Permeability of dormant spores of *Bacillus subtilis* to malachite green and crystal violet

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The permeability of dormant spores of *Bacillus subtilis* to malachite green (MG) and crystal violet (CV) was examined by using potassium trichloro(η^2 -ethylene)platinum(II) (KTPt) as an electron-opaque marker for the dyes. The spores were treated with the dyes and other substances at 30 °C for 30 min or at 80 °C for 5 min. When the spores were incubated in 50 mM-MG solution at 30 °C and in 50 mM-CV solution at 30 °C or 80 °C, many small electron-dense precipitates, which were chemical complexes of dyes and platinum, were seen, mainly around the boundary between the inner and outer coat regions. The spores treated under the above conditions were not stained. Treatment with 50 mM-MG alone or a mixture of 25 mM-oxalic acid and 50 mM-CV at 80 °C made the spores stainable and dye-TPt precipitates were observed mainly in the outer pericortex region. Pretreatment with 25 mM-oxalic acid and 5% (v/v) phenol at 80 °C followed by 50 mM-CV treatment at 30 °C gave the same results as above. It was considered from these results that the inner coat itself might function as the primary permeability barrier to MG and CV, and that a secondary barrier to the dyes might exist around the cortex region.

Introduction

Bacterial endospores are well known for their extreme resistance and dormancy. Previous studies related to the permeability of dormant spores to disinfectants (Sykes, 1970; Thomas & Russell, 1974), polymyxin B (Tochikubo *et al.*, 1986), lysozyme (Gould & Hitchins, 1963; Gould *et al.*, 1970) and germinants (Koshikawa *et al.*, 1984; Vary, 1973; Watabe *et al.*, 1974) indicate that a spore coat or an outer pericortex membrane may play an important role as a penetration barrier. Moreover, indirect evidence for the existence of a penetration barrier at or near the outer edge of the cortex has been obtained by electron microscopy of unfixed spores embedded in methacrylate (Rode *et al.*, 1962). Recently, we showed by immunoelectron microscopy that the permeability barrier of dormant spores of *Bacillus subtilis* to gentamicin was present outside the cortex (Tochikubo *et al.*, 1988).

Dormant spores cannot be stained by conventional methods for staining vegetative bacterial cells. Many investigators have noticed that acid treatment before staining (Fitz-James *et al.*, 1954; Lechtman *et al.*, 1965; Maneval, 1941; Robinow, 1951) or heat treatment

during staining (Dorner, 1922; Dutton, 1928; May, 1926) is necessary to stain bacterial spores. In this paper, we report how far basic dyes can penetrate into *B. subtilis* spores and why the spores can be stained by the method of Schaeffer & Fulton (1933), which is a simplification of a method proposed by Wirtz (1908).

Methods

Dormant spores. Dormant spores of *B. subtilis* PCI 219, prepared by growing the organism on nutrient agar at 37 °C for 5 d, were harvested and washed five times with sterile deionized water by centrifugation.

Treatment of dormant spores with dyes and other chemicals. Dormant spores (about 2×10^9 ml⁻¹) were suspended in an aqueous solution containing 50 mM-crystal violet hydrochloride (CV; pH 3.9) or 50 mM-malachite green oxalate (MG; pH 2.2) and incubated at 30 °C for 30 min or at 80 °C for 5 min. Dormant spores were also treated at 80 °C for 5 min with 25 mM-oxalic acid (pH 1.7), 50 mM-CV plus 25 mM-oxalic acid (pH 1.8) or 5% (v/v) phenol (pH 4.9), all of which were dissolved in deionized water. Afterwards they were washed at least five times with deionized water by centrifugation. For spores treated with oxalic acid or phenol this was followed by incubation in 50 mM-CV solution at 30 °C for 30 min and washing with deionized water.

