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Location of Peptidoglycan Lytic Enzymes in *Bacillus sphaericus*†

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The level of three peptidoglycan hydrolases was determined in the mother cell compartment and forespores of *Bacillus sphaericus*. Vegetative and sporulating cells contained an LD-carboxypeptidase active only on the vegetative cell wall peptidoglycan, and we have previously shown that sporulation is accompanied by the production of two new enzymes active only on the spore cortex peptidoglycan. These are a γ-D-glutamyl-*meso*-diaminopimelate endopeptidase and a *meso*-diaminopimelate-D-alanine dipeptidase. The LD-carboxypeptidase activity appeared to be located in the membranes of both the mother cells and forespores. Endopeptidase activity was located in the integument fraction of the forespores, and the dipeptidase activity was only found in the forespore cytoplasm. These different locations comply with the probable different functions of these enzymes.

Vegetative cells of *Bacillus sphaericus* contain a peptidoglycan built up of 4-*O*-(*N*-acetyl-β-D-glucosaminyl)-*N*-acetyl-D-muramic acid-peptide (GlcNAc-MurNAc-peptide) repeating subunits in which the peptide has the sequence L-alanyl-γ-D-glutamyl-L-lysyl-D-alanine and is cross-linked 35% between the D-alanyl and L-lysyl residues by D-isoasparaginyl residues (7). C-terminal peptides lack both of the D-alanine residues present in the pentapeptide precursor and have L-lysine C termini (7). The spore cortex of *B. sphaericus* is a peptidoglycan whose structure (12; D. J. Tipper, Bacteriol. Proc., p. 24, 1969) is essentially identical to that of *B. subtilis* spore cortex (20). Its peptide subunits have the sequence L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelyl(L)-D-alanine, directly cross-linked between D-alanyl and diaminopimelyl residues. No amide residues are present, and C-terminal peptides retain either the entire tetrapeptide sequence or retain only the L-alanine residue. *B. subtilis* vegetative cell walls have a very similar diaminopimelate-containing peptidoglycan in which the carboxyl groups at the D center of the diaminopimelate residues are amidated (19).

Several lytic enzymes playing a role in the metabolism of these peptidoglycans have been found in cells of *B. sphaericus* and *B. subtilis* (2-4). Natural substrates for these enzymes in *B. sphaericus* have the following composition: *N*-acetyl-muramyl-L-alanyl-γ-D-glutamyl-X-D-alanine, in which X is either L-lysine [MurNAc-(L-Lys) tetrapeptide] or *meso*-diaminopimelic acid [MurNAc-(*ms*-A_2pm) tetrapeptide]. The activities are (i) a MurNAc-L-Ala amidase which severs the peptide side chain from the MurNAc residue of either substrate; (ii) an LD-carboxypeptidase which severs the L-Lys-D-Ala linkage of lysine-containing substrates or substrates containing amidated *meso*-diaminopimelate, but is without effect on substrates containing unamidated *meso*-diaminopimelate; (iii) a γ-D-glutamyl-(L)*meso*-diaminopimelate endopeptidase which is without effect on substrates containing lysine, but which hydrolyzes the γ-D-Glu-*ms*-A_2pm linkage of *ms*-A_2pm-containing substrates, releasing the dipeptide *ms*-A_2pm(L)-D-Ala; (iv) a dipeptidase which selectively acts on the dipeptides L-Lys-D-Ala and *ms*-A_2pm-D-Ala and which was recently found to appear late in sporulation in cells of *B. sphaericus* (17). Similar activities are found in *B. subtilis*, except that dipeptidase has not yet been characterized.

The LD-carboxypeptidase and γ-D-Glu-*ms*-A_2pm endopeptidase activities were found in particulate preparations (4) and in the culture media of sporulating *B. sphaericus* (16). It has been shown previously that accumulation of endopeptidase activity is closely related to sporulation. It is not found in vegetative cells, and its activity increases during sporulation in *B. sphaericus* (4). Netropsin, a drug which inhibits RNA synthesis by intercalating into DNA and has a preference for regions rich in adenine and thymine (18), selectively inhibits sporulation in *B. subtilis* at concentrations which do not grossly affect macromolecular synthesis. It in-

† This paper is dedicated to E. Lederer on the occasion of his 70th birthday.

hibits synthesis of the endopeptidase, whose activity was also low in a *B. subtilis* asporogenous mutant (5). In contrast, LD-carboxypeptidase synthesis occurs in vegetative cells and is unaffected by netropsin or by the mutation to asporogeny (5).

