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## Appearance of a $\gamma$ -D-Glutamyl-(L)*Meso*-Diaminopimelate Peptidoglycan Hydrolase During Sporulation in *Bacillus sphaericus*

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Particulate preparations from sporulating cells of *Bacillus sphaericus* 9602 contained an endopeptidase activity that hydrolyzed the  $\gamma$ -D-glutamyl-(L)*meso*-diaminopimelic acid linkages found in the spore cortical peptidoglycan of this organism. Diaminopimelic acid did not occur in the vegetative cell wall peptidoglycan, and the  $\gamma$ -D-glutamyl-L-lysine linkages found in this polymer were not hydrolyzed by the endopeptidase. The endopeptidase hydrolyzed (X)-L-alanyl- $\gamma$ -D-glutamyl-(L)*meso*-diaminopimelyl(L)-D-alanyl-D-alanine only after removal of the terminal D-alanine residue. The preparations contained an acyl-D-alanyl-D-alanine carboxypeptidase I activity which converted such pentapeptides into substrates for the endopeptidase and which was inhibited 50% by  $4 \times 10^{-7}$  M benzylpenicillin. This activity also hydrolyzed the analogous pentapeptide substrates containing L-lysine. The preparations also contained an acyl-L-lysyl-D-alanine carboxypeptidase II activity that was not active on the *meso*-diaminopimelic acid-containing analogue. Neither this activity nor the endopeptidase was inhibited by  $10^{-3}$  M benzylpenicillin. The specificities of the carboxypeptidases were consistent with the exclusive presence of L-lysine C-termini in the vegetative peptidoglycan and of *meso*-diaminopimelyl-D-alanine C-termini in the spore cortical peptidoglycan of *B. sphaericus* 9602.

The vegetative cell wall peptidoglycan of *Bacillus sphaericus* strain 9602 consists of a glycan of alternating  $\beta$ -1,4-linked residues of *N*-acetylglucosamine (GlcNAc) and *N*-acetyl muramic acid (MurNAc), all of whose MurNAc residues are substituted by the peptide  $N^{\alpha}$ -L-alanyl-D-isoglutamyl- $N^{\epsilon}$ -(D-isosparaginy)l-L-lysine (see Fig. 1) 65% cross-linked between L-lysine and D-isosparagine residues by D-alanine residues. The C-terminal peptide subunits are devoid of D-alanine residues and therefore have C-terminal L-lysine residues (5). These vegetative cell walls are devoid of diaminopimelic acid. The glycan of the spore cortex peptidoglycan also consists of alternating GlcNAc and muramic acid residues, but 54% of the latter are lactamized, 18% carry a single L-alanine residue, and only the residual 28% carry a tetrapeptide, which has the sequence L-alanyl-D-isoglutamyl-(L)*meso*-diaminopimelyl(L)-D-alanine (see Fig. 1). These tetrapeptides are cross-linked 20% between D-alanine and (D)*meso*-diaminopimelic acid (*m*-DPM). This structure is probably devoid of L-lysine and is virtually identical to that found in *Bacillus subtilis* spore cortex (18; D. J. Tipper, *Bacteriol. Proc.*, p. 24,

1969; D. J. Tipper and L. Landbeck, manuscript in preparation).

It has previously been demonstrated that the uridine 5'-diphosphate (UDP)-*N*-acetylmuramyl-L-alanyl-D-glutamate:*m*-DPM ligase necessary for synthesizing the nucleotide pentapeptide precursor of cortical peptidoglycan is absent from vegetative cells and is synthesized in sporulating cells shortly before the appearance of cortical peptidoglycan during synchronous sporulation (16). It has also been demonstrated (D. J. Tipper and P. E. Linnett, manuscript in preparation) that this enzyme activity accumulates in the mother cell cytoplasm of the sporulating cell, but not in its forespores.

After formation of the sporulation septum during the process of bacterial endospore formation, the directed proliferation of membrane results in engulfment of the forespore, which becomes separated from the mother cell cytoplasm by two opposed membranes, the inner and outer forespore membranes (reviewed in 12, 15). In *B. sphaericus*, the engulfment process takes place during the latter stages of the terminal swelling which occurs approximately between hours 2 and 3 of sporulation (J. Gau-

thier, D. J. Tipper, and S. C. Holt, manuscript in preparation). Germ cell wall is laid down between these membranes adjacent to the inner forespore membranes, and cortex is laid down between the germ cell wall and the outer forespore membrane. Since all of the outer forespore membrane is elaborated de novo either during the process of spore septum formation or during the subsequent engulfment process, it would not be surprising to find that this membrane contained components unique to sporulating cells.

The most reasonable hypothesis for the mechanism of synthesis of the unique muramic lactam and C-terminal L-alanine components of the spore cortex (reviewed in 15, 18) states that they are the result of modifications of a polymer of disaccharide pentapeptide, synthesized from precursors produced in the mother cell cytoplasm by enzymes within the outer forespore membrane. One would expect these unique modification enzymes to be located within the outer forespore membrane and, more precisely, on its cortical side.

We began our investigation in the hope of demonstrating unique enzymes in sporulating cells that would be responsible for producing the disaccharide-L-alanine moieties present in the cortical peptidoglycan. The isolation of forespores and of forespore membranes is difficult except under unusual circumstances and has not been successfully achieved in *B. sphaericus*. We have investigated the presence, within the total membrane fraction of sporulating cells of *B. sphaericus*, of enzymes capable of hydrolyzing cortical peptidoglycan components. A unique enzyme activity has been found in the particulate preparations from sporulating cells which results in the formation of C-terminal D-glutamate residues, an activity which could be involved in subsequent liberation of C-terminal L-alanine residues, although such an event has not been demonstrated. No evidence was found for activities causing the production of muramic lactam residues.

As part of these studies, we also investigated the mechanisms which determine the presence of C-terminal lysine residues in the vegetative peptidoglycan and of C-terminal D-alanine residues in tetrapeptides of the cortex. Our data on the specificities of the D-alanine carboxypeptidases in these preparations is quite consistent with the C-terminal structures of the vegetative and cortical peptidoglycans. The sites in the nucleotide pentapeptide precursors of vegetative cell wall and spore cortex peptidoglycans hydrolyzed by these activities (and also by MurNAc-L-Ala amidase) are indicated in Fig. 1.

(This paper was presented in part at the 1st International Congress of Bacteriology, September 1973, Jerusalem, Israel, and at the Colloque Internationale du CNRS on Regulation de la Sporulation Bacterienne, September 1973, Gif-sur-Yvette, France.)

## MATERIALS AND METHODS

**Chemicals.** [ $^{14}\text{C}$ ]alanine (162 and 63 mCi/mmol) was purchased from New England Nuclear Corp. and from the Commissariat a l'Energie Atomique (Saclay, 91 France). All other chemicals or antibiotics were commercially available.

**Enzymes.** The following enzymes, which hydrolyze specific linkages within cell wall peptidoglycan and its precursors, were used: egg white lysozyme, an endo-N-acetylmuramidase hydrolyzing  $\beta$ -1,4-N-acetylmuramyl-N-acetylglucosamine (MurNAc-GlcNAc) linkages (Sigma Chemical Co., St. Louis, Mo.); *Streptomyces albus* G N-acetylmuramyl-L-alanine amidase, which hydrolyzes the bond between GlcNAc-MurNAc disaccharides and peptides, liberating disaccharides and free peptides (3), and the *Streptomyces* R39 DD-carboxypeptidase, which hydrolyzes C-terminal D-alanyl-D linkages (10). The latter two enzymes were kindly provided by J. M. Ghuysen.

**Analytical methods.** Quantitative analyses of amino acids were carried out with either a Beckman or a Technicon amino acid analyzer or by the fluorodinitrobenzene technique (4). Protein content was determined by the method of Lowry et al. (11), with crystalline bovin serum albumin as standard. Uridine content was estimated by measuring the absorbance of products in water at 262 nm, assuming a molar absorbance of  $10^4$ . Amino terminal groups were determined according to the fluorodinitrobenzene method (4).