Determination of various properties of spores treated with dyes and other chemicals. Stainability of dye- or chemical-treated spores was observed by light microscopy and their refractility was examined by phase contrast microscopy. Germinability was determined by the loss of refractility of spores incubated at 37 °C for 60 min on agar which was

Abbreviations: CV, crystal violet; DAP, diaminopimelic acid; DPA, dipicolinic acid; GlcN, glucosamine; KTPt, potassium trichloro(η^2 -ethylene)platinum(II); MG, malachite green.

dissolved in 50 mM-phosphate buffer (pH 7.2) and contained 50 mM-L-alanine. Lysozyme sensitivity was determined by suspending spores in 50 mM-Tris/HCl buffer (pH 8.4) containing 200 µg lysozyme ml⁻¹ and measuring the decrease in OD₆₅₀ after incubation at 37 °C for 120 min.

Electron microscopy and X-ray microanalysis. Treated spore samples were immersed in 50 mM-potassium trichloro(η²-ethylene)platinum (II) (KTPt) buffered with 50 mM-HEPES (pH 6.8) for 120 min at 37 °C under reduced pressure. After washing three times with 50 mM-HEPES buffer by centrifugation, the pellets were fixed in a mixture of 8% (w/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde buffered with 100 mM-HEPES (pH 6.8) for 3 d at 4 °C under reduced pressure. They were washed twice in the same buffer and then suspended in molten 2% (w/v) agar. After solidification, the agar was cut into 1 mm³ cubes. The agar cubes were dehydrated through increasing concentrations of Durcupan and embedded in modified Durcupan (Kushida, 1979); as a hardener, nonenyl succinic anhydride was substituted for 964 and 960 hardener of the original procedure (Stäubli, 1960). Polymerization was performed by heating at 60 °C for 24 h. After polymerization, sections were cut with a glass knife on a Reichert-Jung Ultracut E ultramicrotome and mounted on Formvar-coated copper grids. The sections were observed without staining in a JEOL JEM-1200EX electron microscope at an accelerating voltage of 80 kV.

X-ray spot microanalysis was performed on a Hitachi H-800 electron microscope equipped with a Kevex-7000 Q energy dispersive system (EDS). Accelerating voltage was 100 kV, beam current 10⁻¹⁰ A and beam diameter 7 nm. Selected area analysis was done on a square section (0.04 µm²) for 100 s. Platinum distribution maps were generated by interfacing the EDS with a scanning transmission electron microscope module and the cells were scanned for 400 s with the Pt (M_{αβ} and L_α) lines.

Biochemical analysis. About 1.3 × 10¹⁰ spores treated with dyes and other chemicals were washed five times with distilled water by centrifugation using polycarbonate tubes treated with silicon to prevent their adherence. Afterwards they were autoclaved at 121 °C for 20 min to measure the amounts of dipicolinic acid (pyridine-2,6-dicarboxylic acid, DPA) and calcium. DPA content was determined by the method of Scott & Ellar (1978). Calcium content was measured by using a Hitachi model 180-50 atomic absorption spectrophotometer. For determination of amino acids, glucosamine (GlcN) and diaminopimelic acid (DAP), each treated spore sample was hydrolysed in 6 M-HCl at 100 °C for 5 h with a Pico-Tag work station and the analyses were performed with a JEOL JLC-300 high-speed amino acid analyser equipped with an LC30-DK20 data-processing system using lithium citrate buffer. Total protein content was calculated from the data obtained in the amino acid analysis.

Chemicals. CV and MG were purchased from Merck, oxalic acid and phenol from Katayama Kagaku Industrial Co., KTPt from Aldrich, lysozyme from Boehringer Mannheim, HEPES from Dojindo Laboratories, paraformaldehyde, glutaraldehyde and nonenyl succinic anhydride from TAAB, and Durcupan from Fluka.

