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Cortex Content of Asporogenous Mutants of *Bacillus subtilis*

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A method for the measurement of muramic lactam, which is specifically located in the cortical peptidoglycan of bacterial spores, was developed as a quantitative assay method for spore cortex content. During sporulation of *Bacillus subtilis* 168, muramic lactam (i.e., spore cortex) began to appear at stage IV of sporulation and continued to increase over most of the late stages of sporulation. Spore cortex contents of various *spo* mutants of *B. subtilis* were surveyed. Cortex was not detected in mutants in which sporulation was blocked earlier than stage II sporulation. Spores of a *spo* IV mutant had about 40% of the cortex content of the wild-type spores. One *spo* III mutant had a low amount of cortex, but four others had none.

Peptidoglycan in the cortex of bacterial spores may have a critical role in production and maintenance of the dehydrated spore cytoplasm, from which many characteristic spore properties result (2, 6). Spores contain two peptidoglycan layers (germ cell wall and spore cortex) (1). Although only cortex is thought to be a critical structure in the maintenance of the sporulating state, most of the data so far reported were not specific to spore cortex (1, 7). The similarity of the chemical nature of both peptidoglycans made them difficult to differentiate. Electron microscopy showed that cortex was synthesized at stage IV of sporulation and continued into stage V and even stage VI (7). Although cortex formation is one important marker for the determination of sporulating stage, especially in studies of genetics of sporulation-deficient mutants (12), only electron microscope analysis was available; of course, this method is not quantitative.

Warth and Strominger (9) found a unique spore constituent, muramic lactam, in the cortical peptidoglycan and reported that one out of two muramic acid residues in the spore cortex was present as muramic lactam (10). Therefore, the detection of muramic lactam is a specific and reliable chemical method to detect the presence of cortex in the spore. Using this method, Wickus et al. (11) reported that spore cortex synthesis of *Bacillus cereus* and *B. megaterium* began to appear during the late stages of sporulation at about the same time as dipicolinic acid (DPA) synthesis.

In this paper it will be reported that the detection method for muramic lactam is a useful quantitative assay for the amount of spore cortex. The cortex content of several sporulation-deficient mutants of *B. subtilis* has also been examined.

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MATERIALS AND METHODS

Bacterial strains and cell growth. *B. subtilis* SMY (wild type) and *spo* mutants were generously provided by P. Schaeffer. Schaeffer medium (8) was used for bacterial growth and sporulation throughout the experiments. L-Lysine or uracil was added to a final concentration of 100 μg/ml, if necessary. Growth of bacteria was carried out at 37 C with vigorous shaking and was followed by a Klett-Summerson photoelectric colorimeter. Sporulation was observed by a Zeiss phase-contrast microscope. For chemical studies, 10-ml portions of the culture were taken up at intervals, and cells were harvested by centrifugation at 12,000 × *g* for 10 min. After washing with 10 ml of 1 mM $MgCl_2$, cells were resuspended in 1.2 ml of 1 mM $MgCl_2$ (concentrated cell suspension) and kept frozen until use.

Chemicals. [^{3}H]$NaBH_4$ (500 mCi/96 mg) was obtained from New England Nuclear Corp., Boston, Mass. 2,6-Dipicolinic acid was purchased from Sigma Chemical Co., St. Louis, Mo.

Determination of muramic lactam and DPA content in sporulating cells. The procedure was modified from that reported previously (1). Aliquots of the concentrated cell suspension (50 μl, corresponding to 0.4 ml of the culture) were autoclaved at 120 C for 15 min. Freshly prepared [^{3}H]$NaBH_4$ solution (70 μl; 0.3 M; 3 to 10 mCi/mmol) was added, and the mixture was left in a hood for 24 h at room temperature. One milliliter of 5% acetic acid was added, and the mixture was left for 1 h or longer. After centrifugation at 12,000 × *g* for 10 min, 250 μl of 6 N HCl was added to the precipitate and the tubes were sealed. Acid hydrolysis was carried out at 108 C for 44 h. More than 90% of muramic lactam was reduced and released under these conditions (A. D. Warth, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1969).

