JOURNAL OF BACTERIOLOGY, Aug. 1984, p. 624–632
0021-9193/84/080624-09$02.00/0

Vol. 159, No. 2

Resistance, Germination, and Permeability Correlates of *Bacillus megaterium* Spores Successively Divested of Integument Layers†

TOMIHIKO KOSHIKAWA,‡ TEOFILA C. BEAMAN, H. STUART PANKRATZ, SATOSHI NAKASHIO, THOMAS R. CORNER, AND PHILIPP GERHARDT*

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48824

Received 7 November 1983/Accepted 9 May 1984

A variant strain that produced spores lacking exosporium was isolated from a culture of *Bacillus megaterium* QM-B1551. Two additional spore morphotypes were obtained from the parent and variant strains by chemical removal of the complex of coat and outer membrane. Among the four morphotype spores, heat resistance did not correlate with total water content, wet density, refractive index, or dipicolinate or cation content, but did correlate with the volume ratio of protoplast to protoplast plus cortex. The divestment of integument layers exterior to the cortex had little influence on heat resistance. Moreover, the divestment did not change the response of either the parent or the variant spores to various germination-initiating agents, except for making the spores susceptible to germination by lysozyme. The primary permeability barrier to glucose for the intact parent and variant spores was found to be the outer membrane, whereas the barrier for the divested spores was the inner membrane.

Investigation of the mechanisms by which bacterial spores achieve resistance and initiate germination has been complicated by the use of model types of spores that possess superfluous integument layers, such as spores of *Bacillus cereus* T, *Bacillus subtilis* 168, and *Bacillus megaterium* QM-B1551. Consequently, it appeared desirable to obtain structurally simplified dormant spores derived from a single, well-studied strain to serve as models for further investigation.

In this paper, we report the isolation from *B. megaterium* QM-B1551 of a variant strain that produces spores devoid of the thick peripheral structure which we term exosporium. Both the parent and the variant spores were chemically divested of the coat and outer membrane, the variant spore thus becoming a cortex-encased protoplast. The four morphotype spores, all dormant and heat resistant, were examined for fine structure by electron microscopy, studied for biophysical and biochemical correlates of heat resistance, tested with chemical agents that initiate germination, and used to distinguish the permeability roles of the outer and inner membranes.

MATERIALS AND METHODS

Organisms. *B. megaterium* QM-B1551 (ATCC 12872) was obtained from James C. Vary, University of Illinois Medical Center, Chicago. From this parent strain, an apparently naturally occurring variant that produced spores lacking exosporium (EX^- variant) was isolated by chance selection during serial transfer. The genetic basis was not studied. However, tests conducted at the American Type Culture Collection, Rockville, Md., indicated that the variant has identifying characteristics like those of authentic *B. megaterium* QM-B1551 (Robert L. Gherna, personal communication). The variant is available from the Collection under accession number 33729.

Spore production. Dormant spores of the parent and variant *B. megaterium* strains were produced essentially by the procedure of Shay and Vary (34). Vegetative cells grown overnight on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) were inoculated into 300 ml of supplemented nutrient broth (33) and incubated with aeration by shaking for 7 h at 30°C. The culture was then transferred into 10 liters of the same medium in a fermentor and incubated for 24 h at 30°C with constant stirring (400 rpm) and aeration (11 liters of air per min). The resulting spores were harvested by centrifugation (5,000 × *g* for 5 min) and washed by centrifugation about 15 times with cold, sterile, deionized water; each time, the supernatant liquid was decanted and debris was removed from the surface of the pellet. The spores were stored in water at 4°C and washed twice daily by centrifugation. The four morphotype spores all remained viable, refractile, and heat resistant when stored in this way.

Electron microscopy. Specimens were prepared, stained, sectioned, and examined by electron microscopy essentially as described previously (4).

Volume fractions. Estimates of the volume percentage of the spore occupied by the protoplast and the sporoplast (essentially, the protoplast plus cortex; actually, the protoplast plus primordial cell wall plus cortex plus coat underlayer) were obtained by calculation from measurements on electron micrographs, care being taken to select only longitudinal center sections of spores, as described previously (3).

Chemical treatment. Spores of the parent and the variant were both chemically treated to remove the complex of coat and outer membrane (C^- OM^-), essentially as described by Fitz-James (14) and Vary (39). Clean spores (10 g, wet weight) were suspended in a freshly made solution of 0.5% sodium dodecyl sulfate–0.1 M dithiothreitol–0.1 M NaOH (pH 10). The suspension was shaken (150 rpm) for 2 h at 37°C, and the spores were harvested by centrifugation (5,000

* Corresponding author.

† Publication no. 11050 of the Michigan Agricultural Experiment Station.

‡ Present address: Faculty of Pharmaceutical Sciences, Osaka University, Osaka, Japan.

× g for 10 min). The resulting spores were then washed and stored as described above.

Determination of heat resistance. Heat resistance of each of the four morphotype spores was determined at 70, 80, 90, and 100°C and expressed as a *D* value, as described previously (3).

Water properties. Determinations of wet density and water content by direct gravimetric and volumetric measurements were made as described previously (3), except for the initial centrifugation conditions. The parent spores were centrifuged for 30 min at 11,000 × g, and the variant spores were centrifuged for 30 min at 4,400 × g.

Refractometry. Determinations of average apparent refractive index were obtained by photometric immersion refractometry as described previously (19). Changes in optical density (OD) were monitored by means of a double-beam spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) operated at a wavelength of 700 nm with deionized water as a blank.

Chemical analyses. Dipicolinic acid was determined by the method of Janssen et al. (25). Hexosamine was determined by the method of Cessi and Piliego (11) after hydrolysis of the spores in 3 N HCl for 4 h at 95°C. Mineral element analyses (Ca^{2+}, Mg^{2+}, Mn^{2+}, K^+, Na^+) were made by use of atomic absorption spectroscopy (27a).

Initiation of germination. Initiation of germination was studied with various chemical agents and determined by measuring the decrease in OD, essentially as described by Vary (39), so as to be comparable. A concentrated suspension of spores (OD, 6.0) was heat activated for 10 min at 60°C, chilled in an ice bath, and diluted with initiation medium contained in a cuvette to a final OD of 0.6, which was within the proportionality range of the spectrophotometer. Final reagent concentrations were as follows: 5 mM Tris buffer; 16 mM phosphate buffer; 10 mM each D-glucose, L-leucine, L-proline, and KNO_3; 2 mM inosine; 200 μg of lysozyme per ml. The decrease in OD was measured at 660 nm, with readings taken initially and after 30 min of incubation at 30°C. The results were expressed as the percentage of decrease in OD and were confirmed by observation of the loss in spore refractility by use of a phase-contrast microscope. An OD decrease of greater than 45% was associated with greater than 90% conversion to phase-dark spores, and an OD decrease of less than 10% was associated with less than 10% conversion to phase-dark spores. The determinations were replicated two to four times, and average values were reported.

