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Relationship Between Cortex Content and Properties of *Bacillus sphaericus* Spores

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The muramic lactam content of spores of *Bacillus sphaericus* mutants defective in *meso*-diaminopimelic acid synthesis increased almost linearly with an increase of *meso*-diaminopimelic acid concentration in the medium. Since muramic lactam content is a measure of cortex content, the amount of cortex in spores of the mutants can be easily varied by changing the *meso*-diaminopimelic acid concentration in the medium. Characteristic properties were tested in spores containing different amounts of cortex. Critical amounts of cortex were associated with different spore properties. Refractility and dipicolinic acid accumulation in the spores both required about 20% of the maximum cortex content (although refractility is independent of dipicolinic acid content). For xylene and octanol resistance, about 25% of the maximum cortex content was required. However, for heat resistance, more than 90% of the maximum cortex content was required; heat resistance was, therefore, more closely related to the amount of cortex than to the amount of dipicolinic acid in the spore. Furthermore, for accumulation of Ca^{2+} in the spore, the existence of both cortex and dipicolinic acid in the spore was essential.

The bacterial spore has two peptidoglycan layers. One is a thin inner layer called the germ cell wall, which has a role at the time of germination of the spore (5). The other layer is called cortex and occupies about a half of the spore volume. Much data suggest that characteristic spore properties, such as refractility and heat resistance, might result from a low water content in the spore cytoplasm (dehydrated cytoplasm). The strength and elasticity of the cortex peptidoglycan might have an important role in producing and maintaining these characteristic spore properties (1).

In a previous paper (Y. Imae and J. L. Strominger, J. Biol. Chem., in press), the isolation of conditional spore cortexless mutants of *Bacillus sphaericus* was reported, and some properties of these mutants were examined. It was shown that assay of muramic lactam content of sporulating cells is the best quantitative measure of spore cortex content (6; Imae and Strominger, manuscript in preparation). In the present paper, data will be presented which show that there are critical amounts of cortex required for different properties of the spores.

MATERIALS AND METHODS

Chemicals. [^{3}H]$NaBH_4$ (500 mCi/96 mg) and $^{45}CaCl_2$ (13.0 mCi/mg) were obtained from New England Nuclear Corp., Boston, Mass. DL-Diaminopimelic acid (DL-Dpm; mixture of DD-, LL-, and *meso*-isomers) was obtained from Sigma Chemical Co., St. Louis, Mo. Xylene (mixture of *ortho*-, *meta*-, and *para*-isomers) and 1-octanol were purchased from Fisher Scientific Co., Fair Lawn, N.J.

Bacterial strain. L-Lysine-requiring mutants of *B. sphaericus* 9602 were described in a previous paper (Imae and Strominger, in press). A group II mutant (mutant 32-3) has only a defect in *meso*-Dpm synthesis and forms normal spores in its presence. A group III mutant (mutant 21-1), which is blocked earlier in the L-lysine pathway, is defective in both *meso*-Dpm and dipicolinic acid (DPA) syntheses; it forms abnormal spores in the presence of Dpm alone and normal spores in the presence of Dpm and DPA.

Growth of bacteria. Mutants were grown in SPL medium consisting of SP medium (Imae and Strominger, in press) plus 100 μg of L-lysine per ml. DL-Dpm was added to the culture, if necessary. Most of the bacterial growth described in this paper was carried out in test tubes containing 3 ml of medium.

Assay of ^{45}Ca accumulation in the sporulating cells. Ca^{2+} content in SP medium is roughly 1 mM. For the assay of ^{45}Ca accumulation, cells were grown in the presence of ^{45}Ca at a final concentration of 0.45 μCi/μmol per ml. After cultivation, 0.1-ml aliquots of culture were added to 1.0 ml of ice-cold 5% trichloroacetic acid, and the solution was kept for 10 min in ice. Cells were collected on a membrane filter (Millipore Corp., HA, 0.45 μm) and washed three times with 5 ml of ice-cold 5% trichloroacetic acid. After drying under infrared light, radioactivity was measured in a toluene-based scintillator.