HCl was removed in vacuo over NaOH. Water (0.2 ml) was added and the procedure was repeated. After the addition of 20 μl of water, the sample was spotted on a Whatman 3MM paper. ^{3}H-labeled reduction product of muramic lactam was separated by high-voltage paper electrophoresis at pH 3.9 (pyridine-acetic acid-water, 7.5:25:1,000) for 1.5 h at 40 V/cm. An authentic ^{3}H-labeled reduction product of muramic lactam was prepared in this laboratory (9). Radioactivity on the paper was located using a Packard radiochromatogram scanner model 7201.

RESULTS

Quantitative determination of cortex content in the spore. When cortical peptidoglycan of the spore was reduced by [^{3}H]NaBH$_4$, ^{3}H label was incorporated into the lactam ring of muramic lactam and produced two kinds of reduction products. The major product is a cyclic secondary amine, and the minor one is acyclic primary amino alcohol (9). Since both products showed almost the same mobility on the paper electrophoresis (major one, M_{GlcN} = 0.89; minor one, M_{GlcN} = 0.85 [9]), both products were assayed together throughout the experiments. An authentic marker of ^{3}H-labeled reduction product of muramic lactam migrated 34 cm toward anode under the experimental condition (Fig. 1a). Treatment by [^{3}H]NaBH$_4$ of mature spores, harvested 24 h after initiation of the sporulation, gave a clear peak at the same region as that of the authentic marker (Fig. 1b). Some radioactivity remained at the origin, and also a small amount of radioactivity was found at the region of C-1 reduced muramic acid (at about 5 cm toward anode (Warth, Ph.D. thesis). Practically no radioactivity migrated to the region of C-1 reduced glucosamine (38 cm toward anode). On the other hand, the same treatment of cells that were harvested at a very early stage of sporulation (1.25 h after initiation of sporulation) did not give any peak at 34 cm (Fig. 1c). As in the case of mature spores, some radioactivity remained at the origin and some was found at about 5 cm towards the anode.

Total radioactivity recovered after acid hydrolysis of the samples and the amount of the radioactivity found after separation of the reduction product of muramic lactam are shown in Table 1. When the sample was obtained in a very early stage of sporulation ($T_{1.25}$), about 10,000 counts of ^{3}H label per min were recovered after acid hydrolysis of the sample (obtained from 1 ml of culture). Less than 1% of this radioactivity was found in the region of the reduction product of muramic lactam. In the case of mature spores, about 30,000 counts/min per ml of the culture were obtained after acid hydrolysis, and about 30% of this radioactivity was recovered as a reduction product of muramic lactam. Thus, muramic lactam in the cortical peptidoglycan of the spore was quite selectively labeled by [^{3}H]NaBH$_4$ reduction.

When the amount of mature spores was varied during [^{3}H]NaBH$_4$ reduction, a linear relationship was obtained between the samples used and the radioactivity found in the region of the reduction product of muramic lactam (Fig. 2). Therefore, this assay method is not only quite specific for muramic lactam but also reasonably quantitative for assay of cortical peptidoglycan in the spore.

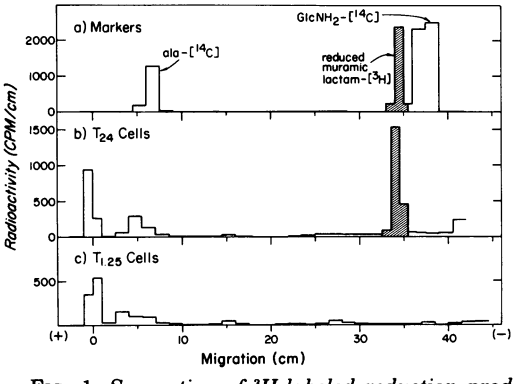

FIG. 1. *Separation of ^{3}H-labeled reduction product of muramic lactam by paper electrophoresis. Authentic markers were D-[^{14}C]alanine (2,200 counts/min), D-[^{14}C]glucosamine (7,700 counts/min), and authentic ^{3}H-labeled reduction product of muramic lactam (3,100 counts/min). Sporulating cells were harvested at $T_{1.25}$ and T_{24}, described in the legend to Fig. 3. Cells were treated with [^{3}H]NaBH$_4$, hydrolyzed, and subjected to paper electrophoresis.*