Permeability. Permeability measurements were made with ^3H-labeled water and ^{14}C-labeled D-glucose, with correction for the amount of interstitial water obtained from a similar measurement with dextran of high molecular weight ($\overline{M}_n$, 2,000,000), as described previously (19). Equilibrium uptake of the labeled water and glucose was attained within 1 h and maintained for at least 24 h by all of the morphotype spores (data not shown). The suspension of spores and solution was routinely allowed to equilibrate for 2 h at 4°C. The native parent and the C^- OM^- parent spores were then centrifuged at 11,000 × g for 30 min, whereas the EX^- variant and the EX^- C^- OM^- variant spores were centrifuged at 4,400 × g for 30 min. In both cases, the supernatant solution was clarified by centrifugation at 33,000 × g for 30 min. Radioactivity was measured with samples of the supernatant solution appropriately diluted in aqueous scintillation fluid (New England Nuclear Corp., Boston, Mass.) and counted in a scintillation counter (Beckman Instruments). The results were expressed as the volume percentage of the spore permeated by the labeled water or glucose on a wet-weight basis (R^w; expressed as milliliters per 100 g of wet spore = grams per 100 g of wet spore) or on a wet-volume basis (R^v; expressed as milliliters per 100 ml of wet spore). R^w and R^v were interconvertable by use of the appropriate wet-density value. The R^w was equivalent to the water content on a wet-weight basis, which was directly comparable to that obtained by the gravimetric method. The R^v for glucose was converted to a glucose-impermeable volume percentage (100 − $R^v_{glucose}$) for comparison with the physical volume percentage calculated from the measurements made on electron micrographs.

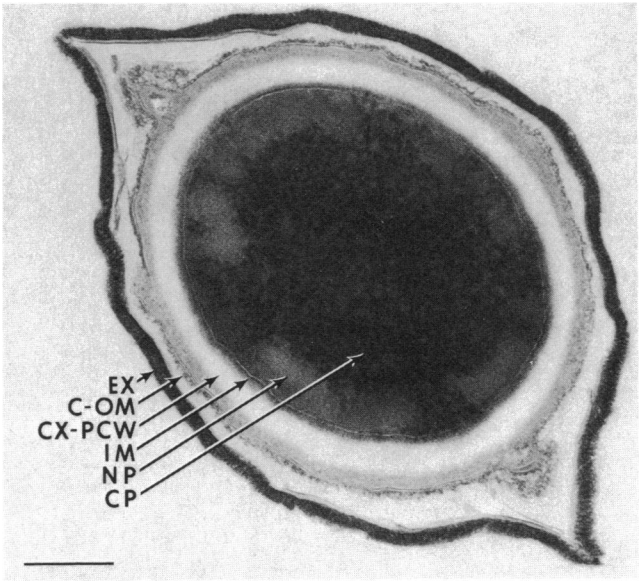

FIG. 1. Stained sectioned parent spore of *B. megaterium* QM-B1551 shown by transmission electron microscopy. This morphotype spore contained all of the usual structural components: exosporium (EX), complex of coat and outer membrane (C-OM), complex of cortex and primordial cell wall (CX-PCW), inner membrane (IM), DNA-containing nucleoplasm (NP), and ribosome-containing cytoplasm (CP). The protoplast consists of the cytoplasm and nucleoplasm enclosed by the inner membrane; the integument consists of everything outside of the protoplast. Bar, 200 nm.

RESULTS

Fine structure. The parent strain of *B. megaterium* QM-B1551 produced spores that contained all of the usual components in stained and sectioned fine structure (Fig. 1) and were consistent in appearance with prior electron micrographs of this strain (4, 17) and morphologically similar strains (2, 15). The outstanding feature was a thick, loose-fitting, peripheral structure with apical openings. This structure often is termed outer coat but should be termed exosporium (see Discussion). The atypical exosporium of this spore lacked the thick, hairlike nap outside of the basal layer, which is present in the typical exosporium of spores of *B. megaterium* Mg19 (4) and of *B. cereus*, *B anthracis*, and *B. thuringiensis* (18, 20, 21, 29).

Electron microscopy of the unstained intact parent spore of *B. megaterium* QM-B1551 revealed that the exosporium in itself was electron translucent (Fig. 2A).

After treatment of the parent spore with alkaline sodium dodecyl sulfate and dithiothreitol to remove the complex of

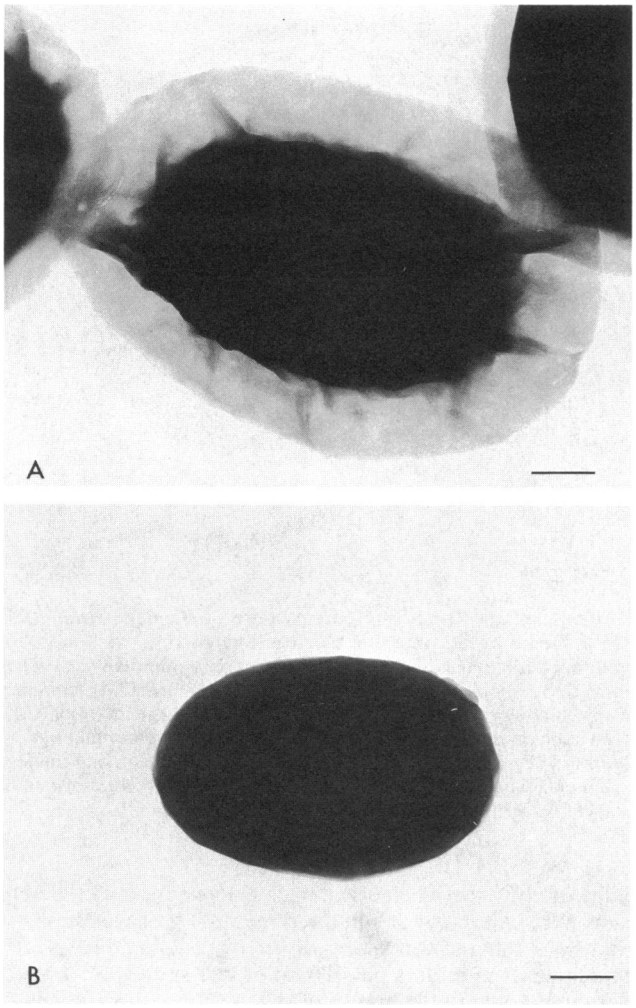

FIG. 2. Unstained intact spores. (A) Parent strain; (B) variant strain. Bars, 200 nm.

coat and outer membrane, there resulted a cortex-encased protoplast surrounded by exosporium (Fig. 3).

