JOURNAL OF BACTERIOLOGY, June 1968, p. 1979-1986
Vol. 95, No. 6

Correlation Between Spore Structure and Spore Properties in *Bacillus megaterium*

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Received for publication 19 February 1968

The spores of six strains of *Bacillus megaterium* were divided into two distinct groups on the basis of germination. Three of the strains germinated in a mixture of L-alanine and inosine (AL type spores), and three strains germinated in a mixture of glucose and potassium nitrate (GN type spores); recriprocal germination in the respective solutions did not occur. The AL spores and the GN spores were morphologically distinct. Other differences between the two spore groups included germination inhibition characteristics, dipicolinic acid content, hexosamine content, phosphorus and magnesium content, spore coat features, ion exchange properties, and heat resistance. A correlation appears to exist between spore morphology and certain other spore properties in strains of *B. megaterium*.

Two distinct spore germination types have been described in the literature for *Bacillus megaterium*. One type, exemplified by *B. megaterium* QM B1551, responds germinatively to glucose and to a variety of inorganic salts, including potassium nitrate (12, 30). A second type, exemplified by *B. megaterium* ATCC 19213 (formerly designated "Texas" strain), responds germinatively to L-alanine and adenosine or inosine (29, 31).

Two distinct morphological types of *B. megaterium* spores have also been described (8). One type, exemplified by *B. megaterium* strain Penn, is characterized by a prominent equatorial fold or ridge (8); this is characteristic also of *B. megaterium* QM B1551 (20, 35). The second type, exemplified by *B. megaterium* ATCC 19213, lacks the prominent equatorial fold and presents a veined, beaded spore coat surface (35).

There exist in *B. megaterium*, therefore, at least two distinct morphological types and two distinct spore germination types. In the present work, we have examined spores of six strains of *B. megaterium* which were selected for study on the basis of germination properties. Three strains belonged to the glucose-nitrate germination type, and three strains belonged to the alanine-inosine germination type. A correlation is shown to exist in these six strains of *B. megaterium* between spore morphology on the one hand and spore germination characteristics and certain spore properties on the other hand.

MATERIALS AND METHODS

B. megaterium strains. Strain "Starka" (40) was obtained from J. Starka, Charles University, Prague, Czechoslovakia; strain "Penn" (8) from P. C. Fitz-James, University of Western Ontario, London, Canada; and strain QM B1551 (12) from H. S. Levinson, U.S. Army Natick Laboratories, Natick, Mass. The remaining three strains bear ATCC numbers and were obtained from the American Type Culture Collection, Rockville, Md.

Spore production. Spores of the six strains were produced on the same agar medium according to the methods described previously (27). Incubation was for 40 hr at 30 C for each strain. Harvested spores were washed seven times with large volumes of cold demineralized water, lyophilized, and stored in desiccators over calcium chloride. The preparations were essentially devoid of vegetative cells and dark (germinated) spores.

Spore germination. Spores in demineralized water were heated for 60 min at 60 C prior to germination tests (4). Such "heat-shocked" suspensions were maintained at 4 C when they were not in use. Spores were added to freshly prepared nonsterile germination solutions (1 mg of spores per 5 ml of germination solution). Germination was evaluated by Powell's (26) optical density (OD) reduction method with the aid of a Klett-Summerson photoelectric colorimeter with a no. 54 filter. Initial readings were 150 to 200 Klett units. Germination data are presented as percentages of reduction of OD with the initial reading taken as 100%. Germination inhibitors were added in appropriate concentrations to suspensions of spores in solutions containing the germinants appropriate for the respective spore types.

Electron microscopy. Following the lead of Franklin and Bradley (9) and others (8, 35), appraisal of spore morphology was limited to the examination of carbon replicas of the spore surfaces. Details of the specific method are given in a previous publication (35). Shadowing was with platinum at an angle of approximately 35°, and the cellular material was

dissolved from the carbon replicas with 0.5% sodium hypochlorite. Specimens were viewed with a Hitachi HS-7S electron microscope with double condenser and at a 50-kv accelerating voltage. Micrographs were taken on contrast grade Kodak projector slides.