Paper electrophoreses were carried out on Whatman no. 1 and 3MM papers at pH 4.1 in pyridine-acetic acid-water (2.5:9:1,000, vol:vol:vol) at 40 V/cm, using either a Gilson model D or a Pherograph apparatus.

Thin-layer chromatography was performed on cellulose powder MN 300 G in the following solvents: (A) 1-butanol-acetic acid-pyridine-water (30:6:20:24); (B) ethyl acetate-pyridine-acetic acid-water (18:50:4:28); and (C) pyridine-*tert*-amyl alcohol-water (35:35:30).

Radioactive spots were detected by autoradiography with Kodirex films (Kodak, Inc.). The radioactive areas were removed from the plates, and the powder was homogenized in a scintillation liquid containing, in 1 liter of toluene, 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-[2]-(5-phenyloxazolyl)benzene. For suspension of the powder, a Polyson Ultraschall sonicator was used. Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer (model 2010).

With nonradioactive substrates, the endopeptidase activity was quantitated by the appearance of amino terminal groups (4) and followed qualitatively by the appearance of ninhydrin-positive spots after chromatography.

**Substrates.** Uridine-diphospho-N-acetylmuramyl-

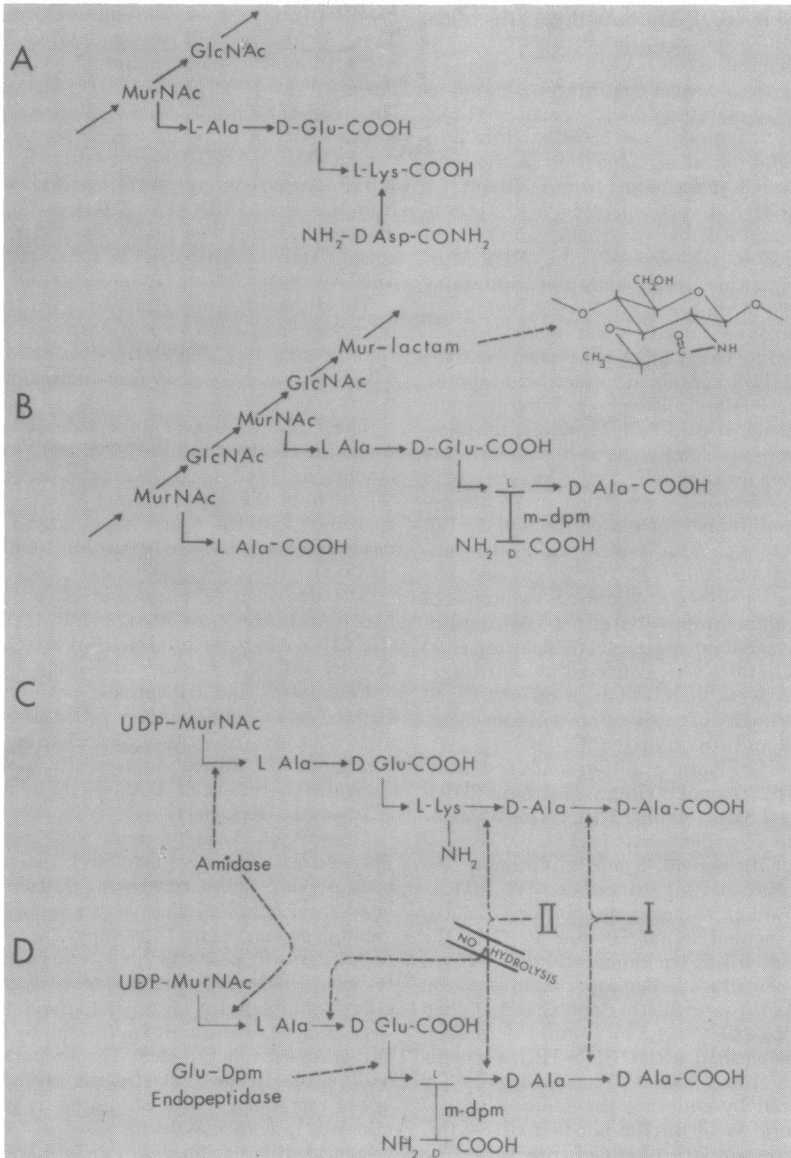


FIG. 1. (A) Structure of the uncross-linked monomer of mature *Bacillus sphaericus* vegetative cell wall peptidoglycan. A D-alanyl residue cross-links such tetrapeptides between L-lysine and D-isoasparagine. (B) The MurNAc-L-Ala, MurNAc tetrapeptide and muramic lactam subunits of the spore cortex peptidoglycan. The girder-like unit is m-DPM with its L and D centers arranged as indicated. The insert shows muramic lactam in  $\beta$ -1,4 linkage to adjacent GlcNAc residues (not shown). (C) The UDP-MurNAc pentapeptide precursor of structure A. (D) The precursor of structure B. The bonds in these precursors and their partial hydrolysis products (not shown, see text) hydrolyzed by DD-carboxypeptidase I (I), L, D-carboxypeptidase II (II), the Glu-DPM endopeptidase and amidase are indicated. The L-D bonds not split by carboxypeptidase II (when the D-residue is carboxy-terminal) are also indicated. Hydrolysis of these precursors by amidase or Glu-DPM endopeptidase requires prior removal of their UDP moieties.

L-alanyl- $\gamma$ -D-glutamyl-L-lysyl-D-alanyl-D-alanine (UDP-MurNAc-[Lys] pentapeptide) was isolated from *S. aureus* strain Copenhagen after incubation in the presence of benzylpenicillin (6).

Uridine-diphospho-N-acetylmuramyl-L-alanyl- $\gamma$ -

D-glutamyl-(L)meso-diaminopimelyl(L)-D-alanyl-D-alanine (UDP-MurNAc-[DPM] pentapeptide) was isolated from *B. cereus* T after incubation in the presence of ristocetin (7).

Uridine-diphospho-N-acetylmuramyl-L-alanyl- $\gamma$ -

D-glutamyl-L-lysine (UDP-MurNac-[Lys] tripeptide) was isolated from *S. aureus* after incubation in the presence of D-cycloserine (6).

Uridine-diphospho-*N*-acetylmuramyl-L-alanyl- $\gamma$ -D-glutamyl-(L)-*meso*-diaminopimelic acid (UDP-MurNac-[DPM] tripeptide) was isolated from *Lactobacterium plantarum* after incubation in the presence of D-cycloserine. It was a gift from R. Plapp.

UDP-MurNac-(DPM) pentapeptide D-[ $^{14}$ C]Ala-D-[ $^{14}$ C]Ala was obtained by enzymatic addition of D-alanyl-D-alanine to the corresponding UDP-MurNac-(DPM) tripeptide. A preparation containing alanine racemase, D-Ala-D-Ala synthetase, and D-Ala-D-Ala adding enzyme activities was made from *B. sphaericus* according to the following procedure. Cells were grown until 30% contained refractile forespores. The cells from a 900-ml culture were harvested and suspended in 20 ml of cold 0.01 M potassium phosphate buffer, pH 7.5. Samples of cell suspension (4 ml) were disrupted for 10 min by using the microtip of a Branson J 17-V sonifier. Immersion in an alcohol bath at  $-10$  C maintained the temperature of the cell suspension at 0 to 4 C. The broken-cell suspensions were centrifuged (15 min,  $25,000 \times g$ ), and the supernatant fractions were mixed with 3 volumes of cold, saturated ammonium sulfate (pH 7.5) containing  $10^{-3}$  M ethylenediaminetetraacetic acid. After 30 min at 0 C, precipitates were collected by centrifugation, and pellets were dissolved in 2 ml of 0.01 M phosphate buffer, pH 7.5, to give a protein concentration of 15 mg/ml and stored at  $-20$  C.