The variant strain produced spores that resembled the parent spores except for the absence of exosporium (Fig. 2B and 4A). Variant spores were sometimes observed in which the peripheral layer of the coat was separated from what we identified as the outer membrane (see Discussion), so that these two structures could be better distinguished (Fig. 4B).

After chemical treatment of the variant spore to remove the coat and outer membrane, there remained only a cortex-encased protoplast (Fig. 5).

Heat resistance. The relationship between heat resistance of the parent spore (expressed as log D) and temperature of exposure was linear between 100 and 70°C, enabling extrapolation to a D_{47} value for comparison with that measured for the germinated spore and vegetative cell (Fig. 6). The dormant spore was more than 10^6-fold more resistant than the germinated spore or vegetative cell. Similar results (data not shown) were obtained with the other morphotype spores.

Heat resistance varied to a small extent among the four morphotype spores, the D_{100} value decreasing from 0.44 to 0.033 min as integument layers were successively divested (Table 1). The D_{100} value of the native parent spore (0.44

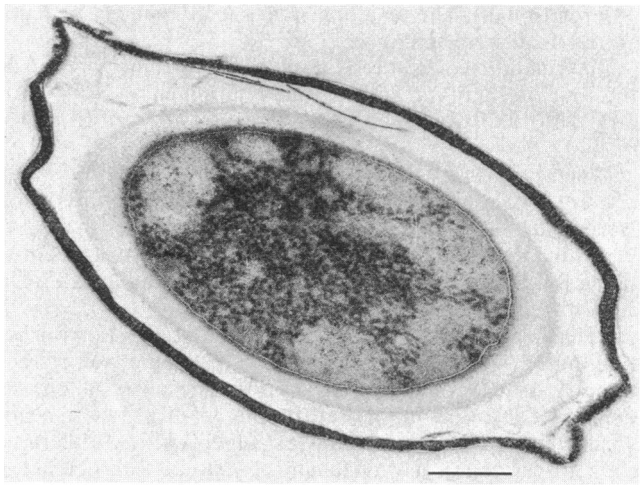

FIG. 3. Parent spore after treatment with alkaline sodium dodecyl sulfate and dithiothreitol to remove the coat and outer membrane. The resulting spore is a cortex-encased protoplast surrounded by exosporium. Bar, 200 nm.

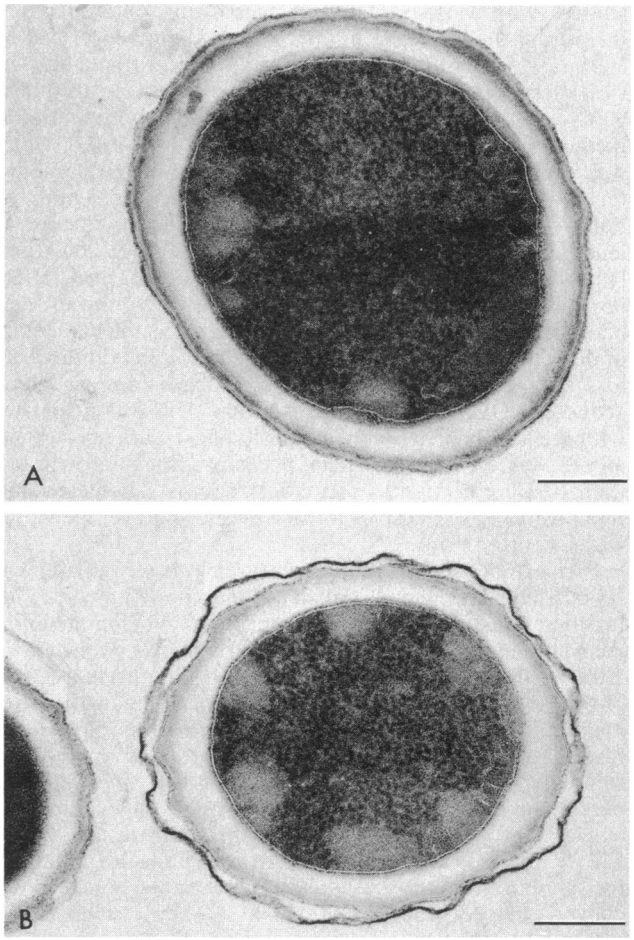

FIG. 4. Variant spore. (A) Usual appearance with the integument layers all contiguous; (B) unusual appearance with the peripheral layer of the coat separated from the outer membrane. Bars, 200 nm.

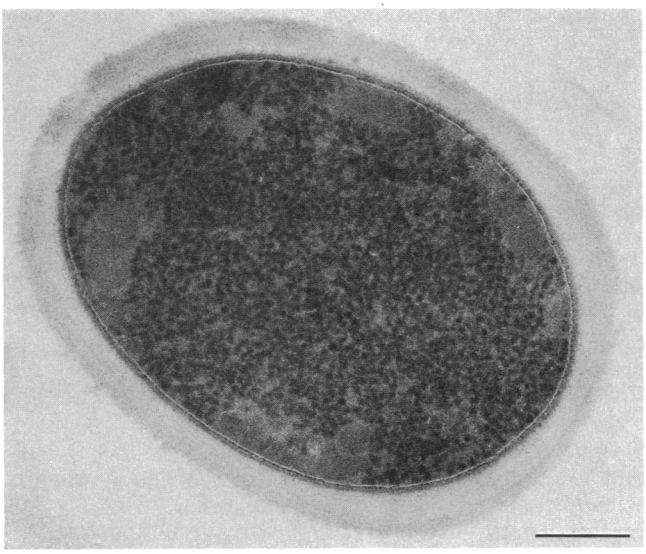

FIG. 5. Variant spore after treatment with alkaline sodium dodecyl sulfate and dithiothreitol to remove the coat and outer membrane. The resulting spore is a cortex-encased protoplast. Bar, 200 nm.

min) was about 10^3-fold less than that of a smooth strain of *B. stearothermophilus* (579 min) (3).

Although the exosporium and coat thus had little influence on the retention of heat resistance and dormancy by the spores, an intact cortex surrounding the protoplast was apparently necessary. EX^- C^- OM^- variant spores were treated with lysozyme in the presence of 2.5 M sucrose and 40 mM $CaCl_2$ essentially as described by Fitz-James (14). There resulted free protoplasts devoid of cortex which remained essentially dormant for 3 to 4 h, retaining 80% of their original refractility and 90% of their original dipicolinate content. Thereafter, however, the protoplasts essentially germinated, losing their refractility and dipicolinate. Efforts were unsuccessful to maintain dormancy in the protoplasts for a longer period by increasing the osmotic pressure with sucrose, decreasing the water activity (down to 0.4) with glycerol, or providing mechanical support with gels of agar (up to 9%) or gelatin (up to 18%).