TABLE 1. *Relationship between total radioactivity incorporated from [^{3}H]NaBH$_4$ and the radioactivity in the reduction product of muramic lactam*[a]

Sporulating cells	Total radioactivity recovered after acid hydrolysis (counts/min per ml of culture)	Radioactivity recovered as the reduction product of muramic lactam (counts/min per ml of culture)
$T_{1.25}$	9,600	40
T_{24}	30,250	8,300

[a] *B. subtilis* wild type (SMY) was grown in Schaeffer medium at 37 C, and cells were harvested at 1.25 and 24 h after initiation of sporulation. Cells were treated with [^{3}H]NaBH$_4$ (about 4 mCi/mmol). After acid hydrolysis, samples were dried up and then dissolved in water. A sample of the hydrolysate was spotted directly on a Whatmann 3MM filter-paper disk to measure the total radioactivity. Another aliquot was also spotted on a Whatmann 3MM paper and subjected to paper electrophoresis. The region of the reduction product of muramic lactam was cut out, and the radioactivity was measured.

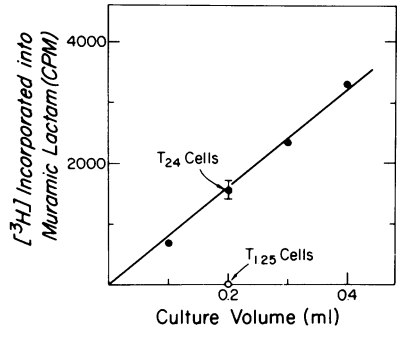

FIG. 2. *Linearity of muramic lactam assay. The amount of the sporulating cells was varied as indicated. $T_{1.25}$ and T_{24} cells were prepared as described in the legend to Fig. 1.*

Cortex synthesis during sporulation of wild-type *B. subtilis*. *B. subtilis* SMY (wild type) was grown in Schaeffer medium at 37 C with vigorous shaking. At intervals, bacterial growth, numbers of refractile and heat-resistant spores, DPA content in the spore, and cortex content were measured (Fig. 3). Initiation of sporulation was conventionally determined as the time point when the logarithmic growth of bacteria slowed down. Refractile spores began to appear about 4 h after initiation of sporulation, roughly coincident with the last increase of turbidity. Nonrefractile forespores were first detected about 3 h after initiation of sporulation (data not shown).

Cortex syntheis (measured by muramic lactam content) began to appear at about 4 h after initiation of sporulation, coincident with the appearance of refractile spores and with the accumulation of DPA in the sporulating cells (Fig. 3). Heat-resistant spores appeared slightly later (about 5 h after initiation of sporulation). Cortex and DPA contents in the spore increased in parallel, followed by the increase of heat-resistant spores. Clearly, cortex synthesis started at the beginning of the stage IV of sporulation and increased throughout the later stages of sporulation.

Cortex synthesis of sporulation-deficient mutants of *B. subtilis*. Sporulation mutants of *B. subtilis* blocked at various stages of sporulation (4) were grown in Schaeffer medium at 37 C. Growth curves and sporulation of the mutants are shown in Fig. 4. Mutants of *spo* Oa, *spo* Ob, and *spo* II have a block in early stages of sporulation. They did not show any detectable formation of forespores and also did not show the second increase of turbidity. *spo* III mutants have a block around the end of stage III of sporulation. One of these mutants (94U) showed the production of nonrefractile forespores, accompanied with a slight increase of turbidity (Fig. 4A). In four other *spo* III mutants (Fig. 4B), the percentage of forespores in the culture was lower and there was no detectable later increase of turbidity. A *spo* IV mutant that is blocked around the end of stage IV of sporulation produced a phase-white but nonrefractile spore, accompanied with a significant increase of turbidity (Fig. 4A).