Other methods. Muramic lactam content and

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DPA content were measured as previously described (Imae and Strominger, in press). Xylene resistance and 1-octanol resistance were measured after exposure to 10% solvent for 10 min at 22 C after vigorous shaking in a Vortex mixer. Cells were then diluted with medium and plated on SPL medium agar plates. Heat treatment was carried out at 80 C for 10 min.

RESULTS

Dpm concentration in the medium and cortex content of the mutant spores. As reported previously (Imae and Strominger, in press), group II and group III mutants of *B. sphaericus*, which are defective in *meso*-Dpm synthesis, require *meso*-Dpm in the medium to produce cortex-containing spores. To know the relationship between DL-Dpm concentration in the medium and cortex content of the spore (measured by muramic lactam content), group II and group III mutants were grown at 30 C in various amounts of DL-Dpm in the medium for about 17 h (just before the release of free spores). This time point was selected to optimize the cortex content of the sporulating cells because, as soon as free spores are released, some fraction of the spores begin to germinate (D. J. Tipper, personal communication), resulting in a decrease in cortex content of the culture.

Muramic lactam content of the sporulating cells of both mutants increased almost linearly with the increase of DL-Dpm concentration in the medium to more than 1,000 μg/ml (Fig. 1). These data are most probably explained by the idea that *B. sphaericus* does not have a transport system for Dpm and that the rate-limiting step for cortex formation must be the permeation of Dpm. Therefore, the cortex content of the mutant spores can be easily controlled simply by varying Dpm concentration in the medium.

Effect of cortex content on characteristic spore properties. Sporulation of a group II mutant of *B. sphaericus* was carried out in the presence of various concentrations of DL-Dpm in the medium. After about 17 h at 30 C, characteristic spore properties were analyzed. The relationship between DL-Dpm concentration in the medium and percentage of refractile spores or DPA accumulation in the spore is shown in Fig. 2. The percentage of refractile spores among total spores increased dramatically at about 200 μg of DL-Dpm per ml in the medium. At about 400 μg/ml, almost all of the spores produced were completely refractile. At a lower concentration of Dpm, some fraction of the total spore was completely refractile, but some were still nonrefractile. The difference between re-

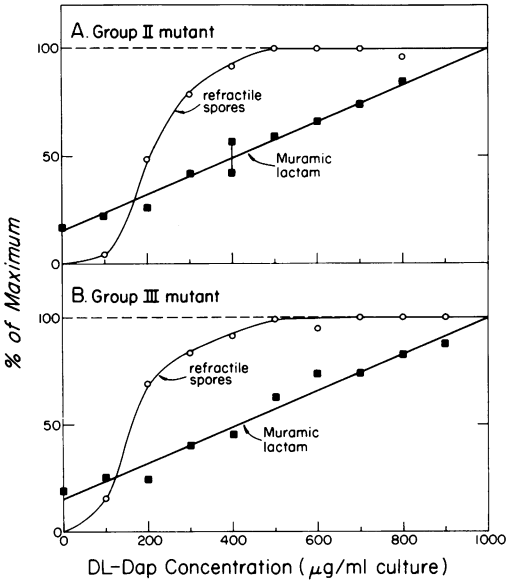

FIG. 1. *Relationship between DL-Dpm (i.e., Dap) concentration in the culture medium and muramic lactam content of sporulating cells of B. sphaericus. Cells were grown in SPL medium at 30 C for 17 h in the presence of various amounts of DL-Dpm. The percentage of refractile spores was observed microscopically, and muramic lactam content was measured as described in the text. Data are expressed as a percentage of the maximum value that was obtained in the presence of 1,000 μg of DL-Dpm per ml. Percent sporulation was more than 98% in all cultures, and percent refractile spores was 90 to 95% at 1,000 μg of DL-Dpm per ml. (A) A group II mutant (mutant 32-3). ^3H counts incorporated into muramic lactam were 4,700 counts/min per 0.4 ml of culture at 1,000 μg of DL-Dpm per ml. This value is about 30% of that of wild-type spores. (B) A group III mutant (mutant 21-1). ^3H counts were 4,400 counts/min per 0.4 ml of culture at 1,000 μg of DL-Dpm per ml. This value is also about 30% of the wild-type value.*