Results

Electron microscopy of B. subtilis spores treated with MG and CV

Thin sections of untreated spores immersed only in KTPt solution demonstrated no electron-dense precipitates (Fig. 1a), whereas those of spores treated with MG or CV at 30 °C for 30 min and subsequently immersed in

KTPt solution showed small electron-dense precipitates, which seemed to be the MG-TPt or CV-TPt deposits, preferentially localized in the outer coat region but not in the inner coat, cortex and core regions (Fig. 1b). When the spores were treated with MG at 80 °C for 5 min, large amounts of MG-TPt deposits were mainly observed in the outer pericortex region (Fig. 2a), suggesting that MG could penetrate the spore coat but not the cortex; in this thin section the contour of the core was clear in company with crystal-like substances.

On the other hand, when spores were treated with CV alone at 80 °C for 5 min the CV-TPt deposits were observed only in the outer coat region in the same manner as the spores treated at 30 °C (data not shown). This difference between the two dyes seemed to be due to oxalate because of its presence in the MG reagent and it was therefore assumed that oxalate must play an important role in penetration of MG into the outer pericortex region. To clarify this, spores were incubated in a mixture of CV and oxalic acid at 80 °C for 5 min. The CV-TPt precipitates were formed between the inner coat and cortex regions, as in the spores treated with MG at 80 °C (Fig. 2b); pretreatment with oxalic acid at 80 °C for 5 min and subsequent treatment with CV showed the same result (data not shown) and crystal-like substances were also observed in a thin section of the spores treated with oxalic acid alone (data not shown). Pretreatment with phenol at 80 °C for 5 min in place of oxalic acid, followed by CV treatment at 30 °C for 30 min, also showed the same result as MG treatment at 80 °C and CV/oxalic acid treatment at 80 °C (Fig. 2c).

To ascertain whether the small electron-dense precipitates formed in the outer pericortex region contained Pt, X-ray spot microanalysis was performed on a thin section of a spore treated with MG at 80 °C for 5 min. The X-ray spectrum indicated the presence of Pt (data not shown). Additionally, the distribution of Pt in thin sections of spores treated with MG at 30 °C and 80 °C was examined. Spores treated at 30 °C showed the presence of Pt mainly in the coat region, whereas those treated at 80 °C showed the presence of Pt throughout the spore, including the core and cortex regions (data not shown); the distribution map of spores treated with KTPt alone at 30 and 80 °C indicated that Pt mainly localized in the coat region, and that of spores treated with oxalic acid at 80 °C showed the presence of Pt throughout the spore. These results suggest that when the spores were stained with MG at 80 °C, the Pt label could penetrate into the core region but the dye could only penetrate to the outer pericortex region.

To check the reproducibility and repeatability of the above results, the same experiments were carried out three times and 500 thin sections of each preparation of treated spores were examined. The proportions of thin

Fig. 1. Electron micrographs of thin sections of untreated spores (a), and spores treated with CV at 30 °C for 30 min (b). Arrowheads in (b) indicate CV-TPt precipitates. A highly magnified picture of the integument (b, inset) clearly shows that platinum deposits are formed in the spore coat region. OSC, outer spore coat; ISC, inner spore coat; OPCX, outer pericortex; CX, cortex; CR, core. Bars represent 200 nm (inset, 100 nm).

sections showing large amounts of MG- or CV-TPt deposits in the outer pericortex region were $71.6 \pm 1.9\%$ with MG treatment at 80 °C, $73.1 \pm 2.3\%$ with CV/oxalic acid treatment at 80 °C and $87.2 \pm 3.3\%$ with phenol/CV treatment at 80 °C (mean $\pm$ SD), suggesting that the results obtained are reproducible.