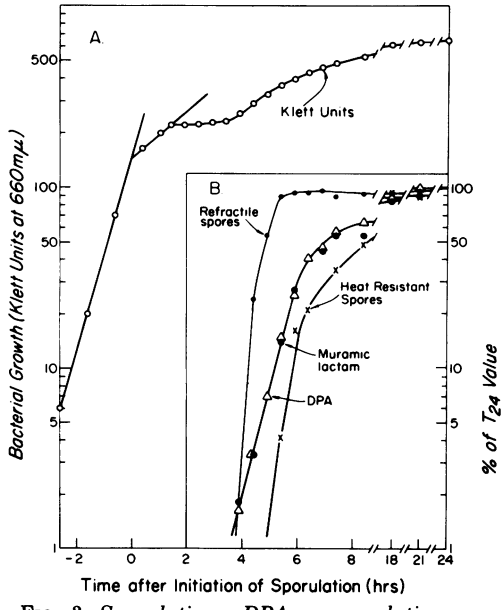

FIG. 3. *Sporulation, DPA accumulation, and muramic lactam content of B. subtilis wild type (SMY). Cells were grown in Schaeffer medium at 37 C with vigorous shaking. At T_{24}, sporulation efficiency was 84% and the refractile spores were 79% of the total cells. Heat-resistant spores (treatment at 80 C for 10 min) were 78% of the total viable cells. DPA content was 35.7 μg/ml of the culture and ^3H counts incorporated into muramic lactam was 6,400 counts/min per 0.4 ml of culture.*

Mutants of *spo* Oa, *spo* Ob, and *spo* II did not make any cortex (measured as muramic lactam) after initiation of sporulation (Fig. 5A). One *spo* III mutant (*spo* III 94U) synthesized a small amount of cortex, about 20% of wild-type content (Fig. 5A). The other four *spo* III mutants had no detectable cortex (Fig. 5B). The *spo* IV mutant, on the other hand, produced forespores that contained about 40% of wild-type cortex content. Although cortex synthesis begins at about stage IV of sporulation, *spo* III 94U can synthesize some cortex and must be blocked later than the other *spo* III mutants.

None of these mutants could accumulate DPA in sporulating cells (Table 2). This result is of special interest in the case of *spo* III 94U and *spo* IV 11T, which have 20 and 40% of the