Spore fractions. Spore integuments (coats) were prepared by mechanical rupture of spores in the Nossal disintegrator (24). A rubber-cushioned glass tube, positioned within the customary metal breakage cartridge, was loaded with 50 mg of spores, 7 ml of water, 4 g of no. 16-220 glass beads (VirTis Co., Inc., Gardiner, N.Y.), and 3 drops of octanol. Eight breakage runs, each 30 sec with interspersed ice bath chilling to control temperature rise, sufficed for complete spore breakage. The coats, recovered by 10-min centrifugation at 10,000 × *g*, were washed exhaustively with water and incubated overnight in tris(hydroxymethyl)aminomethane (Tris) buffer, *p*H 7.2, containing 50 μg of lysozyme per ml. After further washing, these "coat" preparations were employed for chemical analyses.

Guanine plus cytosine (GC) content. For extraction of deoxyribonucleic acid (DNA) from spores, a modification of the dry spore breakage method (37) was employed to minimize depolymerization of DNA. Lyophilized spores and 4 g of glass beads were loaded into the breakage tube. Ten treatments in the Nossal disintegrator, each of 15-sec duration, sufficed for good spore breakage. DNA was isolated from the broken spores according to the method of Marmur (21), and determinations of mean densities followed the procedure of Schildkraut, Marmur, and Doty (38).

Chemical analyses. Dipicolinic acid was determined according to Janssen, Lund, and Anderson (14); total phosphorus determinations were by the method of Fiske and Subbarow (7); hexosamine was determined by a modification (13) of the method of Elson and Morgan (6). Magnesium was determined by atomic absorption spectrophotometry employing the Evans model EEL-140 atomic absorption spectrophotometer (Fisher Scientific Co., Pittsburgh, Pa.). Both ashed and untreated spore samples were analyzed at 285 mμ with 6.5 mg of $LaCl_3$ per ml present to suppress phosphate interference.

Titrations. Titrations of spore suspensions with dilute nitric acid to *p*H 4.0 end point were carried out according to methods described previously (1, 2, 33). The spores, 100 mg in 20 ml of demineralized water, were kept in suspension with a magnetic stirrer during the 3-hr titration. Acid additions were performed manually.

RESULTS

Selection of strains. The six strains chosen for study fell into two distinct germination types (Table 1). Three strains germinated in a mixture of L-alanine and inosine (AL strains), and three strains germinated in a mixture of glucose and KNO_3 (GN strains); reciprocal germination in the respective solutions did not occur during the 60-min incubation period employed.

Spore morphology by carbon replication. The six strains fell into two distinct morphological types (Fig. 1). The three AL strains exhibited a veined surface with indications of a superficial beaded ultrastructure pattern (35). In contrast, the three GN strains exhibited the well-known prominent equatorial ridge and polar knob appearance (8, 20, 35). A separation of the six spore strains into two discrete morphological types and a correlation between this morphological character (Fig. 1) and the germination behavior (Table 1) are apparent.

TABLE 1. *Bacillus megaterium strain designations and spore germination characteristics*

Strain	Strain designation[a]	Germination response[b]	
		In L-alanine-inosine mixture	In glucose-nitrate mixture
B. megaterium ATCC 19213	1AL	+	−
B. megaterium strain Starka	2AL	+	−
B. megaterium ATCC 7051	3AL	+	−
B. megaterium QM B1551	1GN	−	+
B. megaterium strain Penn	2GN	−	+
B. megaterium ATCC 12872	3GN	−	+

[a] These designations are employed to identify the strains throughout the remainder of this report.

[b] Germination measured after incubation for 60 min at 37 C in the germination solutions (concentrations listed in Table 3). Germination (+) criteria were a reduced OD of spore suspensions and the presence of dark spores in preparations viewed with the phase-contrast microscope.

Consistent morphological differences between the two spore types were noted upon germination. This is illustrated in Fig. 2 which contrasts the appearance of strain 2AL and strain 2GN germinated spores. Germination of AL strain spores invariably resulted in a loss of spore rigidity and a collapse and flaccidity of the spore coat without obvious coat rupture. The coats of GN strain spores, on the other hand, ruptured upon germination presumably along the equatorial ridge in characteristic cleavage fashion, as described previously (20).