Since the endopeptidase and dipeptidase are active only on the cortical peptidoglycan, their functions should be unique to sporulation, and concomitant control of their synthesis and of sporulation was anticipated. Because their natural substrate is presumed to be nascent cortical peptidoglyan, it was also anticipated that they would be located within the developing forespore. In contrast, the LD-carboxypeptidase should function exclusively in vegetative peptidoglycan metabolism and should be found in membranes responsible for the synthesis of this polymer. These include membranes of vegetative cells and sporangia and possibly the inner forespore membrane, which presumably makes cell wall primordium and is responsible for wall synthesis during outgrowth. To clarify the functions of these peptidoglycan hydrolases, we have attempted to define their location in the mother cell or forespore compartments of sporulating cells of *B. spaericus* and have also measured their activity in mature spores. The results are consistent with the proposed functions of these enzymes.

MATERIALS AND METHODS

Organism. *B. sphaericus* 9602, the medium used, and the conditions for obtaining maximum sporulation have been described previously (4, 14). The proportion of refractile forespores (percentage of morphology) was estimated by phase-contrast microscopy, and cells were harvested at different phases of growth and sporulation. Free spores were obtained as described by Kingan and Ensign (8); the rather slow release of spores from the sporangia was speeded up considerably when cells were harvested at the time when spore release had just begun (2 to 5% free spores), suspended (10%, wt/vol) in 20 mM Tris-hydrochloride buffer (pH 8), and then shaken at 135 rpm at 32°C. Under these conditions spore release was complete within 2 h.

Isolation of forespores. The method was essentially that described by Singh et al. (11). Samples of sporulating cells (3 g, wet weight) were washed with warm (37°C) buffer (0.6 M sucrose, 0.1 M potassium phosphate [pH 7.0], 16 mM $MgSO_4$). The cell pellet was suspended in 40 ml of warm buffer and incubated with 100 mg of lysozyme for 10 min at 37°C.

The lysozyme-treated cells were washed twice with 30 ml of cold buffer (0.6 M sucrose, 0.05 M Tris-hydrochloride [pH 8], 0.15 M NaCl, 16 mM $MgCl_2$) and sonically treated (1 min; apparatus from Measuring & Scientific Equipment, Ltd.) to release forespores. The sample was centrifuged (10 min, 6,000 × *g*), and the sonic treatment was repeated on the pelleted forespores. The combined supernatant fractions contained mother cell fragments and soluble components.

Radioactive substrates. The sites of acylation of *ms*-A_2pm are indicated by (L) or (D) preceeding *meso*-diaminopimelyl, indicating which of the α-aminoacyl centers is substituted. Similarly, (L) or (D) following *meso*-diaminopimelyl indicates which carboxyl group of *ms*-A_2pm is involved in acylation.

The specific activity of substrate I {*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelyl-(L)-D-[^{14}C]alanine} was 1.1 × 10^6 cpm/μmol. The specific activity of substrate II {*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-L-lysyl-D-[^{14}C]alanine} was 1.6 × 10^6 cpm/μmol. They were obtained as described previously (4). The tetrapeptide L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelyl-(ωNH_2)(L)-D-[^{14}C]alanine (substrate III; 2.2 × 10^6 cpm/μmol) was obtained by a transpeptidation reaction between the nonradioactive peptide isolated from *Streptomyces mediterranei* and D-[^{14}C]alanine (1). The dipeptide *meso*-diaminopimelyl-D-[^{14}C]alanine (substrate IV; 1.1 × 10^6 cpm/μmol) was isolated after incubation of the MurNAc-(*ms*-A_2pm) tetrapeptide with a purified γ-D-Glu-*ms*-A_2pm endopeptidase preparation (16).

Unlabeled substrate. Substrate V [*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)*meso*-diaminopimelic acid] was obtained from its UDP derivative by hyrolysis with 0.02 N HCl at 100°C for 7 min. The UDP derivative was isolated from *Lactobacterium plantarum* after incubation in the presence of D-cycloserine.

Enzyme preparations. Cells, forespores, and spores were broken either in an Ultra-Turrax shaker or in a Braun MSK homogenizer.