Wet-density values for the four morphotype spores were unusually high, and their total values for water content correspondingly were unusually low, relative to their low values for heat resistance (Table 1) and in comparison with those of other spore types. For example, the native parent spores had a wet density of 1.20 g/ml, a water content of 50%, and a D_{100} value of 0.44 min; in comparison, spores of rough-type *B. stearothermophilus* have a similar wet density (1.204 g/ml) and water content (50.7%) but have a D_{100} value (124 min) that is much greater (3).

The four morphotype spores all appeared brightly refractile by phase-contrast light microscopy. Values for the average apparent refractive index ($\bar{n}$), determined with bovine serum albumin, for the four morphotype spores were unusually high, relative to their low heat resistance values (Table 1) and in comparison with those of other spore types (19). When determined with glucose, however, the $\bar{n}$s for the morphotype spores were more consistent with those of other spore types. For example, the native parent spores had a glucose $\bar{n}$ of 1.434 and a D_{100} value of 0.44 min; in comparison, spores of calcium-deficient *B. cereus* T have a glucose $\bar{n}$ of 1.450 and a D_{100} value of 1.00 min (19). The $\bar{n}$ determined with bovine serum albumin reflects the entire spore, whereas the $\bar{n}$ determined with glucose reflects only the structures beneath the outer membrane (see permeability results).

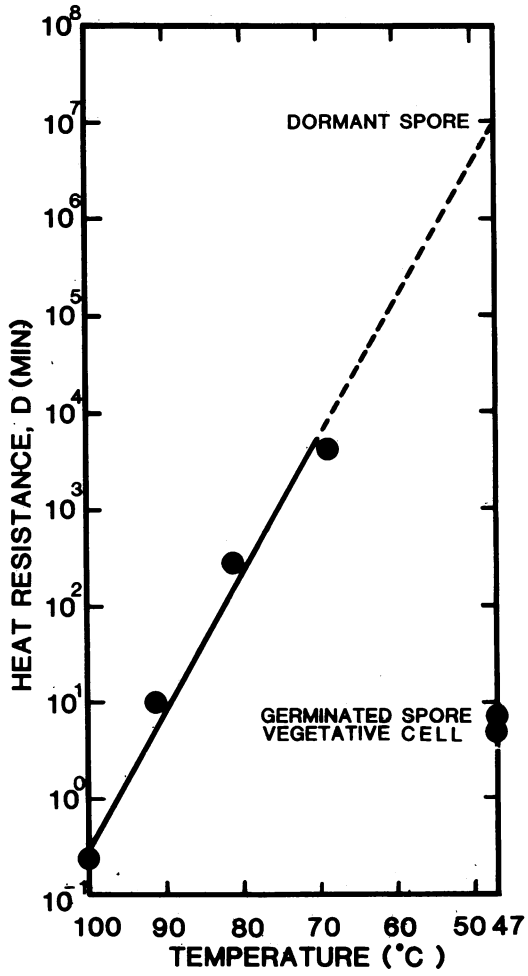

FIG. 6. Heat resistance of the dormant spore extrapolated to a D_{47} value (10^7 min = 19 years) compared with heat resistance of the germinated spore (D_{47}, 7 min) and the vegetative cell (D_{47}, 5 min), all of the parent strain.

The volume ratio of protoplast to protoplast plus cortex was determined for each of the four morphotype spores and correlated with the D_{100} values. The results are shown in

TABLE 1. Heat resistance, wet density, water content, and average apparent refractive index of morphotype spores

Morphotype spore	Heat resistance (min)		Wet density[a]	Water content[b]	Refractive index[c]	
	D_{90}	D_{100}			BSA	Glucose
Native parent	9.1	0.44	1.20	50	1.416	1.434
C^- OM^- parent	4.6	0.38[d]	1.20	58	1.421	1.432
EX^- variant	3.3	0.14[d]	1.29	32	1.405	1.440
EX^- C^- OM^- variant	0.91	0.033[d]	1.30	38	1.391	1.424

[a] Expressed as grams of wet spore per milliliter of wet spore.
[b] Obtained by gravimetric measurement; expressed as grams of water per 100 grams of wet spore.
[c] Determined with bovine serum albumin (BSA) and glucose.
[d] Obtained by least-squares extrapolation from measurements at 90, 80, and 70°C.

Fig. 7, together with previous results for five other types of spores (3). An exponential increase in heat resistance correlated with a decrease in the protoplast/protoplast-plus-cortex ratio over almost five decades of D_{100} values for the seven types of spores.

Concentrations of minerals and dipicolinic acid were determined in the native parent and EX⁻ variant spores (Table 2). Specific mineralization is a major determinant of heat resistance in spores (27a). Dipicolinate occurs in high concentrations, but its function remains uncertain. Both morphotype spores contained about 0.5 μmol of calcium per mg, and the ratio of calcium to dipicolinate was about 0.5. The magnesium content in the parent spore was higher than that in the variant spore, whereas the reverse was true for potassium. The two morphotype spores contained a total of 1.12 and 1.15 μeq of cations per mg (dry weight) of spores, respectively. Both morphotype spores contained similarly high amounts of dipicolinate (about 1 μmol/mg), equivalent to about 16% of the spore dry weight. A correlation of these constituents with heat resistance was not evident.