The incubation for alanine addition contained, in 10 ml: 100 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 8.5), 100 mM KCl, 25 mM  $MgCl_2$ , 25 mM adenosine 5'-triphosphate (pH 6), 0.005 mg of pyridoxal phosphate per ml, 3 mM glutathione, 0.5 mM UDP-MurNac-(DPM) tripeptide, 1.2 mM L-[ $^{14}$ C]alanine (100  $\mu$ Ci), and enzyme solution (75 mg of protein). After incubation for 60 min at 37 C, the solution was made 5% in trichloroacetic acid, stirred for 30 min, and centrifuged. The supernatant fraction was extracted six times with ether at 0 C and applied to a column of Dowex 1  $\times$  2 (1.5 by 30 cm) in 0.01 HCl. The column was washed with 0.01 N HCl; then the nucleotides were eluted with 0.15 M NaCl in 0.01 N HCl and detected by counting the radioactivity of samples. The total yield for the purified UDP-MurNac pentapeptide was 70% of added tripeptide. The nucleotide product was used as a 3.4-mM solution at a specific radioactivity of  $1.6 \cdot 10^7$  counts per min per  $\mu$ mol.

UDP-MurNac-(Lys) pentapeptide D-( $^{14}$ C)Ala-D-( $^{14}$ C)Ala was prepared in the same way from the corresponding UDP-MurNac-(Lys) tripeptide. The final 0.1-mM solution had a specific radioactivity of  $5 \cdot 10^8$  counts per min per  $\mu$ mol.

UDP-MurNac-L-Ala- $\gamma$ -D-Glu-(L)-*m*-DPM(L)-D-[ $^{14}$ C]Ala (UDP-MurNac [DPM] tetrapeptide) was obtained from the corresponding labeled UDP pentapeptide. The removal of the C-terminal D-[ $^{14}$ C]alanine was performed with the D-alanyl-D-alanine carboxypeptidase of *Streptomyces* R39: 200 nmol of UDP-MurNac-(DPM) pentapeptide labeled on both D-alanine residues (specific radioactivity,  $2.5 \times 10^8$  counts per min per  $\mu$ mol) was treated in 20 mM Tris buffer (pH

8) with the R39 carboxypeptidase. The resulting UDP-MurNac tetrapeptide was separated from the free D-[ $^{14}$ C]alanine by passage through a column of Sephadex G-15 (1.5 by 16 cm) in water. The total yield was 90%, and the final 1.25-mM solution had a specific radioactivity of  $1.1 \times 10^8$  counts per min per  $\mu$ mol.

UDP-MurNac-L-Ala- $\gamma$ -D-Glu-L-Lys-D-[ $^{14}$ C]Ala (UDP-MurNac-[Lys] tetrapeptide) was obtained from the labeled UDP-MurNac(Lys) pentapeptide as described above. The final 0.85-mM solution had a specific radioactivity of  $1.5 \times 10^8$  counts per min per  $\mu$ mol.

The different *N*-acetylmuramyl pentapeptides and tetrapeptides (MurNac peptides) were obtained from the corresponding UDP derivatives by hydrolysis with 0.02 N HCl at 100 C for 7 min followed by neutralization.

The DPM or Lys pentapeptides labeled on both D-alanine residues and the DPM or Lys tetrapeptides labeled on their single D-alanine residue were obtained from the corresponding MurNac peptides by treatment with the *N*-acetyl-muramyl-L-alanine amidase from *Streptomyces albus* G in 10 mM Tris buffer (pH 8.8).

The disaccharide tetra- and tripeptides GlcNac-MurNac-L-Ala- $\gamma$ -D-Glu-(L)-*m*-DPM(L)-D-Ala and GlcNac-L-Ala- $\gamma$ -D-Glu(L)-*m*-DPM were isolated from the cell wall of *B. megaterium* (17).

Free tetra- and tripeptides, L-Ala- $\gamma$ -D-Glu-(L)-*m*-DPM(L)-D-Ala and L-Ala- $\gamma$ -D-Glu-(L)-*m*-DPM were also obtained from two disaccharide peptides by treatment with *N*-acetylmuramyl-L-alanyl amidase.

Synthetic tripeptide L-Ala- $\alpha$ -Glu-(L)-*m*-DPM was a gift from P. Dezelee (17).

**Growth of *B. sphaericus* 9602 and harvesting procedures.** The organism and the medium have been previously described (16). Cultures were inoculated from heat-activated spores grown at 32 C with vigorous aeration and transferred twice in mid-exponential growth phase before sporulation was allowed to occur. This gives rise to highly synchronous and almost complete (at least 95%) sporulation. Samples were assayed for turbidity at 600 nm by using a Jouan Junior spectrophotometer. The proportion of terminally swollen cells and refractile prespores (percentage of morphology) was estimated by phase-contrast microscopy with a Wild microscope. Cells were harvested at different phases of growth and sporulation. They were rapidly chilled and immediately centrifuged in a Jouan K 63 F centrifuge. The cell pastes were washed with 10 mM Tris buffer (pH 8) containing 20 mM  $MgCl_2$  and 0.1 mM dithiothreitol (DDT).

**Particulate enzyme preparations.** A 1.3-g amount of each cell sample was shaken with 13 ml of glass beads (0.17-mm diameter) and 5 ml of 10 mM Tris buffer (pH 8), 20 mM  $MgCl_2$ , 0.1 mM dithiothreitol in a Braun MSK homogenizer. Cells were usually 90% broken in 5 min. The pellets sedimenting in 1 h between 6,000 and  $30,000 \times g$  were homogenized in the same Tris buffer as above to give a protein concentration of 8.5 mg/ml.

**Assays for endopeptidase and carboxypeptidase activity.** (i) **Assays with radioactive substrates.** The assays for the maximal release of D-[ $^{14}$ C]alanine and *m*-DPM(L)-D-[ $^{14}$ C]Ala from MurNac-(*m*-DPM)

pentapeptide or tetrapeptide contained, in 30  $\mu$ liters: 0.04 mM substrate, 15 mM Tris buffer (pH 8), 8 mM  $MgCl_2$ , and 10 to 40  $\mu$ g of protein per nmol of substrate. Incubations were performed at 37 C for 15 h. The reaction was stopped by immersion in a boiling water bath for 3 min. After centrifugation the supernatant fluid was analyzed by thin-layer chromatography on cellulose plates in solvent A.

The assays for the release of free D-[ $^{14}C$ ]Ala or of Lys-D-[ $^{14}C$ ]Ala from MurNAc-(Lys) pentapeptide or tetrapeptide were performed in an identical fashion, except that final chromatography was performed in solvent (ii), in which the relative mobilities of substrate, free alanine, and dipeptide Lys-Ala are 100, 75, and 55, respectively.

The radioactive spots were detected by autoradiography, removed from the support, and counted. Activities were expressed as percentage of radioactivity liberated as alanine or *m*-DPM-Ala.

The specific activity of the endopeptidase on the radioactive MurNAc-(*m*-DPM) tetrapeptide and on the radioactive tetrapeptide Ala-Glu-*m*-DPM-Ala was determined as follows. In 20  $\mu$ liters, 12.5 mM substrate, 11 mM Tris buffer (pH 8), 5 mM  $MgCl_2$ , and 7  $\mu$ g of protein per nmol of substrate were incubated at 37 C for 2 h. The specific activities were expressed as nanoequivalents of Glu-*m*-DPM linkages hydrolyzed per milligram of protein per hour.

(ii) **Assays with unlabeled substrates.** The assays for the release of DPM-Ala from MurNAc, or disaccharide, or free tetrapeptides or of DPM from MurNAc, disaccharide, or free tripeptides or of glutamate from D- or L-glutamine contained, in 20  $\mu$ liters: 0.2 mM peptide or glutamine, 10 mM Tris buffer (pH 8), 6 mM  $MgCl_2$ , and 8.5  $\mu$ g of enzyme protein per nmol of substrate. Incubations were performed at 37 C for 6 h. The products of the reaction were analyzed by cellulose thin-layer chromatography in solvents A and B for the peptide substrates and in solvent C for the glutamine substrates. The spots were detected with ninhydrin reagent. The dipeptide *m*-DPM-Ala has a specific orange coloration that clearly distinguishes it from the peptide substrate, which gives a mauve coloration, after subsequent treatment with an acetone solution of  $Cu(NO_3)_2$  (5 g/liter) containing concentrated  $HNO_3$  (0.1 ml/liter).