Identity of strains as B. megaterium. Because of the striking differences noted between the AL strain spores and GN strain spores, with respect both to morphology and germination characteristics, the identity of the six strains as authentic *B. megaterium* was substantiated by conventional

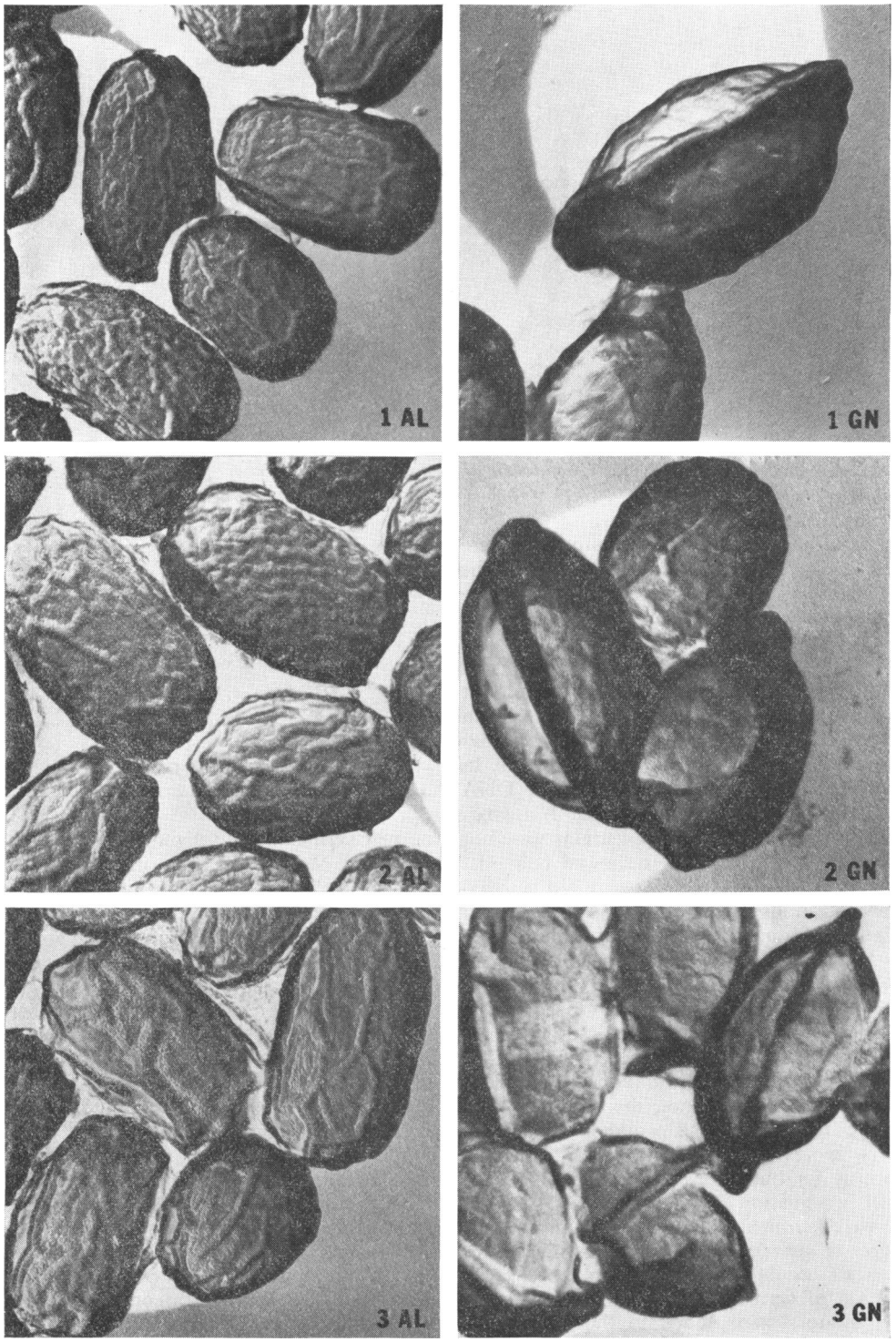

FIG. 1. *Carbon replicas of spores of the six strains of Bacillus megaterium. Note the contrasting appearance of alanine-inosine germination type spores (1AL, 2AL, 3AL) and glucose-nitrate germination type spores (1GN, 2GN, 3GN).* × *30,000.*