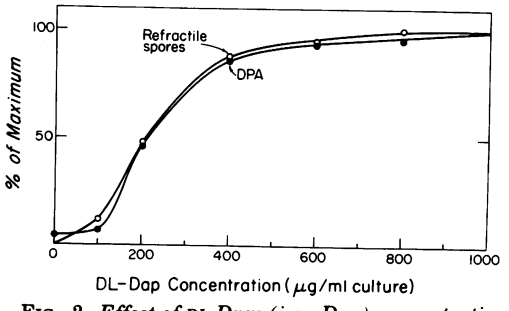

FIG. 2. *Effect of DL-Dpm (i.e., Dap) concentration on the percentage of refractile spores and on DPA content in sporulating cells. A group II mutant (mutant 32-3) was grown as described in the legend to Fig. 1. At 1,000 μg of DL-Dpm per ml, the percentage of refractile spores was 90%, and DPA content was 28.5 μg/ml.*

fractile and nonrefractile spores was very clear. Only a few percent of the spores seemed intermediate between refractile and nonrefractile (Fig. 3). These data strongly suggest that there is a critical cortex content to produce refractility in spores.

The curve of DPA accumulation was parallel with that of refractility in spores (Fig. 2). These data suggest that only refractile spores can accumulate DPA. However, refractility appears in the absence of DPA accumulation (Imae and Strominger, in press).

Resistance against organic solvents (10% final concentration and 10 min of treatment) and heat (80 C and 10 min of treatment) was also measured (Fig. 4). Xylene-resistant spores appeared at a DL-Dpm concentration slightly higher than that required for refractile spore formation. Octanol resistance required a still slightly higher Dpm concentration but, for heat resistance, a much higher concentration of Dpm was required. At 700 μg of DL-Dpm per ml, spores were fully resistant to xylene and octanol, but only 30% of the spores were heat resistant. At 1,000 μg of DL-Dpm per ml almost all of the spores became heat resistant.

It is noteworthy that all of the curves in Fig. 4 appeared parallel, suggesting that each of the

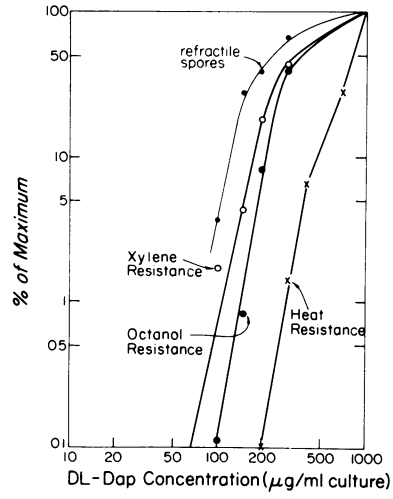

FIG. 4. *Effect of DL-Dpm (i.e., Dap) concentration on resistance of sporulating cells to various treatments. A group II mutant (mutant 32-3) was grown in SPL medium at 30 C for 17 h in the presence of various amounts of DL-Dpm. Xylene and 1-octanol treatment: 10% and 10 min. Heat treatment: 80 C and 10 min. At 1,000 μg of DL-Dpm per ml in the medium, the percentage of refractile spores was 93%, viable cells were 1.7 × 10⁸/ml, xylene-resistant cells were 2.1 × 10⁸/ml, 1-octanol-resistant cells were 2.0 × 10⁸/ml, and heat-resistant cells were 1.9 × 10⁸/ml.*