(iii) **Assays with L- $\gamma$ -glutamyl-p-nitroanilide.** The glutamyl endopeptidase was assayed by the procedure of Orłowski and Meister (13, 14), using L- $\gamma$ -glutamyl-p-nitroanilide. The enzymatic release of p-nitroaniline was followed at 410 nm. The assay for the release of p-nitroaniline contained, in 300  $\mu$ liters: 0.2 mM L- $\gamma$ -glutamyl-p-nitroanilide, 10 mM Tris buffer (pH 8), 7 mM  $MgCl_2$ , and 14  $\mu$ g of protein per nmol of substrate. Incubations were performed at 37 C for 6 h; then 600  $\mu$ liters of 1.75 N acetic acid was added to stop the reaction. After centrifugation the absorbance was measured at 410 nm in comparison with absorbance of increasing amounts of p-nitroaniline ranging from 12 to 60 nmol.

## RESULTS

**Enzyme preparations.** The growth curve of *B. sphaericus* is shown in Fig. 2. The formation

of the spore cortex was followed by estimating the proportion of terminally swollen cells and refractile forespores. Six samples were harvested: I at the mid-log phase; II at the start of sporulation after completion of vegetative division and just before the first appearance of terminal swelling; III at the time of spore engulfment; and IV, V, and VI when 40, 70, and 100%, respectively, of cells contained refractile forespores. The corresponding particulate enzyme preparations were obtained as described in Materials and Methods. All these preparations were assayed for their endopeptidase and carboxypeptidase activities.

The release of *m*-DPM-D-Ala from MurNAc-(DPM) tetrapeptide by enzyme preparation V (30  $\mu$ g of protein per nmol of substrate), under the standard assay conditions described in Materials and Methods, was linear with time until 60% of the substrate had been hydrolyzed (2 h), and was 90% complete by 4.5 h. The hydrolysis of this substrate during incubation for a fixed time (2 h) under these conditions was propor-

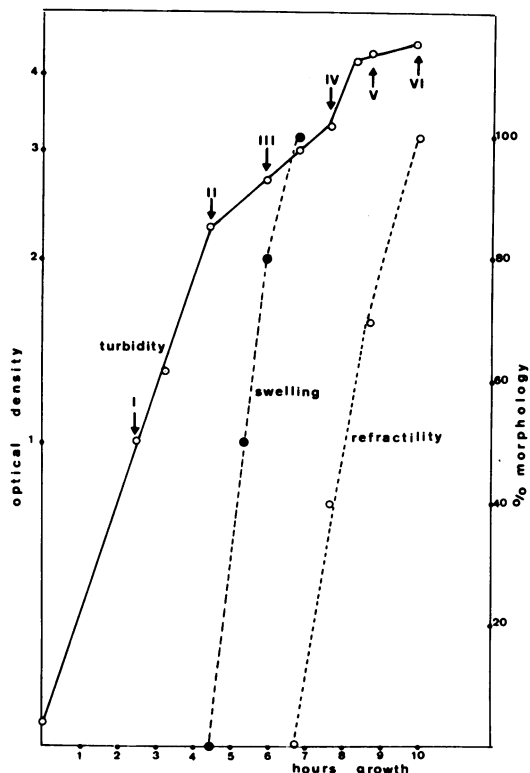


FIG. 2. Growth curves of *Bacillus sphaericus* 9602. Samples were assayed for turbidity at 600 nm (O), for the percentage of terminally swollen cells (●), and for the percentage of cells containing refractile forespores (○). Samples I to VI were harvested at the indicated times.

tional to the amount of enzyme V preparation added over the range from 3 to 30  $\mu\text{g}$  of protein per nmol of substrate.

**Assays on DPM-containing MurNac penta- or tetrapeptides.** The ability of the six particulate preparations (I to VI) to hydrolyze MurNac(DPM) pentapeptide D- $^{14}\text{C}$ Ala-D- $^{14}\text{C}$ Ala was determined (Fig. 3). The autoradiogram (Fig. 3A) illustrates the separation of labeled products (free alanine and *m*-DPM-D-Ala) from substrate. This experiment resulted in only about 25% hydrolysis of the substrate, since the incubation conditions were suboptimal (2 mM  $\text{Mg}^{2+}$ ; 2  $\mu\text{g}$  of enzyme per nmol of substrate). After incubation, the boiled enzyme control contained only the substrate. Under optimal conditions (see Materials and Methods), all enzyme hydrolyzed approximately 84% of the substrate to produce free alanine (D-alanyl-D-alanine carboxypeptidase activity) since, of the total radioactivity present in the reaction mix after such incubation, an average of 42% was present as free alanine (Fig. 3B), and complete release of the terminal D-alanine from the pentapeptide substrate would release 50% of its radioactivity as free alanine. The enzymes from sporulating cells also produced increasing amounts of a second product, which was identified as the dipeptide DPM-Ala; on hydrolysis it gave equimolar amounts of DPM and alanine, it was electrophoretically neutral, and DPM was demonstrated to be *N*-terminal by dinitrophenylation. No free DPM-D-Ala-D-Ala was seen, and the amount of free D-alanine always exceeded the amount of dipeptide liberated (Fig. 3B). Thus, the particulate preparations from *B. sphaericus* exhibit two activities on the spore cortex precursor: a D-alanyl-D-alanine carboxypeptidase and a Glu-DPM endopeptidase activity.

The penicillin sensitivity of these enzymes was determined by using preparation V (which is one of the most active) and the radioactive DPM pentapeptide precursor (see Fig. 4A). Both activities were 100% inhibited by  $10^{-5}$  M benzylpenicillin and were 12% inhibited by  $2.10^{-8}$  M penicillin (Fig. 4A). Fifty percent inhibition was obtained with  $4.10^{-7}$  M penicillin (not shown). Benzylpenicillin is known to inhibit D-alanyl-D-alanine carboxypeptidases of various bacterial species (7-10), and this is apparently also true in *B. sphaericus*, since no free alanine was produced in the presence of  $10^{-5}$  M benzylpenicillin. It was, therefore, possible that the inhibition of the Glu-DPM endopeptidase activity by benzylpenicillin was due to the inability of the enzyme to hydrolyze MurNac pentapeptide unless it had first been

hydrolyzed to MurNac tetrapeptide by the DD-carboxypeptidase, which would also account for the lack of a DPM-D-Ala-D-Ala product. This possibility was tested by following the action of preparation V on the MurNac-(DPM) tetrapeptide in the presence of various amounts of benzylpenicillin. The release of the dipeptide DPM-Ala was followed by autoradiography. The hydrolysis of the MurNac tetrapeptide was not affected by even  $10^{-3}$  M benzylpenicillin (Fig. 4B). No free D-alanine was released, so no activity capable of hydrolyzing DPM-D-alanine linkages was detected.

**Assays on Lys-containing MurNac penta- or tetrapeptides.** The specificity of both carboxypeptidase and endopeptidase was investigated with lysine-containing substrates. MurNac(Lys) pentapeptide-D- $^{14}\text{C}$ Ala-D- $^{14}\text{C}$ Ala was hydrolyzed by all of the six particulate enzyme preparations by D-alanine carboxypeptidase activities with the release of D- $^{14}\text{C}$ Ala only. No radioactive spot of a dipeptide L-Lys-D- $^{14}\text{C}$ Ala was ever detected. The release of D-Ala was completely inhibited by  $10^{-5}$  M benzylpenicillin. Again only D- $^{14}\text{C}$ Ala was released from MurNac(Lys) tetrapeptide labeled in D-alanine. By definition, this release must be due to a D-alanine carboxypeptidase II activity. No L-Lys-D-Ala was released from the tetrapeptide, and the carboxypeptidase II was not inhibited by even  $10^{-3}$  M benzylpenicillin. Thus at least two carboxypeptidases are present in these particulate preparations: a penicillin-sensitive carboxypeptidase I or acyl-D-alanyl-D-alanine carboxypeptidase and a penicillin-resistant carboxypeptidase II that catalyzes the hydrolysis of the L-lysyl-D-Ala bond. The carboxypeptidase I activity acts either on DPM- or lysine-containing substrates. The carboxypeptidase II activity acts only on the lysine-containing substrates.