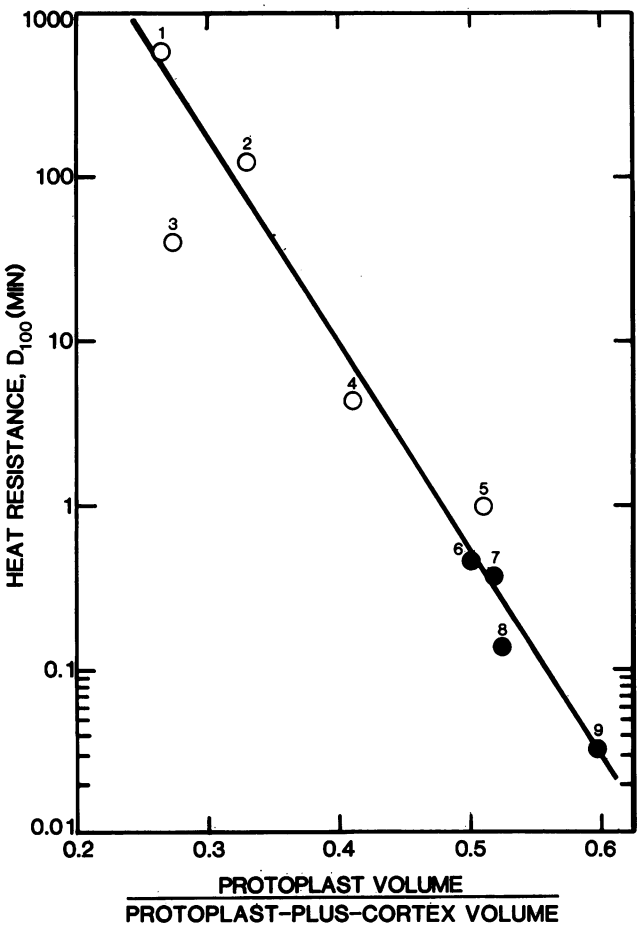

FIG. 7. Heat resistance of *B. megaterium* morphotype spores (●) and various other types of spores (○) (3) correlated by least-squares analysis with volume ratio of protoplast to protoplast plus cortex. The numbers correspond to the following types of spores: 1, *B. stearothermophilus* smooth; 2, *B. stearothermophilus* rough; 3, *B. subtilis* niger; 4, *B. cereus* T, high calcium; 5, *B. cereus* T. low calcium; 6, *B. megaterium* native parent; 7, *B. megaterium* C⁻ OM⁻ parent; 8, *B. megaterium* EX⁻ variant; 9, *B. megaterium* EX⁻ C⁻ OM⁻ variant.

TABLE 2. Mineral and dipicolinic acid contents of morphotype spores

Ion	Content (μmol/mg of spore [dry wt]) in:	
	Native parent	EX⁻ variant
K	0.18	0.34
Na	0.03	0.08
Ca	0.48	0.54
Mg	0.41	0.17
Mn	0.01	0.02
Dipicolinate[a]	0.91	1.01

[a] In the EX⁻ C⁻ OM⁻ variant, the dipicolinate content was 1.07 μmol/mg of spore (dry weight).

Dipicolinate content was also determined in the EX⁻ C⁻ OM⁻ variant spore (1.07 μmol/mg of spore [dry weight]). Because of the reduced dry-weight basis, this value was greater than that in the other two morphotype spores. Little dipicolinate was lost by chemical removal of the coat and outer membrane.

Germination. The four morphotype spores were examined for their responses to various germination-initiating agents after heat activation. The responses were more pronounced in phosphate (Table 3) than in Tris buffer (data not shown), but the patterns were similar. The native parent spore responded to glucose, leucine, proline, and glucose plus KNO_3, as was expected (31, 38). The EX⁻ variant spore, like several naturally occurring spores that lack exosporium (31), did not respond to these agents, but unlike the similar natural morphotype spores (31), also did not respond to alanine and inosine. This lack of response by the EX⁻ variant spore was not changed by chemical removal of the coat and outer membrane or by the addition of exosporium isolated from the C⁻ OM⁻ parent spore. Both the EX⁻ and EX⁻ C⁻ OM⁻ variant spores initiated germination (and grew out) in the complex of agents that are present in supplemented nutrient broth; however, 21 amino acids together, alone, or in various combinations were found to be ineffective (data not shown). Thus, the chemical germination requirements of the variant spore remain undefined.

The four morphotype spores were also examined for their response to lysozyme (Table 3), which can initiate germination only if it has access to the cortex. The enzyme then digests the cortex peptidoglycan, releasing its physical containment of the protoplast and causing the avalanche of

TABLE 3. Response of morphotype spores to germination-initiating agents in phosphate buffer

Agent	% Decrease in OD after 30 min in:			
	Parent		Variant	
	Native	C⁻ OM⁻	EX⁻	EX⁻ C⁻ OM⁻
None	11	4	12	5
D-Glucose	55	58	5	3
L-Leucine	46	18	4	3
L-Proline	52	20	4	2
D-Glucose + KNO_3	56	68	4	2
D-Glucose + L-leucine	56	68	4	3
L-Alanine	10	6	5	2
Inosine	13	5	5	5
L-Alanine + inosine	17	13	3	4
Sporulation nutrient broth	55	70	33	37
Lysozyme	3	41	18	42

events associated with germination. The native parent spore did not respond to lysozyme, as was expected; the C^- OM^- parent spore did so, however, because lysozyme had access to the cortex after penetrating the exosporium through apical openings. Likewise, the EX^- variant spore did not respond to lysozyme, but the EX^- C^- OM^- variant spore did so because lysozyme had direct access to the cortex. Consequently, it was evident that the complex of coat and outer membrane functioned to prevent the penetration of lysozyme into the cortex of the intact parent and variant spores. In the complex, the peripheral layer of the coat apparently functioned as the primary permeability barrier to lysozyme, on the basis of previous observations on macromolecular sieving by spores (22).

Permeability. The morphotype spores were also used to distinguish the roles of the outer and inner membranes in spore permeability to a model small molecule. Equilibrium permeability measurements were made with ^{14}C-labeled D-glucose, on the basis of both wet-spore weight ($R^w_{glucose}$) and wet-spore volume ($R^v_{glucose}$), and similarly with ^3H-labeled water (Table 4). Glucose might be thought to be unsuitable because of possible degradation by metabolism or uptake as a germinating agent; dormant spores are metabolically inactive, however, and germination did not occur under the restrictive conditions used in the permeability measurements. Furthermore, ^{14}C-labeled D-ribose and ^3H-labeled 2-deoxy-D-glucose, neither of which initiates germination or is metabolized after germination by another agent, gave the same results.

In the native parent spore, glucose penetrated 43% of the spore volume (Table 4), an observation consistent with penetration of the large amount of peripheral integument (exosporium and coat) on this morphotype (see Fig. 1). In the EX^- variant spore, glucose penetrated only 11% of the spore volume, an observation consistent with penetration of the small amount of peripheral coat layer on this morphotype (see Fig. 4). Thus it was the outer pericortex membrane, not the inner pericytoplasm membrane, that apparently functioned as the primary permeability barrier to glucose in these morphotype spores.

Indeed, when the outer membrane and coat were chemically divested from the parent and variant spores, glucose penetrated to a much greater extent of the spore volume (53% in the C^- OM^- parent and 28% in the EX^- C^- OM^- variant). In these morphotype spores, glucose apparently penetrated through the cortex to the inner membrane, which now served as the primary permeability barrier.

An effort was made to quantify these comparisons of permeability function with spore structure. The respective $R^v_{glucose}$ was converted to the glucose-impermeable percentage volume ($100 - R^v_{glucose}$) for comparison with the physical percentage volumes occupied by the protoplast and by the protoplast plus cortex as determined from electron microscopy measurements. The glucose-impermeable volume corresponded roughly with the physically measured volume of the protoplast plus cortex in the native parent and EX^- variant spores and with the volume of the protoplast in the C^- OM^- parent and EX^- C^- OM^- variant spores (Table 5). The glucose-impermeable volume was only roughly comparable with the physically measured volume, partly because of the inaccuracy in calculating volumes from measurements on electron micrographs, but mainly because the $R^v_{glucose}$ reflects only the volume occupied by water within a compartment, whereas the physically measured volume reflects the volume occupied by solids as well as water. Consequently, the $R^v_{glucose}$ underestimates and the glucose-impermeable value overestimates the corresponding physically measured value. For example, with the EX^- variant, the glucose-impermeable value, corrected so as to take into account the volume occupied by solids as well as water, was 81%, which corresponded much better with the physically measured volume of 72% for the sporoplast than with that of 38% for the protoplast. For the EX^- C^- OM^- variant, the corrected value was 56%, which corresponded much better with the physically measured volume of 60% for the protoplast than with that of 100% for the sporoplast.