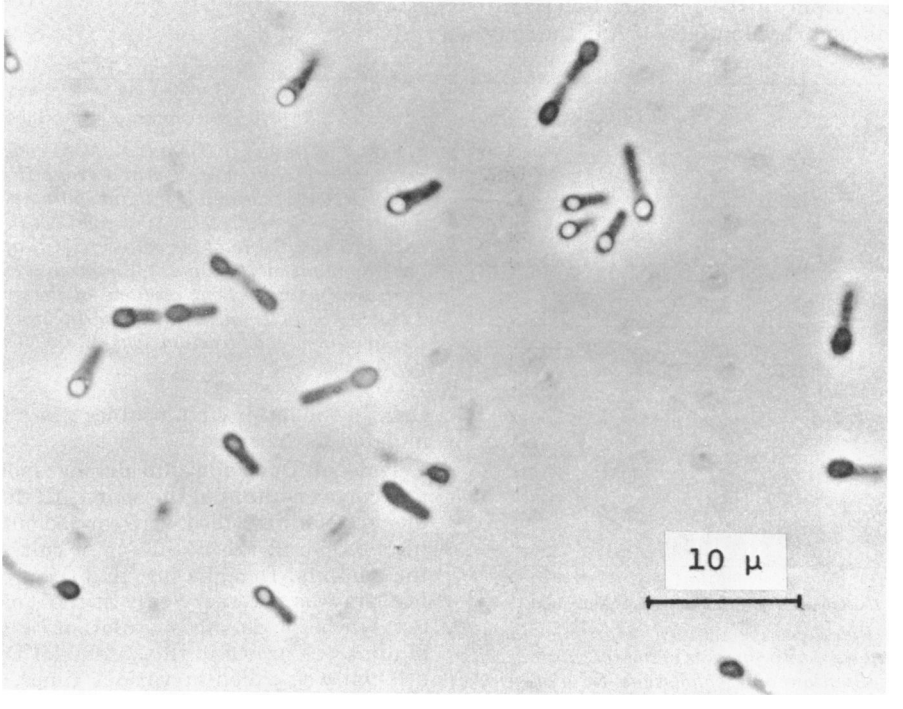

FIG. 3. *Phase-contrast micrograph of sporulating cells of a group II mutant (mutant 32-3) in the presence of 150 μg of DL-Dpm per ml. Cells were grown at 30 C for 17 h.*

properties resulted from the appearance of some new constituent at a critical level.

These results can also be examined as a function of muramic lactam content in the spore (Fig. 5). The striking difference between the amount of cortex required for refractility, DPA accumulation, and xylene or octanol resistance and that required for heat resistance is evident.

Effect of cortex content on the accumulation of ^{45}Ca. ^{45}Ca accumulation in the spore during sporulation of the wild-type strain of *B. sphaericus* started at about 4 h after initiation of sporulation and coincided with the appearance of refractile spores (Fig. 6). The accumulation of ^{45}Ca in group II mutant spores was also dependent on the presence of cortex in the spore (Fig. 7). ^{45}Ca accumulation was observed at Dpm concentrations higher than 200 μg/ml in the medium. The accumulation did not seem to parallel with DPA content in the sporulating cells. However, this difference could be due to the different assay method for these compounds; for ^{45}Ca accumulation, cells were washed with 5% trichloroacetic acid, whereas cells were washed only by 1 mM $MgCl_2$ in the case of the DPA assay.

The presence of not only cortex but also DPA was essential for the accumulation of ^{45}Ca in the spore (Fig. 8). This could be shown by using a group III mutant that formed cortex in the addition of Dpm to the medium but required DPA addition to accumulate DPA in the spores.

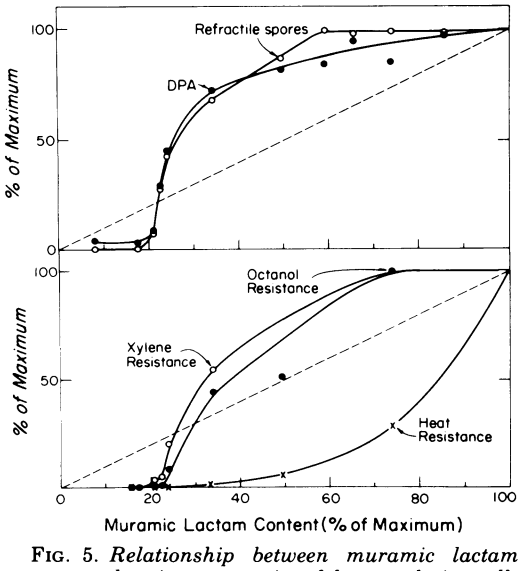

FIG. 5. *Relationship between muramic lactam content and various properties of the sporulating cells of a group II mutant of B. sphaericus. Data presented in the legends to Fig. 1 and 4 were also included.*