Since no Lys-Ala peptide was detected, the  $\gamma$ -D-glutamyl endopeptidase does not act on lysine-containing precursors and seems specific for the Glu-DPM linkages. The properties of this Glu-DPM endopeptidase were further investigated.

**Effect of pH and ionic concentration on the endopeptidase and carboxypeptidase I activities.** The pH, ionic strength, and divalent metal ion optima of the endopeptidase and carboxypeptidase I activities of preparation V are very similar, using MurNac-(DPM) pentapeptide as substrate (Fig. 5). These optima are pH 8,  $\geq 15$  mM Tris buffer, and  $\geq 8$  mM  $\text{MgCl}_2$ .

Preparation V was also assayed with the MurNac-(DPM) tetrapeptide as substrate in the presence of several metal ions at various concentrations (Table 1).  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ , and

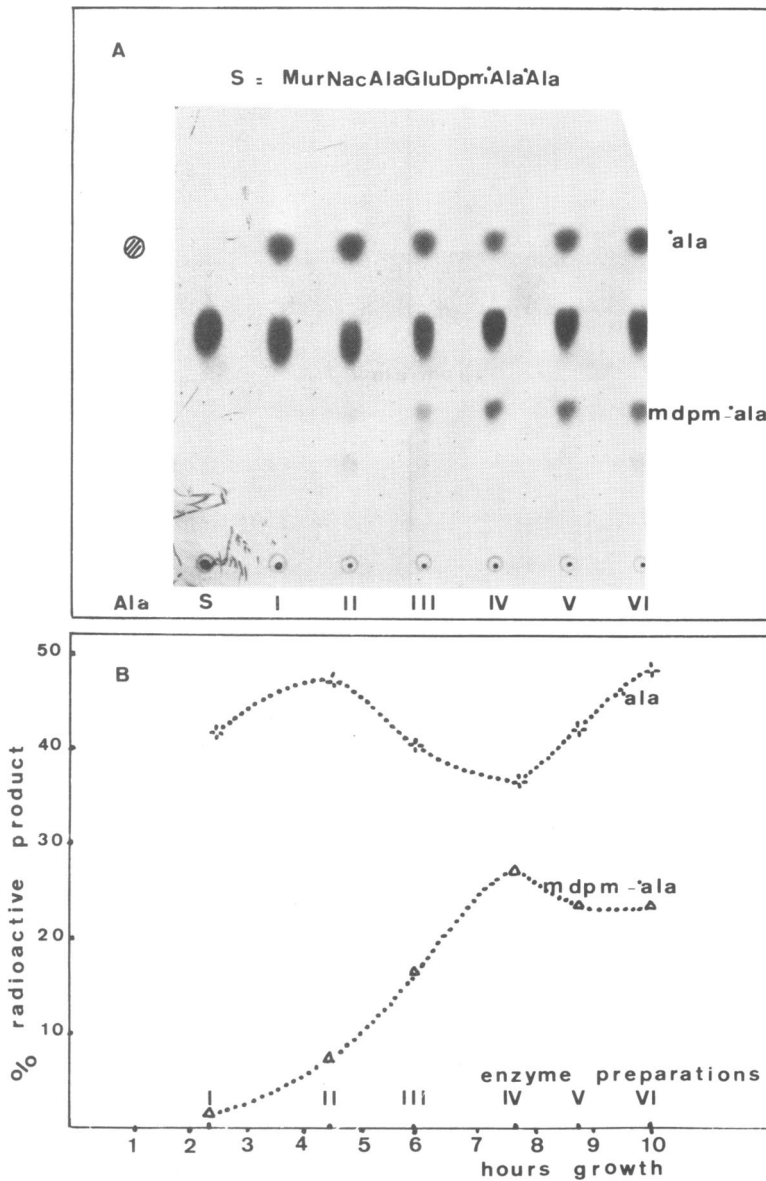


FIG. 3. Variation in the activity of the *D*-alanyl-*D*-alanine carboxypeptidase and of the Glu-DPM endopeptidase during sporulation in *B. sphaericus* with the MurNac-(DPM) pentapeptide as substrate. The  $^{14}\text{C}$  label (\*) was equally distributed between the two *D*-alanine residues. (A) Autoradiogram of a thin-layer chromatogram in solvent A. From left to right samples were: alanine (Ala); substrate with a boiled enzyme control (S); and substrate S with, in linear succession, the preparations I through VI. Incubation was for 10 h, the chromatogram was run for 6 h, and the autoradiogram was exposed for 36 h. (B) Release of free alanine (—) and of the dipeptide DPM-Ala ( $\Delta$ ) expressed as percentage of radioactive product. This indicates the percent of the total radioactivity (in *D*-alanine) present in the initial MurNac pentapeptide substrate, MurNac tetrapeptide (not shown), *m*-DPM-*D*-Ala, and free *D*-alanine at the end of the incubation. These data are from an experiment in which hydrolysis (10 h of incubation) was more complete than that illustrated in Fig. 3A. Note that, after complete carboxypeptidase I action, 50% of the radioactivity would have been in free alanine.

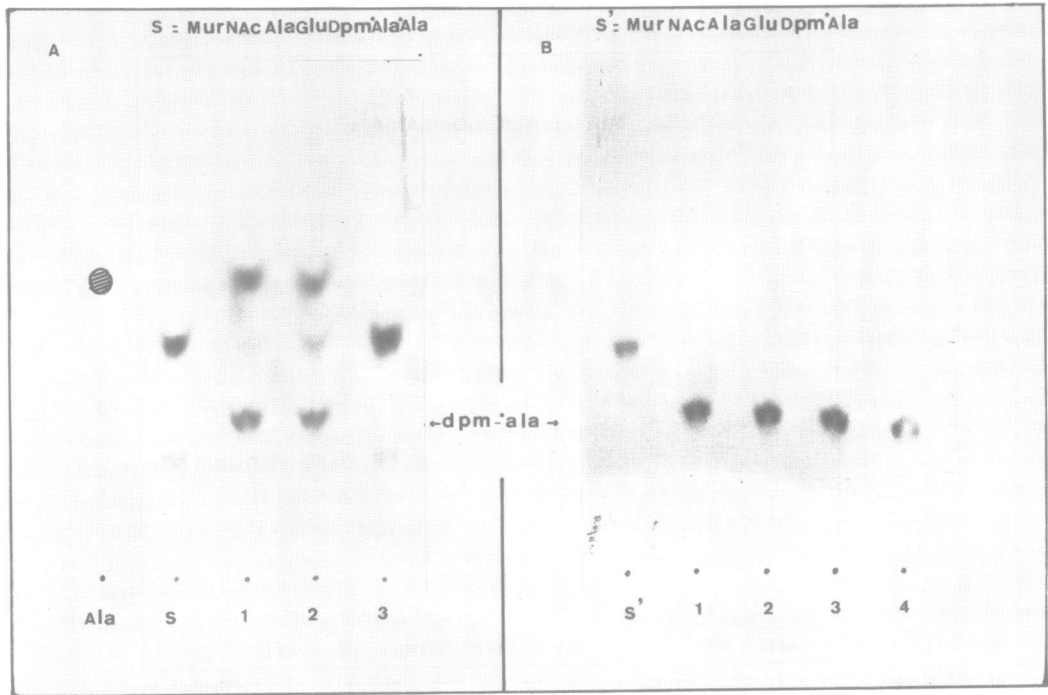


FIG. 4. Penicillin sensitivity of the *D*-alanyl-*D*-alanine carboxypeptidase and of the *Glu*-*DPM* endopeptidase. Autoradiograms of thin-layer chromatograms in solvent A. (A) Action of enzyme preparation V on *MurNac*-(*DPM*) pentapeptide. From left to right samples were: Ala; alanine; S; substrate with boiled enzyme (control); 1, substrate with enzyme V; 2, substrate with enzyme V and  $2 \times 10^{-8}$  M benzylpenicillin; 3, substrate with enzyme V and  $10^{-5}$  M benzylpenicillin. (B) Action of enzyme V on *MurNac*(*DPM*) tetrapeptide (*S'*). Samples were: *S'*; substrate with boiled enzyme (control); 1, substrate with enzyme V; 2, substrate with enzyme V +  $10^{-7}$  M benzylpenicillin; 3, substrate with enzyme V +  $10^{-5}$  M benzylpenicillin; 4, substrate with enzyme V +  $10^{-3}$  M benzylpenicillin.