Altogether, the results indicate that the outer pericortex membrane functioned as the primary permeability barrier to glucose in the native parent and EX^- variant spores, whereas the inner pericytoplasm membrane functioned as the permeability barrier to glucose in the C^- OM^- parent and EX^- C^- OM^- variant spores. The latter situation also occurs in two lysozyme-susceptible strains of *B. megaterium* spores in which the complex of coat and outer membrane is defective (T. C. Beaman, T. Koshikawa, H. S. Pankratz, and P. Gerhardt, FEMS Microbiol. Lett., in press). Furthermore, the results indicate that the exosporium in the parent spore and the coat peripheral to the outer membrane in the native parent or EX^- variant spores did not function as a permeability barrier to glucose.

The permeability measurements also provided an alternative method to the gravimetric method for determining water content, in that the R^w obtained with ^3H-labeled water is equivalent to the total water content of the spore on a wet-weight basis (8; Beaman et al., in press). The values of 58, 56, 36, and 40% obtained by the permeability method (Table 4) were essentially the same as the values of 50, 58, 32, and 38% obtained by the gravimetric method for total water contents of the four morphotype spores, respectively (Table 1). The greatest discrepancy between the two methods (58% versus 50%) occurred with the native parent spore, which was encumbered with the greatest amount of integument layers (Fig. 1).

TABLE 4. Permeability[a] of morphotype spores to [^{14}C]glucose and [^3H]water

Morphotype spore	R^w		R^v	
	Glucose	Water	Glucose	Water
Native parent	35	58	43	71
C^- OM^- parent	44	56	53	67
Ex^- variant	9	36	11	46
Ex^- C^- OM^- variant	21	40	28	51

[a] Milliliters per 100 g (R^w) or 100 ml (R^v) of wet spores.

DISCUSSION

Heat resistance. The divestment of integument layers exterior to the cortex had relatively little influence on heat resistance among the four morphotype spores. In comparison with the D_{47} value of the germinated spore, the extrapolated value of the dormant spore was changed by only about 0.001% by removal of the exosporium, coat, and outer membrane (Table 1). Such a cortex-encased protoplast retains heat resistance also in coatless mutant spores (10) and divested spores (27) of *Clostridium* species. However, neither others nor we have accomplished the isolation of free spore protoplasts devoid of cortex that retain dormancy and heat resistance, despite various efforts; indeed, this may be intrinsically impossible.

The relationship between heat resistance and water con-

TABLE 5. Comparison of volume percentage of the entire spore occupied by structural compartments within morphotype spores, determined from [^{14}C]glucose permeability and electron micrography measurements

Morphotype spore	% of entire spore vol		
	Glucose impermeable[a]	Protoplast + cortex[b]	Protoplast[b]
Native parent	57	56	28
C^- OM^- parent	47	66	34
Ex^- variant	89	72	38
EX^- C^- OM^- variant	72	100	60

[a] $100 - R^{v}_{glucose}$.
[b] Calculated from measurements made on electron micrographs of medially thin-sectioned spores.

tent among the four morphotype spores (Table 1) was complicated by two factors that changed as integument layers were successively divested: (i) the weight basis of the water content changed from an entire spore to a cortex-encased protoplast, and (ii) the protoplast volume increased in itself and as a percentage of the entire spore volume (Table 5). Furthermore, the native parent spore of this strain, paradoxically, had a low water content relative to its low heat resistance. Consequently, the values of heat resistance versus water content for the morphotype spores did not fit the correlation line for five other spore types (3).

Apparently, only the resistance parameters that reflect the spore protoplast and cortex are correlated with heat resistance. Thus, among the parameters studied, it was the volume ratio of protoplast to protoplast plus cortex that correlated with heat resistance among the four morphotype spores of *B. megaterium* (Fig. 7). These results were consistent with similar findings in a wide range of other spore species (1, 3, 24; J. E. Algie and L. S. Tisa, Spore Newslett. 7:20–21, 1981; A. D. Hitchens and R. A. Slepecky, Spore Newslett. 7:103–104, 1981).

The key parameter of spore resistance should be water content of the protoplast. We have now developed a method for determining protoplast water content by use of lysozyme-susceptible spores and shown that the protoplast water content in three such strains of *B. megaterium* spores is sufficiently low to account for their heat resistance (Beaman et al., in press).

Germination. The response of the four morphotype spores to various germination-initiating agents provided heuristic but incomplete evidence about the role of integument layers in the germination process. Glucose, leucine, and proline might be thought to react primarily in some way with the exosporium, inasmuch as the EX^- variant spore (Table 3) and naturally occurring EX^- spores (31) did not respond to these agents; however, the addition of isolated exosporium did not evoke a response to these agents by the EX^- variant spore. Involvement of the coat and outer membrane complex was also discounted, inasmuch as removal of the complex from the wild-type spore did not affect the response. Involvement of the inner membrane was also discounted, inasmuch as glucose (and therefore the other small germinant molecules) permeated as far as the inner membrane in the C^- OM^- parent spore but only as far as the outer membrane in the native parent spore (Table 4), yet both of these morphotype spores responded alike to the germinants. The germination-initiating response is dependent on heat activation, and possibly this might be thought to alter spore permeability. In *B. cereus* T spores, however, heat activation does not alter permeability to germinating agents (alanine and adenosine) or a nongerminating agent (glucose) (7).

Permeability. An outer pericortex membrane apparently exists in addition to the inner pericytoplasm membrane in intact dormant spores. In sectioned *B. megaterium* spores of either the native parent (Fig. 1) (4) or the EX^- variant (Fig. 4A), the outer membrane was identified as double-track dark lines complexed between coat layers. Sometimes the peripheral coat layer was separated from the outer membrane so that the membrane could be better distinguished (Fig. 4B). In the chemically divested spores, of course, the outer membrane and coat, of course, were no longer seen. Similarly, both structures are seen to be defective or absent in lysozyme-susceptible mutant spores (10; Beaman, et al., in press).