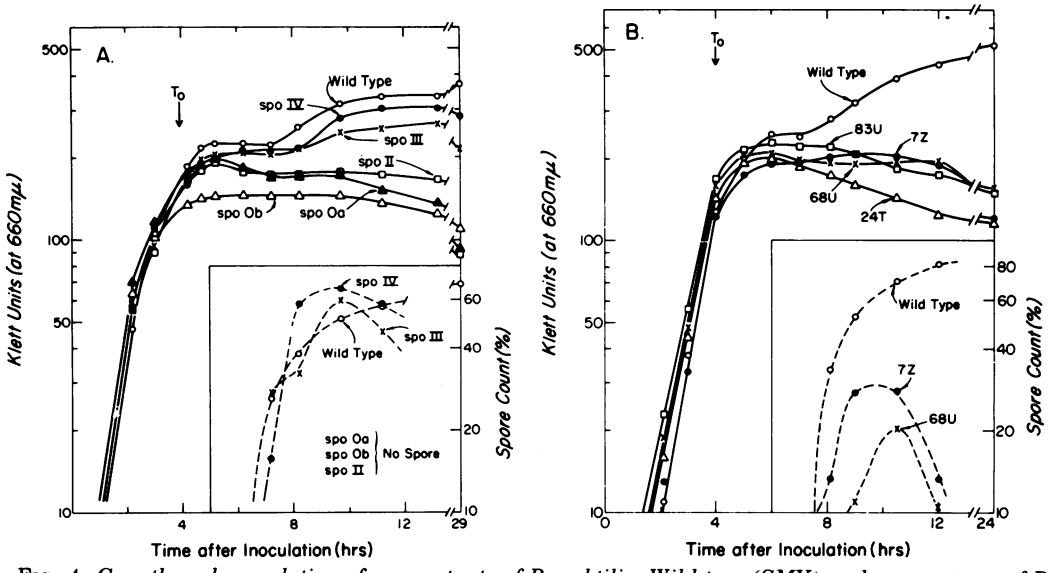

FIG. 4. *Growth and sporulation of spo mutants of B. subtilis. Wild-type (SMY) and spo mutants of B. subtilis were grown, as described in the legend to Fig. 3. Bacterial growth and percentage of forespores are shown. Data for spo Oa 5NA, spo Ob 6Z, spo II$_B$ 4Z, spo III 94U, spo IV 11T, and wild-type SMY are shown in part A, and spo III 7Z lys$^-$, spo III 28T ura$^-$, spo 68U, and spo III 83U are shown in part B.*

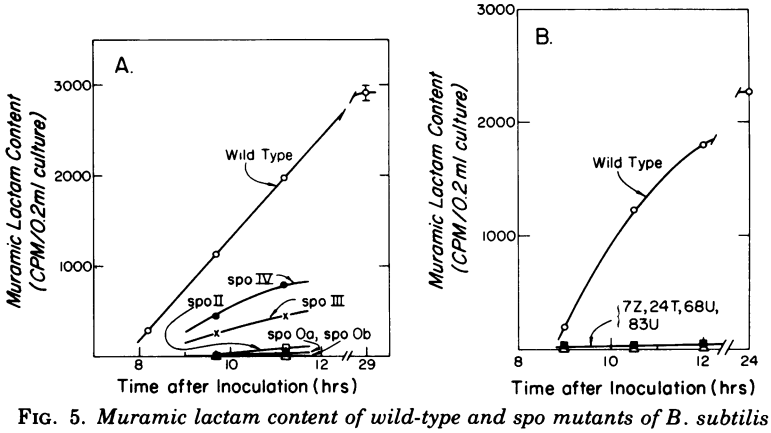

FIG. 5. *Muramic lactam content of wild-type and spo mutants of B. subtilis.*

wild-type cortex content, respectively, since the critical amount of cortex content required for DPA accumulation in *B. sphaericus* was shown to be 25% of the wild-type level (3).

DISCUSSION

The reduction of cortical peptidoglycan by [^{3}H]$NaBH_4$, followed by acid hydrolysis, resulted in the production of ^{3}H-labeled reduction products of muramic lactam, which are easily separable from other radioactive materials. The amount of the radioactivity incorporated into the reduction product of muramic lactam was about 30% of the total radioactivity recovered after acid hydrolysis. This indicates that the reduction method is quite selective for muramic lactam of spore cortex. Although the assay procedure of the spore cortex based on this principle is quite quantitative, there is some difficulty in using it to calculate the absolute amount of cortex in the spore, because the specific activity of [^{3}H]$NaBH_4$ solution varied from day to day due to difficulty in weighing small amounts for dilution. Therefore, the assay must be run using a known spore suspension as a standard. A rough calculation from the data of Fig. 1 showed that muramic lactam content in mature spores of *B. subtilis* was about 2 $\mu g/10^9$ spores.

Cortex synthesis during sporulation of *B.*

TABLE 2. *Dipicolinic acid content of spo mutants*

Mutants	DPA content (μg/ml of culture)			
	8.2[a]	9.7	11.2	29
spo Oa 5Na	<1.5	<1.5	<1.5	<1.5
spo Ob 6Z	<1.5	<1.5	<1.5	<1.5
spo II_B4Z	<1.5	<1.5	<1.5	<1.5
spo III 94 U[b]	<1.5	<1.5	<1.5	<1.5
spo IV 11T	<1.5	<1.5	<1.5	<1.5
Wild type (SMY)	3.4	10.7	13.2	24.4

[a] Sampling time after inoculation (hours). Bacterial growth curves were presented in Fig. 4.