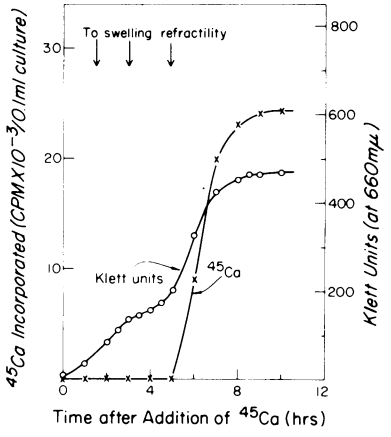

FIG. 6. *^{45}Ca accumulation during sporulation of B. sphaericus. Wild-type cells were grown in SPL medium at 37 C. $^{45}CaCl_2$ was added at the beginning of the cultivation to a final concentration of 0.45 μCi/μmol per ml of culture. Portions of 0.1 ml were taken at intervals, and acid-insoluble accounts were measured.*

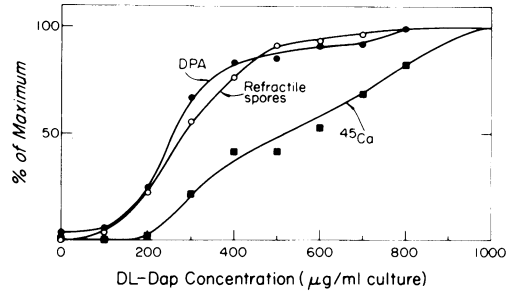

FIG. 7. *Effect of DL-Dpm (i.e., Dap) concentration on the accumulation of ^{45}Ca in a group II mutant of B. sphaericus. A group II mutant (mutant 32-3) was grown in SPL medium at 30 C for 17 h in the presence of 0.45 μCi/μmol per ml of $^{45}CaCl_2$ and of various amounts of DL-Dpm. ^{45}Ca accumulation was measured using 0.1-ml portions of the culture. At 1,000 μg of DL-Dpm per ml, ^{45}Ca incorporated into the acid-insoluble fraction was 12,000 counts/min per 0.1 ml.*

Only in the latter circumstance was ^{45}Ca accumulated in the spore.

Time of Dpm addition during sporulation and cortex content of the spore. All the experiments so far described were carried out by adding DL-Dpm at the beginning of cultivation of the mutants. To make sure that the presence of DL-Dpm was necessary only at the time of cortex synthesis during sporulation, a group II mutant was grown in the absence of Dpm, and DL-Dpm was added at various times, as indicated (Fig. 9A). Cultivation was then continued

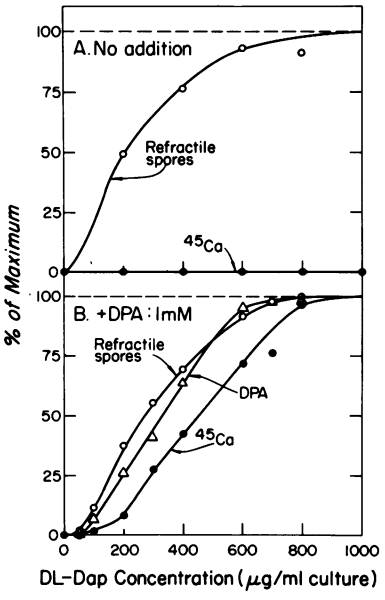

FIG. 8. *Effect of* DL-*Dpm (i.e., Dap) concentration and of DPA on the accumulation of* ^{45}Ca *in a group III mutant of B. sphaericus. A group III mutant (mutant 21-1) was grown in SPL medium at 30 C for 17 h in the presence of various amounts of* DL-*Dpm. (A)* $^{45}CaCl_2$ *was added to a final concentration of 0.45 μCi/μmol per ml of culture.* ^{45}Ca *incorporation into the acid-insoluble fraction at 1,000 μg of* DL-*Dpm per ml was 107 counts/min per 0.1 ml. (B)* $^{45}CaCl_2$ *was added to 0.45 μCi/2 μmol per ml of culture. DPA was added to 1 μmol/ml at the same time.* ^{45}Ca *incorporation was 6,800 counts/min per 0.1 ml of culture at 1,000 μg of* DL-*Dpm per ml.*

