrane has been disrupted. Chemical decoating also eliminated the minimal staining of *cotE gerE* spores with 10-N-nonyl acridine orange suggesting that this weak staining was due to some small amount of coat protein still present on a small percentage of *cotE gerE* spores.

The third significant conclusion is that the great majority of the autofluorescence of dormant spores is due to the autofluorescence of spore coat components. Again the precise identity of the autofluorescing coat components is not known, although one candidate is the dityrosine cross-links that have been reported between coat proteins (Driks 1999). There are also pigments in spore coats as well as oxidase and peroxidase activities that could generate fluorescent molecules in the coats (Driks 1999; Henriques and Moran 2007), and it is notable that the *cotE gerE* spores that had minimal autofluorescence were white when pelleted, in contrast to the salmon/brown color of wild-type, *cotE* or *gerE* spore pellets (Ghosh *et al.*, 2008). Again these autofluorescent species were not removed or even reduced by chemical decoating or by *cotE* or *gerE* mutations, further suggesting that these fluorescent species are associated with the insoluble coat fraction. On a practical note, the presence of significant autofluorescence in spores could hinder analysis of the location of low abundance spore proteins using fusions of such proteins to fluorescent protein reporters, such as one of the many GFP derivatives. It

seems very likely that removal of interfering autofluorescence from spores by using *cotE gerE* spores to do such experiments might be a real advantage in detection of low abundance GFP fusion proteins in spores.

The final important conclusion from our new data is that dormant *Bacillus* spores do not have any notable membrane potential as detected by the binding of DiOC₆(3). The binding of this dye by wild-type dormant spores is presumably due to the non-specific adsorption of the dye to the spore coat layer, as was the case for di-4-ANEPPS. The elimination of most DiOC₆(3) binding to the dormant spores by FCCP is presumably due simply to displacement of the DiOC₆(3) by the hydrophobic FCCP - especially so given the high ratio of FCCP/DiOC₆(3) used in these experiments. The lack of any significant effect on DiOC₆(3) binding by chemical decoating or heat killing of the spores is further strong evidence that the dye binding to dormant spores is not the result of potential across a spore membrane. Even stronger evidence that adsorption of DiOC₆(3) to spores is due to binding to the coat layer and not membrane potential is the almost complete elimination of the binding of this dye to *cotE gerE* spores that lack most coat protein. The absence of membrane potential in dormant spores is, of course, consistent with the absence of metabolism and high-energy compounds in dormant spores.

It is, however, notable that germinated spores and vegetative cells did exhibit membrane potential as detected by FCCP-sensitive DiOC₆(3) binding. This dye binding was seen not only in germinated wild-type spores but also in germinated *cotE gerE* spores, and unlike the case in dormant spores, the binding of this dye was largely eliminated if germinated spores or growing cells were heat killed. Thus as has been suggested (Laflamme *et al.*, 2005a), the generation of membrane potential during spore germination may well be an event that can be used to assess spore viability. The difficulty, however, will be in determining which fraction of any membrane potential-sensitive dye binding is due to membrane potential, and which fraction is due to non-specific adsorption to spore coats.

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Fig. 1a-f.

Fluorescence microscopy of *B. subtilis* spores treated with DAPI. Dormant *B. subtilis* spores of strain PS533 (wt) (a, b), PS3328 (*cotE*) (c), PS4149 (*gerE*) (d) and PS4150 (*cotE gerE*) (e, f), either intact (a, c, d, e) or chemically decoated (b, f), were stained with 50 mg l⁻¹ DAPI and the spores were photographed as described in Methods. The scale bar in (a) is 5 μ m and all photographs were at the same magnification and were taken at the same exposure. Unstained spores were not visible by fluorescence microscopy under these conditions.

Fig. 2a-f.

Fluorescence microscopy of intact and chemically decoated *B. subtilis* spores treated with acridine orange. Dormant *B. subtilis* spores of strains PS533 (wt) (a,b), PS3328 (*cotE*) (c), PS4149 (*gerE*) (d) and PS4150 (*cotE gerE*) (e,f) either intact (a, c, d, e) or chemically decoated (b, f) were stained with 10 mg l⁻¹ acridine orange and the spores were photographed as described in Methods. The scale bar in (a) is 5 μ m and all photographs were taken at the same magnification and exposure. Unstained spores were not visible by fluorescence microscopy under these conditions.

Fig. 3a-d.