[b] The other *spo* III mutant (7Z, 24T, 68U, and 83U) were identical.

subtilis was about 1 h later than the appearance of nonrefractile forespores and about 1 h earlier than the appearance of heat-resistant spores. These data are consistent with the data obtained in *B. cereus* and *B. megaterium* (11). All the data showed that cortex synthesis begins at the stage IV of sporulation (or late in stage III) and continues into later stages of sporulation. Cortex content of *spo* mutants supported this idea. *spo* III mutants did not make any cortex, with the exception of *spo* III 94U, and cortex content of a *spo* IV mutant was only about 40% of the wild-type level.

We have reported, using conditional cortexless mutants of *B. sphaericus*, that only about 25% of cortex content of wild-type level is enough to make spores refractile (3; Y. Imae and J. L. Strominger, J. Biol. Chem., in press). However, *spo* III 94U and *spo* IV 11T mutants of *B. subtilis* remain nonrefractile, despite their content of sufficient cortex. There are several possible explanations for this discrepancy. Although in the case of *B. sphaericus* the mutants were defective only in cortex synthesis and other spore structures were normal (Y. Imae, M. B. Strominger, and J. L. Strominger, manuscript in preparation), *spo* mutants of *B. subtilis* are not specific cortexless mutants but may be blocked in synthesis of other spore components required for refractility. The additional defect cannot be the failure to accumulate DPA, however, because refractility is not dependent on DPA accumulation, at least in *B. sphaericus* (Y. Imae and J. L. Strominger, in press). It is also possible that the amount of cortex required for development of refractility is larger in *B. subtilis* than in *B. sphaericus*.

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LITERATURE CITED

1. **Fitz-James, P., and E. Young.** 1969. Morphology of sporulation, p. 39-72. *In* G. W. Gould and A. Hurst (ed), The bacterial spore. Academic Press Inc., New York.
2. **Gould, G. W., and G. J. Dring.** 1974. Mechanisms of spore heat resistance. Adv. Microb. Physiol. **11**:137-164.
3. **Imae, Y., and J. L. Strominger.** 1976. Relationship between cortex content and properties of *Bacillus sphaericus* spores. J. Bacteriol. **126**:907-913.
4. **Ionesco, H., J. Michel, B. Cami, and P. Schaeffer.** 1970. Genetics of sporulation in *Bacillus subtilis* Marburg. J. Appl. Bacteriol. **33**:13-24.
5. **Janssen, E. W., A. J. Lund, and L. E. Anderson.** 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science **127**:26-27.
6. **Lewis, J. C., N. S. Snell, and N. H. Burr.** 1960. Water permeability of bacterial spores and the concept of a contractile cortex. Science **132**:544-545.
7. **Murrell, W. G.** 1969. Chemical composition of spores and spore structures, p. 215-273. *In* G. W. Gould and A. Hurst (ed), The bacterial spore. Academic Press Inc., New York.
8. **Schaeffer, P., J. Millet, and J-P. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc.Natl. Acad. Sci. U.S.A. **54**:704-711.
9. **Warth, A. D., and J. L. Strominger.** 1969. Structure of the peptidoglycan of bacterial spores: occurrence of the lactam of muramic acid. Proc. Natl. Acad. Sci. U.S.A. **64**:528-535.
10. **Warth, A. D., and J. L. Strominger.** 1972. Structure of the peptidoglycan from spores of *Bacillus subtilis*. Biochemistry **11**:1389-1396.
11. **Wickus, G. G., A. D. Warth, and J. L. Strominger.** 1972. Appearance of muramic lactam during cortex syntheis in sporulating cultures of *Bacillus cereus* and *Bacillus megaterium*. J. Bacteriol. **111**:625-627.
12. **Young, F. E., and G. A. Wilson,** 1972. Genetics of *Bacillus subtilis* and other gram-positive sporulating bacilli, p. 77-106. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D. C.