(i) Ultra-Turrax method. A 2-g (wet weight) sample of cells was shaken with 2.5 ml of glass beads (diameter, 0.17 mm) and 2.5 ml of 50 mM Tris-hydrochloride buffer (pH 8)–20 mM $MgCl_2$–0.2 mM dithiothreitol at 4°C for 1 min. After cooling for 3 min, the cycle was resumed. Cells were 80% broken in 10 min of effective breakage.

Intact cells, forespores, and cell debris were sedimented at 6,000 × *g* for 10 min, and the supernatant was centrifuged, first at 30,000 × *g* for 20 min and then at 130,000 × *g* for 60 min; the final supernatant was saved.

The pellets obtained by centrifugation at 30,000 × *g* (cell walls plus spore integuments) and 130,000 × *g* (membrane fragments) were washed and then homogenized in the same Tris-hydrochloride buffer as described above, to give protein concentrations of 5 to 8 mg/ml.

(ii) Braun MSK homogenizer. A 2.0-g (wet weight) sample of cells, forespores, or spores was broken by 5 min of shaking with 13 ml of glass beads (diameter, 0.17 mm) and 4.5 ml of 50 mM Tris-hydrochloride buffer (pH 8)–20 mM $MgCl_2$–0.2 mM dithiothreitol.

Particulate enzyme preparations were sedimented as described above. The final supernatant was concentrated by ultrafiltration on an Amicon apparatus (membrane UM 10) until a protein concentration ranging from 3 to 6 mg/ml was obtained.

Sucrose density gradient. The particulate enzyme preparation from the spores sedimenting at 30,000 × *g* was put over the following sucrose gradient

in 0.01 M HEPES (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4): 8 ml of 2.02 M sucrose, 10 ml of 1.44 M sucrose, and 10 ml of 0.77 M sucrose (10). The gradient was centrifuged for 16 h in a Spinco SW25.1 rotor at 25,000 rpm. The gradient was separated into fractions of 1 ml, and the optical density of samples was read at 280 nm.

Enzyme assays. The assays contained the following, in 10 μl: 0.1 or 0.2 mM substrate, 25 mM Tris-hydrochloride buffer (pH 8), 10 mM $MgCl_2$, and 10 μg of protein. Incubations were performed at 37°C for 90 min. The products of the reaction were analyzed by thin-layer chromatography. The radioactive spots were detected by autoradiography, removed from the support, and counted in a Packard Tri-Carb liquid scintillation spectrometer (model 2405), as described previously (4).

LD-Carboxypeptidase activity was expressed as nanomoles of D-[^{14}C]alanine released per milligram of protein per hour from substrate II or III.

Endopeptidase activity was expressed as nanomoles of *ms*-A_2pm-D-[^{14}C]Ala released per milligram of protein per hour from substrate I.

LD-Dipeptidase activity was expressed as nanomoles of D-[^{14}C]alanine released from substrate IV per milligram of protein per hour.

The apparent K_m's of substrates II and III for the LD-carboxypeptidase are 0.8 and 1.2 mM, respectively (2).The apparent K_m of substrate I for the endopeptidase is 0.33 mM (2). Data for the dipeptidase have yet to be determined. Because of limitations in the amount of substrates available, concentrations well below the K_m (0.1 to 0.2 mM) were employed. Under these conditions, the reaction rate was linear for at least 20 min followed by gradual deceleration during the 90-min incubations. Thus, although data do represent relative enzyme activities, they are not specific activities. This does not affect qualitative judgment of localization.

Analytical methods. Protein content was determined by the method of Lowry et al., using bovine serum albumin as standard (9).

Thin-layer chromatography was performed on MN 300 cellulose powder. The solvent 1-butanol–acetic acid–pyridine–water (30:6:20:24) was used with substrates I, III, IV, and V. The solvent ethyl acetate–pyridine–acetic acid–water (25:25:5:15) was used with substrate II.

RESULTS

Enzyme preparations were obtained from whole sporulating cells by shaking with glass beads to disrupt both mother cells and forespores followed by differential centrifugation to pellet unbroken cells (6,000 × *g*), cell wall and forespore integument fractions (30,000 × *g*), and membrane fragments (130,000 × *g*). The final supernatant contained both mother cell and forespore cytoplasmic components. With optimal recovery, forespore soluble protein accounts for about 8% of the total soluble protein of sporulating cells (13), so that the specific activities of enzymes found exclusively in forespore fractions were considerably lower in these whole cell fractions.