$\text{Co}^{2+}$  activated the enzyme. This activation was 50% in  $8.10^{-3}$  M  $\text{Mg}^{2+}$ ,  $10^{-3}$  M  $\text{Mn}^{2+}$ , or  $10^{-4}$  M  $\text{Co}^{2+}$ . Higher concentrations of  $\text{Co}^{2+}$  and  $\text{Ca}^{2+}$  were inhibitory.  $\text{K}^{+}$  had no significant stimulation.

The endopeptidase activity was quickly lost in *N*-2-hydroxyethyl piperazine-*N'*-2'-ethanesulfonic acid buffer (pH 8).

**Specificity profile of the *Glu* *DPM* endopeptidase.** The best substrate is *MurNac*-(*DPM*) tetrapeptide, on which preparation V had a specific activity of 56 neq of *Glu*-*DPM* linkages hydrolyzed per mg of protein per h. The effect of preparation V on other substrates is shown in Fig. 5. Disaccharide tetrapeptide *GlcNac*-*MurNac*-*L*-*Ala*- $\gamma$ -*D*-*Glu*-*m*-*DPM*-*D*-*Ala* was also a good substrate, *m*-*DPM*-*D*-*Ala* being released, and the tetrapeptide *L*-*Ala*-*D*-*Glu*-*m*-*DPM*-*D*-*Ala* was split into its constituent dipeptides, *L*-*Ala*-*D*-*Glu* and *m*-*DPM*-*D*-*Ala*, with a specific activity of 45 neq per mg per h.

The tripeptide *L*-*Ala*-*D*-*Glu*-*m*-*DPM*, the *MurNac* tripeptide *MurNac*-*L*-*Ala*-*D*-*Glu*-*m*-*DPM*, and the disaccharide-tripeptide *GlcNac*-

*MurNac*-*L*-*Ala*-*D*-*Glu*-*m*-*DPM* were all good substrates: in each case free *m*-*DPM* was released (Fig. 6). In contrast, the synthetic peptide *L*-*Ala*- $\alpha$ -*D*-*Glu*-(*L*)-*m*-*DPM* was not hydrolyzed and *D*-glutamine was not a substrate since it was not hydrolyzed to glutamic acid. Similarly *L*-glutamine and *L*- $\gamma$ -glutamyl-paranitroanilide were not hydrolyzed.

**Activity of the  $\gamma$ -*D*-*Glu*-*m*-*DPM* endopeptidase as a function of growth phase in *B. sphaericus*.** The enzyme activity was studied as a function of growth phase in *B. sphaericus*, with the *MurNac* tri- and tetrapeptides as substrates. The endopeptidase activity was linearly proportional to the amount of enzyme added and to the time of incubation over the range used in these assays (see Materials and Methods). The release of the dipeptide *m*-*DPM*-*Ala* or of free *m*-*DPM* was negligible in sample I and in several other preparations from vegetative cells. Dipeptide release increased rapidly in sporulating cells to reach a maximum when 40 to 70% of forespores were refractile (Fig. 7). It subsequently declined, possibly as a

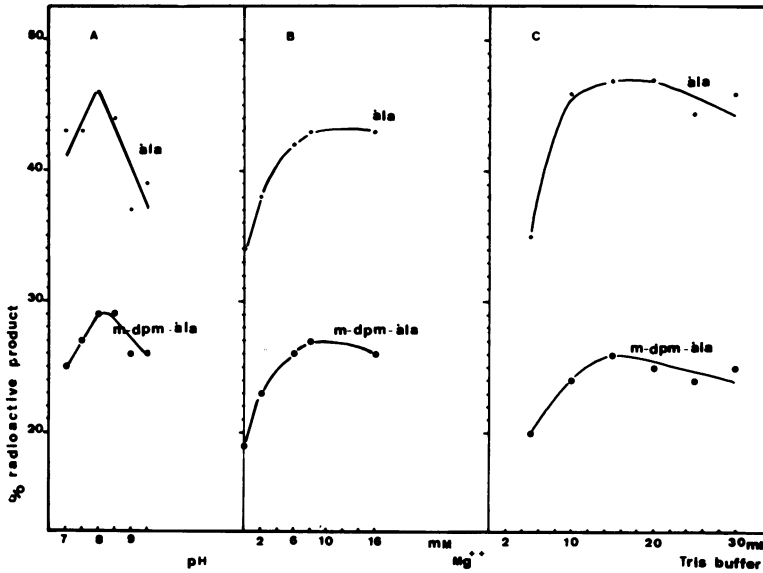


FIG. 5. Alanine (○) and DPM-Ala (●) released from MurNAc(DPM) pentapeptide by enzyme preparation V under varying conditions. As in Fig. 3, data are presented as percentage of the total radioactive D-alanine present in the indicated products and in MurNAc tetra- and pentapeptides (not shown). (A) As a function of pH in 20 mM Tris buffers containing 4 mM Mg<sup>2+</sup>; (B) as a function of the magnesium concentration in 20 mM Tris (pH 8); (C) as a function of the Tris buffer molarity at pH 8, 4 mM Mg<sup>2+</sup>.

TABLE 1. Effect of cations on the Glu-DPM endopeptidase activity with the MurNAc-(DPM) tetrapeptide as substrate

Cation added <sup>a</sup>	Relative rate of hydrolysis <sup>b</sup>		
	8 × 10 <sup>-3</sup> M	10 <sup>-3</sup> M	10 <sup>-4</sup> M
Mg <sup>2+</sup>	1.54	1.54	1.3
K <sup>+</sup>	1.16	1.26	1.16
Mn <sup>2+</sup>	1.36	1.52	1.42
Ca <sup>2+</sup>	0.64	1.0	1.0
Co <sup>2+</sup>	0.64	1.0	1.42

<sup>a</sup> The incubation mixture contained, in 20 μliters: 6.25 × 10<sup>-5</sup> M radioactive MurNAc-(DPM) tetrapeptide, 13 mM Tris buffer (pH 8), and 2 μliters of the enzyme preparation V (17 μg in protein). The cations were added at the indicated final concentrations as the chloride salts (Mg<sup>2+</sup>, K<sup>+</sup>, Mn<sup>2+</sup>, Ca<sup>2+</sup>) or as the nitrate salt (Co<sup>2+</sup>). Reactions were performed at 37 C for 2 h.

<sup>b</sup> Results are presented relative to the rate of hydrolysis by incubations containing no added cations.

consequence of concomitant sporangial lysis. This pattern was reproducible and paralleled that found with the pentapeptide substrate (Fig. 3B).

DISCUSSION

All bacteria that have been investigated synthesize their peptidoglycan via a UDP-N-acetylmuramyl pentapeptide precursor contain-

ing C-terminal D-alanyl-D-alanine, and *B. sphaericus* is no exception (cf. ref. 7, 16). As anticipated from the structure of the vegetative and cortical peptidoglycans, whose carboxyl terminal peptides lack, respectively, both D-alanyl residues and only the terminal D-alanine residue (Fig. 1), these cells contain D-alanine carboxypeptidase activities.