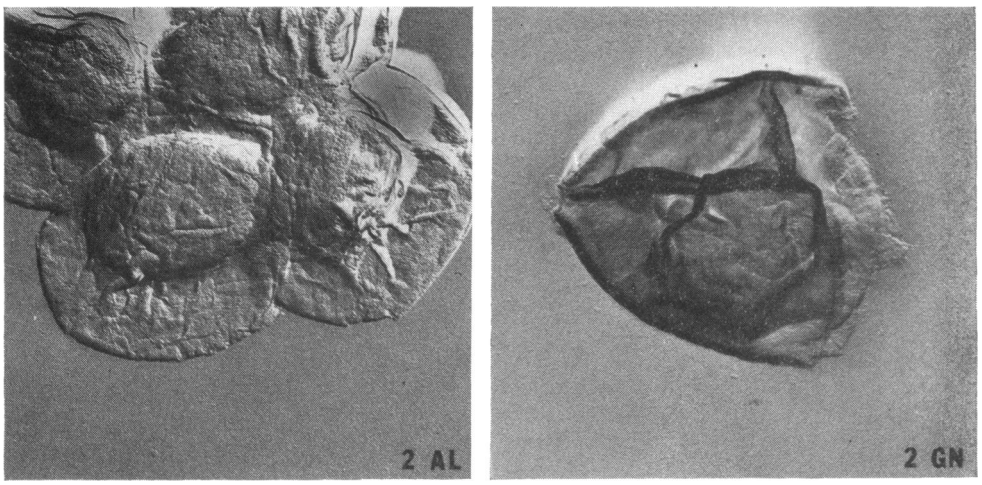

FIG. 2. *Germinated spores, replicas. Contrasting appearance of an alanine-inosine germination type spore (2AL) and a glucose-nitrate germination type spore (2GN).* × *21,000.*

means. For all six strains the vegetative rods were typical in size and shape for *B. megaterium*; the size and shape and position of the spores were characteristic; the cells exhibited a vacuolated cytoplasm when grown on glucose-agar; acid production from glucose, mannitol, xylose, and arabinose was noted; the Voges-Proskauer test was negative; and growth did not occur in an anaerobic environment. A culture of *B. cereus* was used as a control for the preceding tests. In addition, GC contents of the six spore DNA preparations were determined (Table 2). Significant differences between the six strains were not noted. All six DNA samples showed unimodal distributions, and satellites (5) were not detected at concentrations as high as 4 μg of DNA per gradient. We conclude that the six strains are all authentic *B. megaterium*, as judged by conventional taxonomic criteria.

Spore germination differences. Germination differences between AL and GN strain spores were consistent for the two types (Table 3). The GN spores germinated also in aqueous solutions of *n*-butane and *n*-pentanol; the AL spores did not (32). This butane and pentanol germination of GN spores was "fractional," as described previously for butane germination (32) and for "ionic" germination (30). The AL and GN spores alike were germinated by *n*-dodecylamine (28).

Effect of germination inhibitors. A division of the six strains into two groups on the basis of inhibition of germination was noted (Table 4). The inhibitors were added to the appropriate germination solutions for the two respective spore types. Thus, ethyl alcohol, phenethyl alcohol (39), *n*-pentanol (32), and *n*-butane (32) inhibited normal germination in alanine-inosine solutions of all AL strains; the same compounds, under comparable conditions, exerted no inhibitory effect on germination of GN spores in glucose-nitrate mixtures. A separation of the six strains into two types on the basis of germination inhibition is demonstrated.

TABLE 2. *Mean density values and computed GC contents of spore DNA samples*

DNA source (strain)	Mean density (g/ml)	GC (moles %)
1AL	1.6975	38.3
2AL	1.6985	39.3
3AL	1.698	38.8
1GN	1.699	39.8
2GN	1.698	38.8
3GN	1.6985	39.3

Chemical differences. Quantitative chemical differences were observed between the two classes of spores (Table 5). Individually and as a group the AL strain spores contained significantly more dipicolinic acid, less hexosamine (used here as an indicator of mucopeptide content), and markedly lower contents of phosphorus and magnesium.

Differences also in the isolated spore coats of the two classes of spores were noted (Table 6). The AL strain spore coats comprised from 25 to 34% of the spore dry weight, whereas GN strain coats comprised up to 46% of the spore weight. Coats of GN strain spores contained up to 5% total phosphorus, whereas AL strain spores contained less than half that amount (8, 16). Lysozyme was effective in solubilizing virtually all coat attached cortical mucopeptide (hexosamine)

TABLE 3. *Germination response of the two classes of spores*

Germination solution[a]	Germination (% reduction of OD)[b]					
	Strain			Strain		
	1AL	2AL	3AL	1GN	2GN	3GN
Demineralized water	1	2	0	6	3	2
L-Alanine, inosine	64	72	70	6	6	6
Glucose, KNO_3	3	3	0	68	67	73
n-Butane[c]	0	0	0	47	32	23
n-Pentanol[c]	3	1	1	46	39	24
n-Dodecylamine	78	73	76	71	69	76

[a] L-Alanine and inosine, each 1 mM in 33 mM phosphate buffer, *p*H 8; glucose and KNO_3, each 20 mM in water; *n*-butane, 1 atm, and *n*-pentanol, 0.1%, both in water; and *n*-dodecylamine, 0.06 mM in 16 mM phosphate buffer, *p*H 8.