As forespores mature and develop refractility, they also develop resistance to sonic disruption and can be isolated intact by low-speed centrifugation after sonic treatment (13) of sporulating cells made fragile by pretreatment with lysozyme (11).

Forespores of increasing maturity (6) were isolated in this way from a single sporulating culture of *B. sphaericus*. Samples of cultures from mid to late sporulation were harvested at 4, 5.5, 6.5, and 8 h after the end of the exponential growth phase, when morphology had reached, respectively, 15, 50, 70, and 100% refractility. The mother cell fraction (6,000-×-*g* supernatant) was separated into 30,000-×-*g* (cell wall) and 130,000-×-*g* (membrane) particulate preparations and the final 130,000-×-*g* supernatant (cytoplasmic) preparation. Forespores and spores (6,000-×-*g* pellet) were washed by centrifugation until free of contamination by unbroken mother cells and cell debris, as determined by observation in a phase-contrast microscope, and were then broken in the Braun homogenizer. Differential centrifugations gave the same three enzyme fractions isolated from the mother cell fraction: 30,000-×-*g* (integument) and 130,000-×-*g* (membrane) particulate fractions and 130,000-×-*g* (cytoplasmic) supernatant fraction.

Levels of enzymes in whole cells at 5.5 and 8 h after the end of the exponential phase of sporulation. Essentially identical data were given by preparations made by the MSK or Ultra-Turrax procedures (Table 1).

LD-Dipeptidase activity was found only in the cytoplasmic preparation, and LD-carboxypeptidase was found only in particulate preparations. In contrast, both cytoplasmic and particulate (30,000-×-*g*) preparations had endopeptidase activity. Although total dipeptidase activity increased late in sporulation and LD-carboxypeptidase activity remained constant, endopeptidase activity declined at the end of sporulation, as previously shown (4).

Levels of enzymes in forespore and mother cell fractions as a function of time in growth. The results of the assays of these preparations for LD-carboxypeptidase, endopeptidase, and LD-dipeptidase activities are shown in Fig. 1 to 3, with data for mature spores included with the data on forespores.

For the LD-carboxypeptidase activity, data with substrate II exactly paralleled those obtained with substrate III, and data are given for only one of these substrates. As in preparations from whole sporulating cells (Table 1), the LD-carboxypeptidase was present only in the cell

TABLE 1. *Levels of enzymes in whole cells at 5.5 and 8 h after the end of the exponential phase of B. sphaericus growth*

Enzyme	Substrate	Product released	Sp act (nmol/mg per h)					
			Particulate preparation (30,000 × *g*)		Particulate preparation (130,000 × *g*)		Cytoplasmic preparation	
			$T_{5.5}$[c]	T_8	$T_{5.5}$	T_8	$T_{5.5}$	T_8
LD-Dipeptidase[a]	*ms*-A_2pm-D-[^{14}C]Ala (IV)	D-[^{14}C]alanine	0	0	0	0	95	230
LD-Carboxy-peptidase[b]	(ωNH_2)*ms*-A_2pm tetrapeptide (III)	D-[^{14}C]alanine	21	25	33	36	0	0
γ-D-Glu-*ms*-A_2pm endopeptidase[b]	MurNAc(*ms*-A_2pm) tetrapeptide (I)	*ms*-A_2pm-D-[^{14}C]Ala	38.5	24	1	2	24	16

[a] Preparation made with the Ultra-Turrax apparatus.
[b] Preparation made with the Braun homogenizer.
[c] T_n is the time (in hours) after the end of exponential phase.