Acyl-D-alanyl-D-alanine carboxypeptidase I activity is present in vegetative and sporulating particulate enzyme preparations, and like the similar enzyme of *E. coli* (7,8), this activity is highly sensitive to penicillin G. Unlike the *E. coli* carboxypeptidase I, which hydrolyzes only DPM-containing pentapeptide substrates (8), the *B. sphaericus* particulate activity hydrolyzes both DPM- and lysine-containing substrates (Fig. 1). Two distinct activities may be present, but, if so, both are penicillin sensitive and both are present throughout vegetative growth and sporulation at constant relative specific activities (data not shown). Particulate preparations from cells of *B. megaterium* contain a benzylpenicillin-sensitive carboxypeptidase I activity that hydrolyzes m-DPM pentapeptide, and a benzylpenicillin-resistant activity that is active on L-lysine pentapeptide (20). The *B. sphaericus* carboxypeptidase I may be the first such enzyme that does not discriminate between m-DPM and L-lysine-containing substrates.

A second activity is present in the *B.*

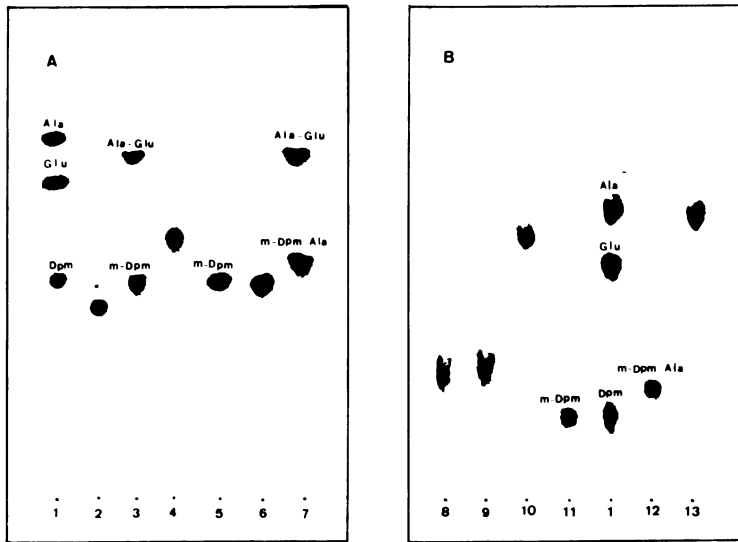


FIG. 6. Thin-layer chromatograms of the products of incubation of enzyme V with various substrates, developed with ninhydrin. (A) Chromatogram in solvent A after two runs; (B) chromatogram in solvent B. The products of the action of enzyme V on various substrates are chromatographed adjacent to controls of untreated substrates. 1, Free alanine, glutamic acid, and DPM (controls); 2, tripeptide Ala- $\gamma$ -D-Glu-*m*-DPM; 3, products from this tripeptide; 4, disaccharide tripeptide, GlcNAc-Ala-Glu-DPM; 5, products from this disaccharide tripeptide; 6, tetrapeptide, Ala-Glu-DPM-Ala; 7, products from this tetrapeptide; 8, tripeptide, Ala- $\alpha$ -D-Glu-DPM; 9, products from this tripeptide; 10, N-acetylmuramyl tripeptide, MurNAc-Ala-Glu-DPM; 11, products from this MurNAc-tripeptide; 13, disaccharide tetrapeptide, GlcNAc-MurNAc-Ala-Glu-DPM-Ala; 12, products from this disaccharide tetrapeptide. Disaccharide-Ala-Glu and MurNAc-Ala-Glu do not react with ninhydrin so are not seen in 5, 11, and 12.

*sphaericus* particulate enzymes that hydrolyzes the L-Lys-D-Ala linkage only after removal of the terminal D-alanine residue from MurNAc-(Lys) pentapeptide; it has no effect on the analogous MurNAc-(DPM) tetrapeptide (Fig. 1). This activity was not inhibited by  $10^{-3}$  M benzylpenicillin and in this sense is analogous to the carboxypeptidase II activity of *E. coli* (8). The substrate specificity of the L-lysyl-D-alanine carboxypeptidase II is such that it explains the presence of carboxyl-terminal L-lysine residues in the vegetative peptidoglycan and the absence of carboxyl-terminal (L)*m*-DPM residues in the cortical peptidoglycan (5). Under the assay conditions used, this enzyme does not appear to hydrolyze L-alanyl-D-glutamate residues, although an L,D-carboxypeptidase capable of hydrolyzing this substrate could convert the product of the D-glutamyl-*m*-DPM endopeptidase activity into the carboxyl-terminal L-alanyl residues found in the cortical peptidoglycan. Perhaps hydrolysis of the L-Ala-D-Glu bond by the carboxypeptidase II activity requires a polymeric substrate. This possibility is being investigated. By analogy, most lytic MurNAc-L-Ala amidases require a polymeric substrate and are inactive on GlcNAc-MurNAc disaccharide peptides. The *S. albus* G enzyme

used in substrate preparations for these studies (see Materials and Methods) is a unique exception.

The discovery of the  $\gamma$ -D-glutamyl-*m*-DPM endopeptidase activity was unanticipated since its function is not immediately apparent. Hydrolysis of pentapeptide substrates requires the prior removal of the carboxyl-terminal D-alanine residue by carboxypeptidase I. Since this activity is 50% inhibited by  $4 \times 10^{-7}$  M benzyl penicillin, similar concentrations of penicillin also inhibit the action of the endopeptidase on pentapeptides. However, hydrolysis of tetrapeptides is not inhibited by even  $10^{-3}$  M penicillin, and it is concluded that the endopeptidase is not inhibited by penicillin. The *in vivo* substrate may be the nascent polymer of disaccharide pentapeptide, the probable immediate precursor of the cortical peptidoglycan. However, since these activities do act on MurNAc peptides, it is also possible that the unique endopeptidase of sporulating *B. sphaericus* cells modifies the membrane-bound lipid intermediate involved in cortical peptidoglycan synthesis. Elimination of the D-glutamate residue might then occur before or after subsequent polymerization.

The specificity of the endopeptidase for DPM

is consistent with a role in sporulation only, since no DPM occurs in the vegetative cell peptidoglycan. The time of appearance of this activity, between samples II and IV, roughly parallels the period during which the forespores become engulfed and spore cortex formation begins and is also consistent with a role in cortex synthesis, as is the absence of this activity from vegetative cells. This activity is also absent from  $90,000 \times g$  supernatant fractions from sporulating cells up to the stage at which they begin to autolyze, and this suggests that this enzyme functions in a membrane localization, which is consistent with the hypothesis (see introduction) on the role of the outer forespore membrane in the unique events involved in spore cortex synthesis. Since sensitivity to this enzyme requires the prior removal of the carboxyl-terminal D-alanyl residue of the pentapeptide, the endopeptidase probably does not hydrolyze cross-links within the cortical peptidoglycan in which the sequence  $m\text{-DPM(L)-D-Ala-(D)m-DPM(D)-OH}$  is structurally analogous to  $m\text{-DPM(L)-D-Ala-D-Ala-OH}$ .

Thus it is unlikely to be involved in germination.

The hydrolysis of the  $\gamma$ -D-glutamyl-*meso*-diaminopimelate linkage of the tripeptide and its muramic and disaccharide derivatives by the *B. sphaericus* particulate enzymes could be due to a D,L-carboxypeptidase different from the D-Glu-*m*DPM endopeptidase. If so, the two activities appear at the same time and in constant ratio during sporulation (Fig. 7).

Sporulating cells of *Bacillus cereus* T have increased activity of soluble  $\gamma$ -L-glutamyl peptidase that hydrolyzes glutathione to give free cysteine (2). This enzyme also hydrolyzes  $\gamma$ -L-glutamyl-*p*-nitroanilide. This is not a substrate for the particulate *B. sphaericus* enzyme, which is probably an unrelated enzyme. *B. subtilis* produces a poly  $\gamma$ -D-glutamate polymer and excretes an enzyme that is capable of hydrolyzing this polymer and L-glutamine. This enzyme also catalyzes, at a higher efficiency, the transfer of  $\gamma$ -glutamyl residues from these substrates to D-glutamine (21). In contrast, the enzyme activity from *B. sphaericus* is particulate and neither hydrolyzes L-glutamine nor catalyzes detectable transpeptidation reactions using as donor L-glutamine, D-glutamine, tetrapeptide, or MurNAc tetrapeptide, and as acceptor D-glutamate, *meso*-diaminopimelate, L-lysine, or glycine. It is concluded that these are also unrelated enzymes.