What we termed the outer membrane may correspond to what Aronson and Fitz-James (2) have termed a double-track or pitted coat layer in sectioned and freeze-etch preparations of *B. cereus* and *B. megaterium* spores. The ordered lattice appearance of the pitted layer surface is not like that usually seen in a bacterial membrane; however, the pitted layer in the dormant spore might be in a crystalline state and resemble the ordered lattice occasionally seen in protoplast membranes (5). Alternatively, the outer membrane may be a different structure than the pitted layer, complexed in the coat, and not seen by freeze-etching because it does not usually cause a fracture plane in the dormant spore (35). Whatever the explanation, the morphological evidence perhaps is less compelling than the biochemical and functional evidence for the existence of an outer membrane in the dormant spore.

Biochemical evidence suggesting the existence of an outer membrane in the dormant spore of *B. megaterium* has been presented and discussed by Crafts-Lighty and Ellar (12), mainly the occurrence of cytochromes, electron transport enzymes and polypeptides in isolated outer integument.

Functional evidence indicates that an outer membrane not only exists but is intact and serves as a permeability barrier in the dormant spore. Rode et al. (32) have shown that, in unfixed spores of *B. megaterium*, methacrylate solution permeates the coat but not beyond a sharply delineated boundary at the juncture of the coat with the cortex; however, the spore treated with a membrane-disrupting fixative no longer possesses this permeability barrier. Another line of evidence for the existence of an outer permeability barrier in the dormant spore has been provided by Carstensen et al. (9), whose dielectric measurements indicate that the cortex is surrounded by a thin outer membrane which insulates against the passage of mobile ions in an electric field. The third and most significant line of evidence for a functioning outer membrane has been provided by permeability studies with three species of spores in which the glucose-impermeable volume corresponds to the physical volume ocupied by the sporoplast (essentially the protoplast plus cortex) rather than by the protoplast alone (19). These findings were now confirmed by the permeability results with the native parent and EX^- variant spores of *B. megaterium* QM-B1551 (Table 5).

Altogether, the evidence thus indicates the existence in the dormant spore of an intact outer membrane (or possibly a membrane-like layer of the coat) functioning as a permeability barrier to small molecules. Only when the outer membrane is defective or removed does the inner membrane become the primary permeability barrier.

Exosporium morphotypes of *B. megaterium* spores. The parent and variant spores of strain QM-B1551 used in this study are representative of similar morphotype spores with

and without exosporium that occur naturally among *B. megaterium* strains. Recognition of the two different morphotype spores was made independently by two laboratories in 1959. Tomcsik and Baumann-Grace (37) used light microscopy to detect an extraperipheral structure (which they termed exosporium) in more than half of 36 strains, by use of negative staining and homologous spore antiserum. Fitz-James and Young (15) used electron microscopy to show that spores of two strains, but not two others, possess an extraperipheral structure (which they termed outer coat) which is thick, loose fitting, and shaped like the hull of an English walnut. This morphological distinction, based on the presence or absence of an extraperipheral structure, was confirmed by Rode (31) and now by us (Fig. 1 and 2A versus Fig. 4 and 2B). Also, Gibson and Gordon, in *Bergey's Manual* (23), recognized two types of *B. megaterium* spores distinguished by fuchsin staining the periphery or not; Fitz-James and Young (15) similarly had distinguished the two types by crystal-violet staining.

Subsequent investigation by Beaman et al. (4) revealed a third morphotype spore among the antiserum-reacting spores studied by Tomcsik and Baumann-Grace (37): the spores of strain Mg19 were shown by electron microscopy to contain a typical exosporium with a thick, hairlike nap, unlike the atypical exosporium of strain QM-B1551. Nadirova and Aleksandrushkina (30) also observed three different types of spore surfaces in 10 *B. megaterium* strains.

Altogether, three morphotypes of spores thus can be distinguished among *B. megaterium* strains: one type (exemplified by strain Mg19) contains a typical exosporium like that of *B. cereus*, a second type (exemplified by strain QM-B1551) contains an atypical exosporium, and a third type (exemplified by strain Texas and our variant strain) does not contain either type of exosporium.

Exosporium terminology. Evidence and historical precedence suggest that the extraperipheral structure on spores of *B. megaterium* strain QM-B1551 and similar morphotype strains should be termed exosporium rather than coat, hull, covering, or other nonspecific terms. Unlike coat, this atypical exosporium (as well as typical exosporium) is loose fitting, has apical openings, and is translucent when unstained (Fig. 1 and 2A). Exosporium differs chemically from coat in not being solubilized by alkaline sodium docecyl sulfate and dithiothreitol (Fig. 3) (2, 26), 1 N NaOH, or hot trichloroacetic acid (15). Furthermore, spores with exosporium are rich in phosphorus (15) and phospholipids (6), including diphosphatidylglycerol, which is the only phospholipid in exosporium isolated from *B. cereus* (28); in contrast, only homogeneous protein characterizes coat (2). Also, the outer peripheral layer (exosporium) differs from the inner layer (coat) of *B. megaterium* and *B. cereus* spores in the mineral matter remaining after microincineration (36).

Historically, de Bary in 1885 (13) first described and Flügge in 1886 (16) first used the term exosporium for the extraperipheral layer of bacterial spores. Tomcsik and Baumann-Grace in 1959 (37) kept the term exosporium for *B. megaterium* spores instead of inventing a new expression to avoid increasing the confusion in the nomenclature of the various spore layers. So now should we.

ACKNOWLEDGMENTS

This work was supported by contracts DAAG 29-80-C-005 and DAAG 29-83-K-0057 from the Biological Sciences Program of the U.S. Army Research Office.

We thank Michelle Diez and James C. Vary for help with the germination tests, Gary R. Bender for the mineral element analyses, Robert L. Gherna for the culture verification, and Robert E. Marquis and the reviewers for the *Journal of Bacteriology* for critical reading of the manuscript.