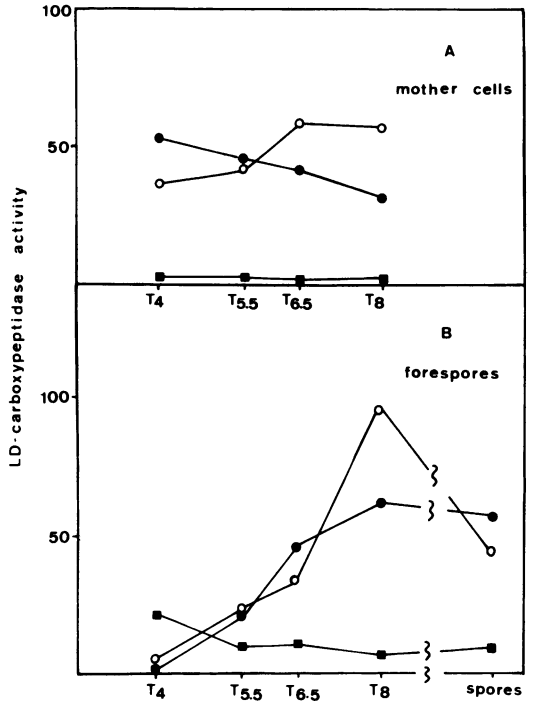

FIG. 1. *(A) LD-Carboxypeptidase activity with substrate II, MurNAc-L-Ala-γ-D-Glu-L-Lys-D-[^{14}C]Ala, in the mother cells of B. sphaericus. (B) LD-Carboxypeptidase activity with substrate III, L-Ala-γ-D-Glu-(L)ms-A_2pm-(ωNH_2)(L)-D-[^{14}C]Ala, in the forespores of B. sphaericus. Symbols: ○, particulate preparation (30,000 × g); ●, particulate preparation (130,000 × g); ■, cytoplasmic preparation. T_n values are the hourly periods after T_0, which represents the end of the exponential growth phase. Enzyme activity is expressed as nanomoles of D-[^{14}C]alanine released per milligram of protein per hour.*

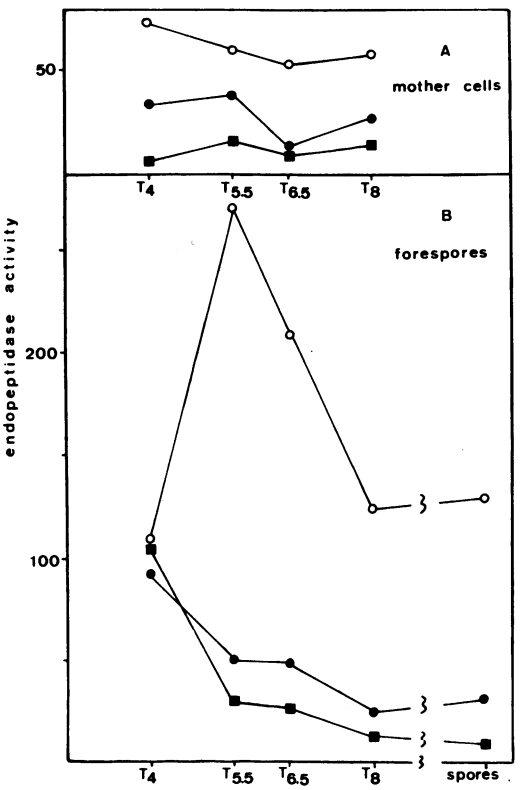

FIG. 2. *(A) Endopeptidase activity with substrate I, MurNAc-L-Ala-γ-D-Glu-(L)ms-A_2pm(L)-D-[^{14}C]-Ala, in the mother cells of B. sphaericus. (B) Endopeptidase activity with substrate I in the forespores of B. sphaericus. Symbols: ○, particulate preparation (30,000 × g); ●, particulate preparation (130,000 × g); ■, cytoplasmic preparation. Enzyme activity is expressed as nanomoles of ms-A_2pm-D-[^{14}C]Ala released per milligram of protein per hour.*

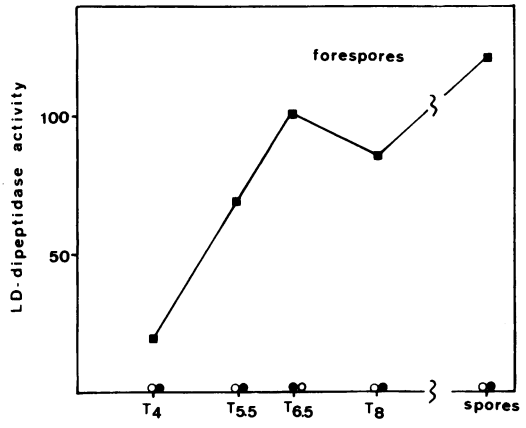

FIG. 3. *LD-Dipeptidase activity with substrate IV, ms-A2pm-D-[14C]Ala, in the forespores of B. sphaericus. Mother cell fractions had no activity. Symbols: ■, cytoplasmic preparation; ● and ○, particulate fractions. T_n values are the hourly periods after T_0, which represents the end of the exponential growth phase. Enzyme activity is expressed as nanomoles of D-[14C]alanine released per milligram of protein per hour.*

wall, integument, or membrane (particulate) preparations of the forespores, spores, or mother cell compartments (Fig. 1). Its activity was roughly constant in the mother cell but increased throughout sporulation in the forespores.