In conclusion, a new enzyme activity has been observed exclusively in sporulating cells of *B. sphaericus*. It hydrolyzes the bond between the  $\gamma$ -carboxyl of D-glutamic acid and the L-amino group of *meso*-deaminopimelic acid, and its action on tetrapeptides produced by the action of carboxypeptidase I should result in the formation of carboxyl-terminal L-alanyl-D-glutamate dipeptides. Since these are not found in the cortical peptidoglycan, the only known substrate in these cells for this enzyme, then it must be assumed that any such dipeptides formed are intermediates either in the formation of lactam residues or in the formation of carboxyl-terminal L-alanine residues. The latter seems more probable and, as already stated, would simply require the activity of an L,D-carboxypeptidase, perhaps that already found capable of hydrolyzing L-lysyl-D-alanine linkages but inactive on L-Ala-D-Glu linkages under the assay conditions used in these studies. Carboxyl-terminal L-alanine residues are liberated from the peptidoglycan of *Bacillus stearothermophilus* by an autolytic enzyme associated with phage-induced lysis after induction of a lysogenic phage with mitomycin C (19). No such L-alanyl-D-glutamate endopeptidase

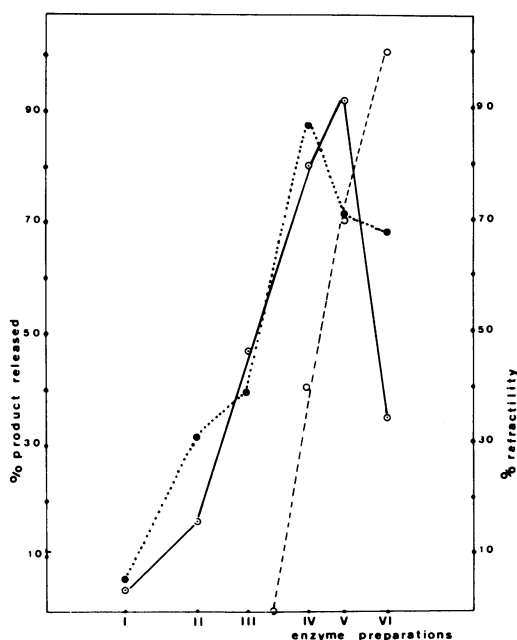


FIG. 7. Variation in activity of the Glu-DPM endopeptidase during sporulation in *B. sphaericus* with MurNAc tri- and tetrapeptides as substrates. Release of DPM from the tripeptide, (●); release of DPM-Ala from the tetrapeptide, (○); percentage of cells containing refractile forespores, (○). Data are presented as percentage of the total radioactive D-alanine or unlabeled DPM present in the indicated products. The rest is present as unmodified substrates.

activity has been found in *B. sphaericus* even after attempts at inducing lysogenic phage with mitomycin C (D. J. Tipper, unpublished observations). The production of free D-glutamate during autolysis of cell walls of *Bacillus psychrophilus* suggests the presence of an associated  $\gamma$ -D-glutamyl-L-lysine endopeptidase, although direct demonstration of this activity has not been reported (1). The *B. sphaericus* enzyme, which is incapable of hydrolyzing lysine-containing substrates, appears to be a different enzyme.

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#### LITERATURE CITED

- Best, G. K., and S. J. Mattingly. 1973. Chemical analysis of cell walls and autolytic digests of *Bacillus psychrophilus*. *J. Bacteriol.* **115**:221-227.
- Cheng, M. M., A. I. Aronson, and S. C. Holt. 1973. Role of glutathione in the morphogenesis of the bacterial spore coat. *J. Bacteriol.* **113**:1134-1143.
- Ghuysen, J. M., L. Dierickx, J. Coyette, M. Leyh-Bouille, M. Guinand, and J. N. Campbell. 1969. An improved technique for the preparation of *Streptomyces* peptidases and N-acetyl-muramyl-L-alanine amidase active on bacterial wall peptidoglycans. *Biochemistry* **8**:213-222.
- Ghuysen, J. M., D. J. Tipper, and J. L. Strominger. 1966. Enzymes that degrade bacterial cell walls, p. 685-699. *In* E. F. Neufeld and V. Ginsburg (ed.), *Methods in enzymology*, vol. 8. Academic Press Inc., New York.
- Hungerer, K. D., and D. J. Tipper. 1969. Cell wall polymers of *Bacillus sphaericus* 9602. I. Structure of the vegetative cell wall peptidoglycan. *Biochemistry* **8**:3577-3587.
- Ito, E., S. G. Nathenson, D. N. Dietzler, J. S. Anderson, and J. L. Strominger. 1966. Formation of UDP-acetyl-muramyl peptides, p. 234-337. *In* E. F. Neufeld and V. Ginsburg (ed.), *Methods in enzymology*, vol. 8. Academic Press Inc., New York.
- Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase, penicillin sensitive enzymatic reactions in strains of *E. coli*. *J. Biol. Chem.* **243**:3180-3192.
- Izaki, K. and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIV. Purification and properties of two D-alanine carboxypeptidases from *Escherichia coli*. *J. Biol. Chem.* **243**:3193-3201.
- Leyh-Bouille, M., J. Coyette, J. M. Ghuysen, J. Idczak, H. R. Perkins, and M. Nieto. 1971. Penicillin sensitive DD-carboxypeptidase from *Streptomyces* strain R<sub>11</sub>. *Biochemistry* **10**:2163-2170.
- Leyh-Bouille, M., M. Nakel, J. M. Frere, K. Johnson, J. M. Ghuysen, M. Nieto, and H. R. Perkins. 1972. Penicillin sensitive DD-carboxypeptidases from *Streptomyces* strains R<sub>11</sub> and K<sub>11</sub>. *Biochemistry* **11**:1290-1298.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265-275.
- Murrell, W. G. 1967. The biochemistry of the bacterial endospore. *Advan. Microbiol. Physiol.* **1**:133-251.
- Orlowski, M., and A. Meister. 1963.  $\gamma$ -Glutamyl-p-nitroanilide, a new convenient substrate for determination and study of L and D  $\gamma$ -glutamyl transpeptidase activities. *Biochim. Biophys. Acta* **73**:679-681.
- Orlowski, M., and A. Meister. 1965. Isolation of  $\gamma$ -glutamyl transpeptidase from hog kidney. *J. Biol. Chem.* **240**:338-347.
- Tipper, D. J., and J. Gauthier. 1972. Structure of the bacterial endospore. *In* L. L. Campbell, H. O. Halvorson, and R. Hanson (ed.), *Spores V*. American Society for Microbiology, Washington, D. C.
- Tipper, D. J., and I. Pratt. 1970. Cell walls polymers of *B. sphaericus* 9602. II. Synthesis of the first enzyme unique to cortex synthesis during sporulation. *J. Bacteriol.* **103**:305-317.
- van Heijenoort, J., L. Elbaz, P. Dezelee, J. F. Petit, E. Bricas, and J. M. Ghuysen. 1969. Structure of the meso-diaminopimelic acid containing peptidoglycans in *Escherichia coli* B and *Bacillus megaterium* K. M. *Biochemistry* **8**:207-213.
- Warth, A. D., and J. L. Strominger. 1969. Structure of the peptidoglycan of bacterial spores: occurrence of the lactam of muramic acid. *Proc. Nat. Acad. Sci. U.S.A.* **64**:528-535.
- Welker, N. E. 1971. Structure of the cell wall of *B. stearothermophilus*: mode of action of a thermophilic bacteriophage lytic enzyme. *J. Bacteriol.* **107**:697-703.
- Wickus, G. G., and J. L. Strominger. 1972. Penicillin-sensitive transpeptidation during peptidoglycan biosynthesis in cell-free preparations from *Bacillus megaterium*. *J. Biol. Chem.* **247**:5307-5311.
- Williams, W. J., J. Litwin, and C. B. Thorne. 1955. Further studies on the biosynthesis of  $\gamma$ -glutamyl peptides by transfer reactions. *J. Biol. Chem.* **212**:427-438.