[b] Reductions in OD of spore suspensions were determined after 60 min of incubation at 37 C; 65 to 75% reduction indicates germination of essentially all spores.

[c] *n*-Butane and *n*-pentanol, which do not induce germination of AL type spores, are in fact inhibitors of "normal" germination of AL type spores (*see* Table 4).

TABLE 4. *Effect of inhibitors on germination of the two classes of spores*

Inhibitor[a]	Germination (% reduction of OD)[b]					
	Strain			Strain		
	1AL	2AL	3AL	1GN	2GN	3GN
None	66	70	72	70	69	73
n-Butane	0	0	0	71	66	76
n-Pentanol	0	0	0	69	64	65
Ethyl alcohol	2	1	1	71	65	75
Phenethyl alcohol	10	18	10	76	70	82

[a] *n*-Butane, 1 atm; *n*-pentanol, 0.1%; ethyl alcohol, 5%; and phenethyl alcohol, 0.06%.

[b] Germination solutions: for AL strains, L-alanine and inosine, each 1 mM in 33 mM phosphate buffer, *p*H 8; for GN strains, glucose and KNO_3, each 20 mM in water. Incubation for 60 min at 37 C.

of AL strain spores, whereas GN strain spores retained significant amounts of hexosamine (up to 3.36% following lysozyme treatment; 16, 43).

Ion exchange properties. It is known that spores may function as ion exchange bodies (1, 33) and that the ionic state of the spore may affect its heat resistance (2) and germination response (33, 36). Based on titration data, employing 0.01 N nitric acid and titration of spore suspensions (100 mg of spores in 20 ml of water) to a *p*H 4.0 end point, the six spore strains were again separable into the two distinct spore classes (Fig. 3). The GN strain spores were characterized by a marked initial consumption of acid (Fig. 3), approximately five times that required by AL strain spores. After the initial rapid rate, spores of all strains exhibited a low, diminishing rate of acid consumption (quantity of acid required to maintain *p*H 4.0 during a 3-hr titration). These data suggest the presence of a greater number of available binding sites for H^+ and the presence of more loosely bound exchangeable cations (e.g., Ca^{++}; 34) on GN spores than on AL spores.

Spore resistance to heat. Since it is known that spore ionic load affects heat resistance (2), the resistance of the six spore strains was examined. Spores were suspended in water and exposed to 80 C in a water bath. Periodically, samples were plated on nutrient agar to assess spore viability. GN spores were significantly more resistant to heat than AL spores (Table 7).

TABLE 5. *Chemical composition differences between the two classes of spores*

Strain	Composition (%)[a]			
	Dipicolinic acid	Hexosamine	Phosphorus	Magnesium
1AL	13.2	4.0	1.3	0.26
2AL	12.2	4.7	1.4	0.29
3AL	11.8	4.5	1.5	0.28
1GN	9.1	6.2	2.9	0.70
2GN	8.7	6.7	2.6	0.76
3GN	8.6	5.6	2.4	0.67

[a] Percentage of spore dry weight.

TABLE 6. *Integument (coat) composition differences between the two classes of spores*[a]

Strain	Integument wt (% of whole spore)	Integument content	
		Total phosphorus (%)	Hexosamine (%)
1AL	25	1.64	0.32
2AL	28	2.02	0.28
3AL	34	2.21	0.37
1GN	46	5.01	2.47
2GN	43	4.66	3.36
3GN	37	4.45	3.02

[a] The isolated integuments were incubated overnight in Tris buffer, *p*H 7.2, containing 50 μg of lysozyme per ml prior to the determinations indicated above.