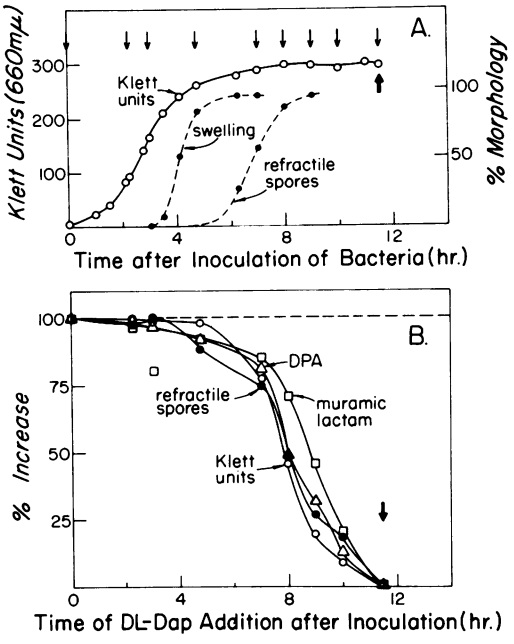

FIG. 9. *Effect of time of addition of Dpm (i.e., Dap) on cortex content and formation of normal refractile spore. A group II mutant (mutant 32-3) was grown at 37 C in SPL medium (main culture). (A) At the time point indicated by thin arrows, 30-ml cultures were removed and* DL-*Dpm was added to a final concentration of 1,000 μg/ml. Cultivation was terminated at 11.5 h after inoculation, as indicated by a thick arrow. Klett units and the percentage of terminal swelling were measured in the main culture. The percentage of refractile spores was measured in the flask to which* DL-*Dpm was added at the beginning of the cultivation. (B) Values were expressed as the percentage of the difference between maximum values obtained from the culture to which* DL-*Dpm was added at the beginning of the cultivation and minimal values obtained from the culture to which* DL-*Dpm was added at the time of harvesting. Maximum values for Klett units, refractile spores, DPA content, and muramic lactam content were 517, 92.3%, 45.3 μg/ml, and 7,000 counts/min per 0.4 ml, respectively. Minimal values were 293, 0%, 2.6 μg/ml, and 1,000 counts/min per 0.4 ml, respectively. The maximum value of muramic lactam content was about 32% of the wild-type value, and the minimum value was less than 5%.*

at 37 C for 11.5 h. Turbidity of the culture, refractile spore content, DPA content, and muramic lactam content were measured. The addition of DL-Dpm up to 5 h after initiation of sporulation resulted in a complete recovery of normal sporulation; values of turbidity increase in the culture due to the refractility of the spores, of percentage of refractile spores, and of DPA content were the same as that of the culture that received DL-Dpm at the beginning of the cultivation. After 7 h or longer of cultivation, addition of DL-Dpm gave a reduced but significant increase of the fraction of normal spores. Cortex content, measured by the amount of muramic lactam, of such sporulating cells was also changed, as well as other properties. Since cortex synthesis should start at around 6 h of cultivation under the experimental condition (Imae and Strominger, in press), this result clearly indicated that DL-Dpm was only necessary at the time of cortex synthesis. However, the addition of DL-Dpm after 7, 8, 9, or even 10 h of starvation resulted in a significant increase of the fraction of normal spores. This may indicate that the cortex synthesizing system is active for some time without substrate (i.e., half-time, 1 to 2 h, from the data of Fig. 9B). Alternatively, it may be that this system remains active, but the synthesis of the spore coat prevents entry of substrate to the site of cortex synthesis. Electron microscopy has

shown that all other spore structures are synthesized normally in the absence of Dpm (unpublished data).