Effect of MG, CV, oxalic acid and phenol on various properties of B. subtilis spores

Spores treated with MG or CV at 30 °C for 30 min or with CV at 80 °C for 5 min remained refractile, unstained and germinable by L-alanine, and were not

Fig. 2. Typical electron micrographs of thin sections of spores treated with MG at 80 °C for 5 min (a), spores treated with a mixture of CV and oxalic acid at 80 °C for 5 min (b) and spores treated with CV after pretreatment with phenol at 80 °C for 5 min (c). Arrowheads indicate MG-TpT or CV-TpT precipitates. A highly magnified picture of the integument (a, inset) clearly shows that platinum deposits are formed in the outer pericortex region. OSC, outer spore coat; ISC, inner spore coat; OPCX, outer pericortex; CX, cortex. Bars represent 200 nm.

Table 1. *Some properties of dye- or chemical-treated spores of B. subtilis*

Spores remained refractile after each of the treatments.

Treatment	Stainability	Germinability by L-alanine	Lysozyme sensitivity
None		+	—
CV			
30 °C, 30 min	Unstained	+	—
80 °C, 5 min	Unstained	+	—
MG			
30 °C, 30 min	Unstained	+	—
80 °C, 5 min	Stained	—	+
Oxalic acid			
30 °C, 30 min	Unstained with CV at 30 °C	+	—
80 °C, 5 min	Stained with CV at 30 °C	—	+
Phenol			
30 °C, 30 min	Unstained with CV at 30 °C	+	—
80 °C, 5 min	Stained with CV at 30 °C	—	+

Table 2. *Contents of DPA, calcium, GlcN, DAP and total protein in spores treated with oxalic acid, phenol or MG*

Treatment	Content (μg per approx. 1.3×10^{10} spores) of:				
	DPA	Calcium	GlcN	DAP	Total protein
None	365.0 ± 13.8 (100)	98.9 ± 12.0 (100)	163.4 ± 7.5 (100)	77.3 ± 1.9 (100)	2711.4 ± 81.6 (100)
Oxalic acid (80 °C, 5 min)	14.3 ± 2.1 (3.9)	15.9 ± 9.4 (16.1)	153.0 ± 8.5 (93.6)	73.9 ± 1.6 (95.6)	2276.3 ± 78.4 (84.0)
Phenol (80 °C, 5 min)	41.8 ± 4.4 (11.5)	13.9 ± 8.8 (14.1)	142.0 ± 9.5 (86.9)	67.1 ± 3.4 (86.8)	2186.7 ± 87.5 (80.6)
MG (80 °C, 5 min)	17.0 ± 3.4 (4.7)	13.8 ± 10.1 (14.0)	138.4 ± 12.9 (84.7)	67.1 ± 9.4 (86.8)	2055.8 ± 221.8 (75.8)

* Each value represents the mean $\pm$ SD of three determinations. Numbers in parentheses are percentages of the control values.

sensitive to lysozyme (Table 1). Pretreatment with oxalic acid or phenol at 30 °C gave the same results. On the other hand, spores that were either dyed green by MG treatment at 80 °C for 5 min, or violet by CV treatment at 30 °C for 30 min following initial pretreatment with oxalic acid or phenol at 80 °C for 5 min, retained their refractility but lost their germinability by L-alanine and became sensitive to lysozyme (Table 1). These results paralleled those obtained by electron microscopy.

Chemical composition of treated spores

The contents of DPA, calcium, GlcN, DAP and protein of spores treated with oxalic acid, phenol or MG-oxalate at 80 °C for 5 min were examined because these chemicals could make them stainable. Considerable amounts of DPA and calcium were lost during treatment with MG at 80 °C and pretreatment with oxalic acid or phenol at 80 °C, but the contents of GlcN and DAP,

which are components of the cortex, and protein were close to those for untreated spores (Table 2). As the measured values shown in Table 2 were based on the original amount of spores treated, some of the losses of GlcN, DAP and protein could be accounted for by incomplete recovery of spores during the centrifugation process. In addition DPA and calcium might be lost to some extent.

Discussion

In this paper we have demonstrated by using a new electron-opaque probe, KTPt, that MG and CV must penetrate the spore coat and reach the outer pericortex region for dyed spores to be observed by light microscopy.