Although mother cell preparations contained some endopeptidase activity, this may have derived from contamination with fractions of forespores disrupted by sonic treatment, since the forespore fractions had a fivefold-higher specific activity (Fig. 2). Except for the earliest forespore fraction, most of this endopeptidase activity was found in particulate fractions, and more particularly in the 30,000-×-*g* precipitate, which consisted of fragmented forespore integuments. In the forespores the activity increased until 5.5 h after the end of the exponential phase and then declined, as already shown for whole cells (4). Mature spores retained about one-half of the maximum specific activity found in forespores (Fig. 2).

The LD-dipeptidase was located strictly in the cytoplasmic preparation from the forespores (Fig. 3). No activity was found in any of the particulate preparations or in the cytoplasm of the mother cells. Some contamination of this cytoplasmic fraction would be expected, but the dipeptidase activity is relatively labile (17) and low levels probably do not survive the sonic disruption of lysozyme-treated cells (see above).

The 30,000-×-*g* particulate preparation derived from mature spores should consist of integuments: fragments of spore coat and cortex which retain between them whatever remains of the outer forespore membrane. Association with and contamination by some of the inner spore membrane would also be expected. To distinguish between integument and inner membrane components, the 30,000-×-*g* particulate preparation was fractionated by centrifugation on a sucrose density gradient. The profile at an absorbance at 280 nm of the resulting sedimentation is given in Fig. 4. Two fractions were obtained. The more abundant, heavier fraction was essentially made of integuments, whereas the lighter fraction consisted of membrane vesicles, as seen by electron microscopy (data not shown). In each fraction, endopeptidase activity was qualitatively assayed with unlabeled substrate V (MurNAc-L-Ala-γ-D-Glu-*ms*-A_2pm) by measuring the concomitant release of *ms*-A_2pm. Endopeptidase was present only in the integument fraction.

DISCUSSION

LD-Carboxypeptidase activity appears to be located in the membrane and cell wall and integument fractions of both the mother cells and the forespores. The wall fractions are certainly

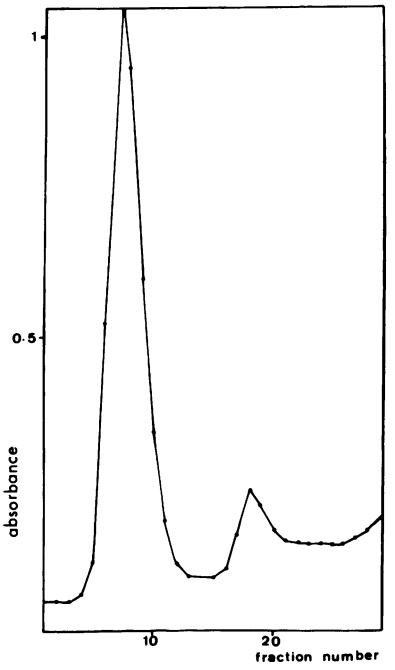

FIG. 4. *Fractionation of the 30,000-×-g envelope preparation from B. sphaericus spores on a discontinuous sucrose gradient (see text). The gradient was separated into fractions of 1 ml, and the absorbance of samples was read at 280 nm. Fraction 1 was the bottom of the tube.*

contaminated with membrane, which is the probable location. This enzyme attacks only uncross-linked peptide subunits of the vegetative cell wall peptidoglycan and only after their terminal D-alanine residues have been removed by DD-carboxypeptidase action, giving a tetrapeptide, as in substrate II. All of the un-cross-linked peptidoglycan subunits in vegetative cells of *B. sphaericus* are degraded in this way (7). Its presence in the mother cell cytoplasmic membrane is therefore expected and is consistent with its functions.