DISCUSSION

Conditional spore cortexless mutants of *B. sphaericus* 9602 have an interesting property: the muramic lactam content of such mutant spores increased almost linearly with DL-Dpm concentration in the medium. This result is probably due to the absence of an active transport system for Dpm. Therefore, the imperfect recovery of muramic lactam content of the mutant spores, even in the presence of a very high concentration of DL-Dpm in the medium (1,000 μg/ml) (Imae and Strominger, in press), is probably due to the insufficient permeation of Dpm into the cell. As a consequence, the cortex content of the mutant spores can be easily varied by changing the DL-Dpm concentration in the medium. This provides a method for controlling cortex content of the spores.

By using this unique property of the mutants, the effect of cortex content on the characteristic properties of the spore could be studied by comparing the properties of the spores produced with different concentrations of DL-Dpm in the medium. The results, summarized in Fig. 5, clearly indicated that there were different critical cortex contents for different characteristic spore properties. At 1,000 μg of DL-Dpm per ml in the medium, mutant spores showed almost the same properties as those of wild-type spores under the experimental conditions, although the cortex content of these mutant spores was only about 30% of the wild-type level. (This is a rough comparison because the cortex content of the mutant spores and that of wild-type spores showed some fluctuations due to the day-to-day variation of the experimental conditions.) Mutant spores required only about 10% of the average cortex content of the wild-type cortex level to yield a 50% refractile spore (Fig. 2 and 5). Since the mutant spores had about 3 to 5% of the cortex content of the wild-type level in the absence of Dpm, probably due to the leakiness of the mutation, the DL-Dpm concentration in the medium required to give 50% of spores refractile was only 150 to 200 μg/ml (Figs. 1 and 2). For DPA accumulation, almost the same cortex content was required. Furthermore, the curves of DPA content in the spores and the percentage of refractile spores as a function of DL-Dpm concentration were very similar (Fig. 2). This may imply that only refractile spores can accumulate DPA in the spores. Since refractile spores are produced without DPA (Imae and Strominger, in press), there is no possibility that only DPA-containing spores showed refractility. The data indicate that very low amounts of cortex were sufficient to make spores refractile and able to accumulate DPA. This may explain properties of a cortexless mutant of *B. cereus* isolated by Pearce and Fitz-James (4), if their mutant was slightly leaky for cortex synthesis. For xylene and octanol resistance, mutant spores required slightly more cortex content than required for refractility, about 10 to 15% of the wild-type level (Fig. 5). For heat resistance, about 30% of the cortex content of the wild-type level was essential; i.e., at least 1,000 μg of DL-Dpm per ml in the medium was necessary to make mutant spores resistant to treatment at 80 C for 10 min.

At about 10% of the cortex content of the wild-type level, spores suddenly changed to round, refractile spores and became resistant to xylene. Thus, increase of the cortex content in the mutant spores did not result in a gradual change of the spore properties, but these changes occurred at a critical cortex content. Although the mechanism of spore refractility and of resistances of spores to various chemical and physical treatments are unknown, the results strongly support the idea that some critical change in the physical state of the spore occurs at a cortex content of about 10% of the wild-type level. This change could be the production of a dehydrated spore cytoplasm, as has been suggested by many people (see reference [1]). A further increase of cortex content may also result in a second change in physical state, perhaps required for extreme dehydration of the spore cytoplasm accompanied by heat resistance. Although these characteristic spore properties could result from dehydration of the spore cytoplasm, the mechanism by which the spore cortex functions to extrude water from the cytoplasm is unknown. In fact, the two theories, the contractile cortex theory (2) and the expanded cortex theory (1), are rather directly opposite.

^{45}Ca accumulation in the spore depended not only on the amount of cortex in the spore but also on the presence of DPA. This result is consistent with the idea that Ca^{2+} is present in the spore as a form of chelate with DPA (3).

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