Fluorescence microscopy of *B. subtilis* spores treated with di-4-ANEPPS. Dormant *B. subtilis* spores of strains PS533 (wt) (a), PS3328 (*cotE*) (b), PS4149 (*gerE*) (c) and PS4150 (*cotE gerE*) (d) were stained with 5 $\mu\text{mol l}^{-1}$ di-4-ANEPPS and the spores were photographed as described in Methods. The scale bar in (a) is 5 μm and all photographs were taken at the same magnification and exposure. Note that in (d) there were >25 spores in the field shown as seen by brightfield microscopy, and that unstained spores were also not visible by fluorescence microscopy under these conditions.

Fig. 4a-f.

Fluorescence microscopy of intact and chemically decoated *B. subtilis* spores treated with 10-N-nonyl acridine orange. Dormant *B. subtilis* spores of strains PS533 (wt) (a,b), PS3328 (*cotE*) (c), PS4149 (*gerE*) (d) and PS4150 (*cotE gerE*) (e,f) either intact (a, c, d, e) or chemically decoated (b, f) were stained with 10 mg l⁻¹ 10 N-nonyl acridine orange and the spores were photographed as described in Methods. The scale bar in (a) is 5 μ m and all photographs were taken at the same magnification and exposure, except that a 2-fold increase in laser intensity in (c) and (d) was compensated for by dividing the intensity values in each image by 2. Note that in (e) and (f) there were >25 spores in the field shown as seen by brightfield microscopy,

that unstained spores were not visible by fluorescence microscopy under these conditions, and that the staining material seen in (e) and (f) is most likely the agarose pad on the slide.

Fig. 5a-d.

Autofluorescence of spores of various *B. subtilis* strains. Unstained dormant *B. subtilis* spores of strains PS533 (wt) (a), PS3328 (*cotE*) (b), PS4149 (*gerE*) (c) and PS4150 (*cotE gerE*) (d) were photographed by fluorescence microscopy as described in Methods. The scale bar in (a) is 5 μ m and all photographs were taken at the same magnification and exposure. Note that in (d) there were >25 spores in the field shown as seen by brightfield microscopy.

Fig. 6a-f.

Flow cytometry of *Bacillus* spores incubated without or with DiOC₆(3). Dormant spores of *B. subtilis* PS533 (a-c) and *B. megaterium* (d-f) were incubated with no DiOC₆(3) (a,d), with DiOC₆(3) (b,e) or with DiOC₆(3) plus FCCP (c,f) and analyzed by flow cytometry as described in Methods.

Table 1Fluorescence intensity of *Bacillus* spores stained with DiOC₆(3)*

Strain: Spore state and treatment	<i>B. subtilis</i>		<i>B. megaterium</i>
	PS533 (wt)	PS4150 (<i>cotE gerE</i>) Peak fluorescence intensity ¹	QM B1551
Dormant	4	3	3
Dormant + DiOC ₆ (3)	95	7	800
Dormant decoated + DiOC ₆ (3)	350	-	600
Dormant + DiOC ₆ (3) + FCCP	17	4	5
Dormant decoated + DiOC ₆ (3) + FCCP	18	-	6
Dormant + DiOC ₆ (3) heated ²	90	-	800
Germinated	5	3	3
Germinated + DiOC ₆ (3)	175	220	700
Germinated + DiOC ₆ (3) + FCCP	30	13	60
Germinated + DiOC ₆ (3) + FCCP-heated ³	45	22	80

* Dormant or germinated spores of various species and strains were incubated with or without DiOC₆(3), FCCP and heat treatment and then analyzed by flow cytometry as described in Methods.

¹ The peak fluorescence intensity was taken as the maximum fluorescence seen in flow cytometric analyses (see Fig. 6) from the majority (> 85%) of the spores in the population. All values shown are averages of at least 3 separate experiments with different spore preparations, and individual values varied by less than 35% from the average. However, when relative levels of fluorescence in spores from the same preparation were compared, these values were within 15%.

² Spores were heated at 95°C for 1 hr prior to dye addition.

³ Germinated stained spores were heated at 80°C for 30 min just prior to flow cytometry.

Table 2Peak fluorescence values for vegetative *B. subtilis* cells stained with DiOC₆(3)^{*}

Cell treatment	Peak fluorescence ¹
None	4
+ DiOC ₆ (3)	155
+ DiOC ₆ (3) and FCCP	8
heat ² — then DiOC ₆ (3)	6
+ DiOC ₆ (3) then heat ²	21

^{*} Vegetative *B. subtilis* PS533 cells were prepared, stained and analyzed by flow cytometry as described in Methods.

¹ Peak fluorescence values in arbitrary units were determined from the position of the major peak (generally > 85% of all cells) of fluorescent cells in flow cytometry. All values are averages of at least duplicate experiments and individual values differed by < 25% from the averages.

² Cells were heated for 30 min at 80°C before or after DiOC₆(3) addition.