Activity of this enzyme increases during sporulation in forespore particulate preparations, although previous experiments (5) indicated that total activity in sporulating cells was roughly constant. However, since the present data show that mother cell activity is also constant, if the activity of mother cells and forespores is combined, an increase is seen during sporulation. The discrepancy may be explained by a decreasing efficiency of forespore breakage and consequent decrease in enzyme recovery as forespores mature, when the breakage technique previously employed for whole cells is used (5).

The presence of LD-carboxypeptidase activity in mature spores is consistent with a need for this activity in the synthesis and modification of vegetative-type peptidoglycan during the outgrowth of germinated spores. The high activity in forespores may be required for synthesis of the cell wall primordium, the inner layer of the cortical region of the spore wall, which is probably made of vegetative-type peptidoglycan (12, 15).

Endopeptidase activity is principally located in the forespore integument fraction; specific activities in other fractions are five times lower (Fig. 2). Endopeptidase is synthesized only in sporulating cells, reaching maximum activity at 5.5 h after the end of the exponential growth phase (4, 5). Its period of synthesis closely precedes and parallels spore cortex formation, which closely follows the development of refractility (6). The much lower endopeptidase activity found in other particulate forespore fractions and in the mother cell fractions is probably due to contamination, but the presence of cytoplasmic activity is not explained. The localization of endopeptidase activity in the 30,000-×-*g* particulate preparation was remarkable, since centrifugation under these conditions routinely sediments integuments, whereas centrifugation at 130,000 × *g* is required to sediment free cytoplasmic membrane fragments. In fact, a minor fraction of free membrane fragments was present with the 30,000-×-*g* pellet, although these fragments are probably not responsible for the endopeptidase activity, since no activity was found in the 130,000-×-*g* cytoplasmic membrane preparation. To verify this supposition, the 30,000-×-*g* preparation from spores was separated into integument and free membrane fractions by centrifugation on a sucrose density gradient. The endopeptidase activity was found exclusively in the heavier integument fraction, which consists of the coats, cortex, and entrained membranes.

Cortical peptidoglycan, but not vegetative cell wall peptidoglycan, is a substrate for the endopeptidase, the reverse of the substrate specificity of the LD-carboxypeptidase. Thus, the endopeptidase can only be involved in cortex metabolism. A potential role for this activity does exist. About 18% of the disaccharide subunits of the *B. sphaericus* spore cortex are substituted by single L-alanine residues (12), as in *B. subtilis* (19). If, as seems probable, the cortex is initially synthesized as a nascent polymer of disaccharide-pentapeptide subunits, the L-alanine C termini could be derived by sequential action of DD-carboxypeptidase, the endopeptidase, and an LD-carboxypeptidase capable of hydrolyzing the L-Ala-D-Glu product produced by the endopeptidase. No D-Glu C termini are found in the cortex, and an LD-carboxypeptidase with the requisite specificity has yet to be found in *B. sphaericus*.

The exclusive localization of *meso*-diaminopimelate ligase activity in the mother cell cytoplasm and the accumulation of L-lysine ligase activity in the forespore cytoplasm (13) suggest that the precursors of cortical and cell wall primordium peptidoglycans are made in the mother cell and forespore compartments, respectively. This is entirely consistent with the location of these two peptidoglycans in the spore (6) and the polarity and proposed functions of the forespore membranes (12, 13, 15). The cortex is immediately adjacent to the outer forespore membrane, which should be responsible for its synthesis from precursor made in the mother cell. The cell wall primoridum is immediately adjacent to the inner forespore membrane, which should be responsible for its synthesis from precursors made in the forespore. Since the outer forespore membrane becomes trapped between the cortex and coat layers of the developing spore, free fragments from this membrane may not be released during forespore breakage, whereas fragments from the inner forespore membrane would be produced. This could explain the differential distribution of endopeptidase and LD-carboxypeptidase activities in the integument and membrane fractions derived from forespores and spores. Alternatively, whereas the LD-carboxypeptidase is located in

the membrane, the endopeptidase may be bound to its cortex substrate.

The function of the dipeptidase is presumably to salvage the *meso*-diaminopimelate-D-alanine released from the cortex by endopeptidase action. The location of the dipeptidase in the forespore cytoplasm suggests that this soluble product of endopeptidase action diffuses into the forespore rather than into the mother cell. After hydrolysis, the free diaminopimelate could only be of use to the cell if it were decarboxylated to lysine or if it diffused back into the mother cell compartment for reincorporation into cortex precursors.

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