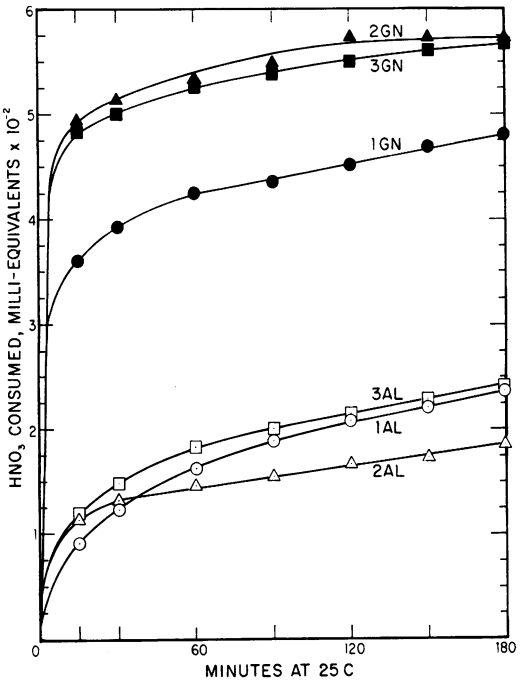

FIG. 3. *Titration of suspensions of spores of six strains of Bacillus megaterium. For each strain the quantity of 0.01 N HNO_3 required to adjust and maintain the spore suspension (100 mg of spores in 20 ml of water) at pH 4.0 is plotted on the vertical axis.*

TABLE 7. *Heat resistance of the two classes of spores*

Strain	Survivors (% of original population)[a]	
	20 min	30 min
1AL	9	6
2AL	21	12
3AL	2	1
1GN	92	86
2GN	97	70
3GN	70	45

[a] Pasteurized (20 min at 65 C) spore suspensions in demineralized water were exposed to 80 C for the indicated intervals. Survivors were determined by prompt plating of appropriate dilutions on the surface of nutrient agar plates. Colonies were counted after 24 hr of incubation at 37 C.

DISCUSSION

The data presented indicate that for the six strains of *B. megaterium* examined a correlation exists between spore morphology and other spore properties, such as germination behavior, heat resistance, chemical composition, and ion exchange characteristics. Although the existence of *B. megaterium* spores differing in morphology (8, 20, 35), in germination behavior (12, 30, 31), in phosphorus content (8, 16, 18), and in metal cation content (42) has been known for some time, this is a first attempt to establish a general relationship between spore surface structure and other spore properties within this species.

Selection of particular parameters for investigation in this work was based on previously demonstrated relationships. For example, relationships between dipicolinic acid and heat resistance (19, 23), germination response (18), and degree of dormancy (15) have been reported. An involvement of spore lytic enzyme (41) and cortical mucopeptide (10, 43) in the germination process seems well established; a relationship between hexosamine content and heat resistance has been reported (2); spores of various species differing in cation content, particularly Mg^{++} to Ca^{++} ratios, exhibit significantly different heat resistance properties (23); exchangeable ionic load may influence germination (33, 36) and heat resistance (1, 2). These, then, are instances wherein chemical properties of spores have been related to certain spore functions.

Specifically, the data presented in this paper were acquired in connection with a broad approach which hopefully may provide some understanding of the prime germination event. Subtle or pronounced differences in spores of strains of a single species might be expected to provide clues with respect to germination sites and mechanisms. Perhaps, attention should now be paid to the respective spore cortical mucopeptides. Germination (22) and germination-like (10) responses of spores to lysozyme indicate the involvement of the cortex in germination. If the cortex is in fact the primary site of action of germinants, one might anticipate that detailed examination of the respective mucopeptides from AL and GN type spores would reveal chemical differences. An immunological approach designed to detect such differences may be feasible, since it is known that cell wall peptide (polymer) of *Staphylococcus aureus* is immunologically active (11).

Intimate details of spore morphology and even prominent spore anatomical features now play a negligible role in taxonomy of *Bacillus* and *Clostridium* (3). Although the morphological differences reported here in diverse strains of *B. megaterium* seem pronounced, they are, in fact, minor when compared to differences that are now known to exist for spores of certain *Clostridium* species. For example, five grossly dissimilar spore morphological types have been reported for *Clostridium bifermentans* (25, 44). It has been advo-

cated that pronounced, distinctive spore morphological features should be considered species specific, and new species of *Clostridium* have, in fact, been described on this basis (17). Whether or not this is justified may be debatable, but at the very least it would appear desirable to take into account pronounced spore anatomical features for comprehensive characterization of present species.

ACKNOWLEDGMENTS

The generosity of Manley Mandel, The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, in determining the GC contents and that of Leodocia Pope for the electron microscopy is gratefully acknowledged. The able assistance of Margaret A. Crawford and Diane P. Yolton is also acknowledged.

This investigation was supported by Public Health Service grant AI-07582 from the National Institute of Allergy and Infectious Diseases and by Contract Nonr 375 (12) from the Office of Naval Research.

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