LITERATURE CITED

1. **Algie, J. E.** 1983. The heat resistance of bacterial spores and its relationship to the contraction of the forespore during sporulation. Curr. Microbiol. **9**:173–175.
2. **Aronson, A. I., and P. Fitz-James.** 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. **40**:360–402.
3. **Beaman, T. C., J. T. Greenamyre, T. R. Corner, H. S. Pankratz, and P. Gerhardt.** 1982. Bacterial spore heat resistance correlated with water content, wet density, and protoplast/sporoplast volume ratio. J. Bacteriol. **150**:870–877.
4. **Beaman, T. C., H. S. Pankratz, and P. Gerhardt.** 1972. Ultrastructure of the exosporium and underlying inclusions in spores of *Bacillus megaterium* strains. J. Bacteriol. **109**:1198–1209.
5. **Beaman, T. C., H. S. Pankratz, and P. Gerhardt.** 1974. Chemical composition and ultrastructure of native and reaggregated membranes from protoplasts of *Bacillus cereus*. J. Bacteriol. **117**:1335–1340.
6. **Bertsch, L. L., P. P. M. Bonsen, and A. Kornberg.** 1969. Biochemical studies of bacterial sporulation and germination. XIV. Phospholipids in *Bacillus megaterium*. J. Bacteriol. **98**:75–81.
7. **Black, S. H., and P. Gerhardt.** 1962. Permeability of bacterial spores. III. Permeation relative to germination. J. Bacteriol. **83**:301–308.
8. **Black, S. H., and P. Gerhardt.** 1962. Permeability of bacterial spores. IV. Water content, uptake, and distribution. J. Bacteriol. **83**:960–967.
9. **Carstensen, E. L., R. E. Marquis, S. Z. Child, and G. R. Bender.** 1979. Dielectric properties of native and decoated spores of *Bacillus megaterium*. J. Bacteriol. **140**:917–928.
10. **Cassier, M., and A. Ryter.** 1971. Sur un mutant de *Clostridium perfringens* donnant des spores sans tuniques a germination lysozyme-dépendante. Ann. Inst. Pasteur (Paris) **121**:717–732.
11. **Cessi, C., and F. Piliego.** 1960. The determination of amino sugars in the presence of amino acid and glucose. Biochem. J. **77**:508–510.
12. **Crafts-Lighty, A., and D. J. Ellar.** 1980. The structure and function of the spore outer membrane in dormant and germinating spores of *Bacillus megaterium*. J. Appl. Bacteriol. **48**:135–145.
13. **deBary, A.** 1885. Vorlesungen Bakterien. Verlag Wilhelm Engelmann, Leipzig, Germany.
14. **Fitz-James, P. C.** 1971. Formation of protoplasts from resting spores. J. Bacteriol. **105**:1119–1136.
15. **Fitz-James, P. C., and I. E. Young.** 1959. Cytological comparison of spores of different strains of *Bacillus megaterium*. J. Bacteriol. **78**:755–764.
16. **Flügge, C.** 1886. Die Mikroorganismen, 2nd ed. Verlag F.C.W. Vogel, Leipzig.
17. **Freer, J. H., and H. S. Levinson.** 1967. Fine structure of *Bacillus megaterium* during microcycle sporogenesis. J. Bacteriol. **94**:441–457.
18. **Gerhardt, P.** 1967. Cytology of *Bacillus anthracis*. Fed. Proc. **26**:1504–1517.
19. **Gerhardt, P., T. C. Beaman, T. R. Corner, J. T. Greenamyre, and L. S. Tisa.** 1982. Photometric immersion refractometry of bacterial spores. J. Bacteriol. **150**:643–648.
20. **Gerhardt, P., H. S. Pankratz, and R. Scherrer.** 1976. Fine structure of the *Bacillus thuringiensis* spore. Appl. Environ. Microbiol. **32**:438–440.
21. **Gerhardt, P., and E. Ribi.** 1964. Ultrastructure of the exosporium enveloping spores of *Bacillus cereus*. J. Bacteriol. **88**:1774–1789.
22. **Gerhardt, P., R. Scherrer, and S. H. Black.** 1972. Molecular sieving by dormant spore structures, p. 68–74. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
23. **Gibson, T., and R. E. Gordon.** 1974. Genus I. *Bacillus* Cohn 1872, p. 529–550. *In* R. E. Buchanan and N. E. Gibbons (ed.),

Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.

24. **Hyun, H. H., J. G. Zeikus, R. Longin, J. Millet, and A. Ryter.** 1983. Ultrastructure and extreme heat resistance of spores from thermophilic *Clostridium* species. J. Bacteriol. **156:**1332–1337.
25. **Janssen, F. W., A. J. Lund, and L. E. Anderson.** 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127:**26–27.
26. **Kawasaki, C., T. Nishihara, and M. Kondo.** 1969. Ultrastructure and its relation to the fractions isolated from spore coat of *Bacillus megaterium*. J. Bacteriol. **97:**944–946.
27. **Labbe, R. G., R. R. Reich, and C. L. Duncan.** 1978. Alteration in ultrastructure and germination of *Clostridium perfringens* type A spores following extraction of spore coats. Can. J. Microbiol. **24:**1526–1536.

27a. **Marquis, R. E., E. L. Carstenson, G. R. Bender, and S. Z. Child.** 1984. Physiological Biophysics of spores, p. 227–240. *In* G.-J. Dring, D. J. Ellar, and G. W. Gould (ed.), Fundamental and applied aspects of bacterial spores. Academic Press, Inc., London.

28. **Matz, L. L., T. C. Beaman, and P. Gerhardt.** 1970. Chemical composition of exosporium from spores of *Bacillus cereus*. J. Bacteriol. **101:**196–201.
29. **Moberly, B. J., F. Shafa, and P. Gerhardt.** 1966. Structural details of anthrax spores during stages of transformation into vegetative cells. J. Bacteriol. **92:**220–228.
30. **Nadirova, I. M., and N. I. Aleksandrushkina.** 1979. The structure of spore surfaces and morphological and physiological properties of some *Bacillus megaterium* strains. Izv. Akad. NaukKaz. SSR Biol. Nauk **1:**88–94.
31. **Rode, L. J.** 1968. Correlation between spore structure and spore properties in *Bacillus megaterium*. J. Bacteriol. **95:**1979–1986.
32. **Rode, L. J., C. W. Lewis, and J. W. Foster.** 1962. Electron microscopy of spores of *Bacillus megaterium* with special reference to the effects of fixation and thin sectioning. J. Cell Biol. **13:**423–435.
33. **Setlow, P., and A. Kornberg.** 1969. Biochemical studies of bacterial sporulation and germination. XVII. Sulfhydryl and disulfide levels in dormancy and germination. J. Bacteriol. **100:**1155–1160.
34. **Shay, L. K., and J. C. Vary.** 1978. Biochemical studies on glucose initiated germination in *Bacillus megaterium*. Biochim. Biophys. Acta **538:**284–292.
35. **Stelma, G. N., Jr., A. I. Aronson, and P. C. Fitz-James.** 1980. A *Bacillus cereus* mutant defective in coat desposition. J. Gen. Microbiol. **116:**173–185.
36. **Thomas, R. S.** 1964. Ultrastructural localization of mineral matter in bacterial spores by microincineration. J. Cell Biol. **23:**113–133.
37. **Tomcsik, J., and J. B. Baumann-Grace.** 1959. Specific exosporium reaction of *Bacillus megaterium*. J. Gen. Microbiol. **21:**666–675.
38. **Vary, J. C.** 1972. Spore germination of *Bacillus megaterium* QM B1551 mutants. J. Bacteriol. **112:**640–642.
39. **Vary, J. C.** 1973. Germination of *Bacillus megaterium* spores after various extraction procedures. J. Bacteriol. **116:**797–802.