When the spores of *B. subtilis* were subjected to MG treatment at 80 °C for 5 min, conditions judged to be almost identical to those of Schaeffer and Fulton's method of spore staining (Schaeffer & Fulton, 1933), followed by immersion in KTPt solution, they were stained green (Table 1) and many small electron-dense precipitates were observed in the outer pericortex region (Fig. 2a). It has already been reported that the TPt anion complexes with CV⁺ to produce a CV-TPt precipitate (Beveridge & Davies, 1983; Davies *et al.*, 1983). Since MG is a triphenyl methane compound like CV, though a weak basic dye, it must be able to form a MG-TPt precipitate. In fact, in our preliminary experiment MG reacted with KTPt to produce a MG-TPt precipitate *in vitro* and it was ascertained by X-ray microanalysis that the small electron-dense precipitates observed in thin sections contained Pt. Therefore it seems reasonable to consider that the small electron-dense deposits formed in the outer pericortex region consist of MG-TPt complex. The same result was also obtained by treatment with a mixture of CV and oxalic acid at 80 °C for 5 min (Fig. 2b) and by pretreatment with oxalic acid or phenol at a high temperature and subsequent CV treatment (Fig. 2c).

Many methods of spore staining other than that of Schaeffer & Fulton (1933), which is a simplification of a method proposed by Wirtz (1908), need a mordant such as chromic acid (May, 1926; Möller, 1891), nitric acid (Lechtman *et al.*, 1965), osmic acid (Wirtz, 1908), ammonia (Lagerberg, 1917), or a mixture of phenol, acetic acid and ferric chloride (Maneval, 1941). In our experiments MG oxalate was employed and pretreatment with oxalic acid or phenol at 80 °C was necessary for spore staining with CV (Table 1). Oxalic acid and phenol also seemed to act as a mordant and the heating step was essential in this case.

When the spores were treated with MG or CV at room temperature and immersed in the KTPt solution, they

were not stained and MG-TPt or CV-TPt precipitates were formed in the outer coat region, especially around the boundary between the inner and outer coat regions (Fig. 1b). This fact seems to suggest the existence of the primary permeability barrier to the dyes between these two regions. Ohye & Murrell (1962) reported that OsO₄ penetrates poorly beneath the outer coat of dormant spores of *Bacillus coagulans*. Alternatively, the inner coat itself may function as the permeability barrier and pretreatment with oxalic acid or phenol at a high temperature may cause partial destruction of the inner coat layer and enable the dyes to reach the outer pericortex region because spores treated with oxalic acid or phenol became susceptible to lysozyme (Table 1). Failure of the dyes to penetrate the cortex suggests the existence of a secondary permeability barrier, that is, an outer membrane, which has been demonstrated biochemically in the spores of *Bacillus megaterium* (Crafts-Lighty & Ellar, 1980), by the morphological observation of variant spores lacking an exosporium (Koshikawa *et al.*, 1984) and by showing the presence of vegetative cell membrane derived proteins in the spore coat of *B. subtilis* (Fujita *et al.*, 1989). However, the localization and the integration of an outer membrane remain unclear with *B. subtilis* spores. Beaman *et al.* (1988) have reported the permeabilization of the outer membrane of *Bacillus stearothermophilus* spores by heat shock at 100 or 80 °C as shown by the susceptibility to lysozyme action and permeation of Nycodenz.

Spores treated with MG oxalate, oxalic acid or phenol at a high temperature lost large amounts of DPA and calcium (Table 2) and became susceptible to lysozyme but retained their characteristic refractility (Table 1). The same phenomena have been reported for the spores of *B. megaterium* (Rode & Foster, 1960) and the fully activated spores of *B. stearothermophilus* (Beaman *et al.*, 1988). Treatment with the above chemicals seemed not to affect the cortex because little loss of GlcN and DAP was observed (Table 2).

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