

Cell Wall Polymers of *Bacillus sphaericus*: Activities of Enzymes Involved in Peptidoglycan Precursor Synthesis During Sporulation

PAUL E. LINNETT¹ AND DONALD J. TIPPER

Department of Microbiology, University of Massachusetts Medical School, Worcester, Massachusetts 01604

Received for publication 24 July 1974

In synchronously sporulating cells of *Bacillus sphaericus* 9602, the specific activities of those enzymes specifically required for the synthesis of the UDP-*N*-acetyl-muramyl-pentapeptide precursor of vegetative cell wall peptidoglycan decay by 50% after the end of exponential cell division, probably as a consequence of dilution by newly synthesized protein. The *meso*-diaminopimelate ligase is the only new activity whose synthesis is required for synthesis of the nucleotide-pentapeptide precursor of spore cortex peptidoglycan. The addition of D-Ala-D-Ala to the nucleotide tripeptide is catalyzed by an enzyme present in both vegetative and sporulating cells, which apparently does not discriminate between lysine- and diaminopimelate-containing acceptors. The activities of the L-Ala and D-Ala-D-Ala ligases and of the D-Ala-D-Ala synthetase increases in parallel with the appearance of the diaminopimelate ligase, indicating coordinate derepression and suggesting operon-like organization of the appropriate structural genes.

In electron micrographs of thin sections of bacterial endospores, the cortex is a prominent feature of the integuments that surround the cytoplasmic "core" of the spore. It disappears early in the process of spore germination, and in this process soluble fragments of peptidoglycan appear in the medium. The peptidoglycan structure of the spore cortex has been confirmed by its demonstrated susceptibility to lysozyme (reviewed in 26, 40, 42).

All bacterial cell wall peptidoglycans contain a polysaccharide built of alternating β -1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues. This polysaccharide is usually substituted on the carboxyl group of every MurNAc residue by a peptide, which may be cross-linked to give a potentially infinite, three dimensional polymer that is principally responsible for the strength of the bacterial cell wall (37, 39). This is true in *Bacillus sphaericus* vegetative cells in which the peptidoglycan is composed entirely of subunits in which GlcNAc-MurNAc disaccharides are linked through their carboxyl residues to L-alanyl-D-iso-glutamyl-L-lysyl-D-alanine peptides which are cross-linked between D-alanine and L-lysine by D-isoasparaginylyl residues (12).

The structure of the cortical peptidoglycan of *B. sphaericus* spores (D. J. Tipper, *Bacteriol. Proc.*, p. 24, 1969) is identical to that of *Bacillus subtilis* (42, 43, cf 40). All contain unique component not yet found in any cell wall peptidoglycan. These are muramic lactam residues and residues of *N*-acetyl muramic acid substituted by single L-alanine residues. They comprise 54 and 18%, respectively, of *B. sphaericus* cortical peptidoglycan. The residual 28% consists of disaccharide units substituted by the tetrapeptide, L-alanyl- γ -D-glutamyl-meso-diaminopimelyl-D-alanine. This structure contains no L-lysyl or D-isoasparaginylyl residues and is cross-linked directly through its meso-diaminopimelyl residues. Meso-diaminopimelic acid (m-Dpm) is not found in vegetative cell walls.

UDP-*N*-acetylmuramyl-pentapeptide is thought to be a common intermediate in the synthesis of all types of peptidoglycan (cf 30, 39). Its immediate precursor, UDP-*N*-acetylmuramyl tripeptide, is a very poor substrate for in vitro peptidoglycan polymerization (10a) and UDP-MurNAc-L-Ala is not incorporated into peptidoglycan by enzymes from sporulating bacilli (P. Linnett, unpublished observations). It is probable, although still not demonstrated, that the three different types of subunits in endospore cortical peptidoglycan are all derived

¹ Present address: Woodstock Agricultural Research Center, Sittingbourne Laboratories, Sittingbourne, Kent, England.

from UDP-MurNAc-L-Ala-D-Glu-meso-Dpm-D-Ala-D-Ala. Synthesis of UDP-MurNAc-pentapeptide has been investigated in several species and, for the most common types of peptidoglycan structure, involves sequential addition of residues of L-alanine, D-glutamate, L-lysine or meso-diaminopimelic acid and the presynthesized dipeptide, D-alanyl-D-alanine to the carboxyl group of UDP-N-acetyl muramic acid. The D-alanyl-D-alanine peptide is dimerized from D-alanine which is produced by racemization of L-alanine. All activities occur in the $100,000 \times g$ supernatant fraction of broken cells (cf 13, 14, 15, 17, 22, 25, 27, 39, 41).

The synthesis of the precursors for vegetative and cortical peptidoglycan synthesis in *B. sphaericus* should thus involve a common intermediate: UDP-MurNAc-L-Ala-D-Glu. In the previous paper in this series (41), it was demonstrated that sporulation in *B. sphaericus* involves the appearance of a new enzyme activity responsible for the addition of meso-diaminopimelate to this nucleotide-dipeptide intermediate.

In synchronously sporulating cultures of *B. sphaericus*, the activity of the enzyme that adds L-lysine to this nucleotide-dipeptide acceptor decays at the end of vegetative growth, while the activity for the addition of meso-diaminopimelate is totally absent during vegetative growth and until 4 h of sporulation have ensued, whereupon this activity appears and increases rapidly in a process that is dependent on continued synthesis of both protein and RNA. This paper describes the changes in the specific activities of the other enzymes involved in the synthesis of the pentapeptide portion of the nucleotide precursors for vegetative cell wall and cortical peptidoglycan synthesis during sporulation in *B. sphaericus*. It was anticipated, in particular, that the addition of D-alanyl-D-alanine to the nucleotide-peptides might involve different enzymes for cortex and cell wall peptidoglycan synthesis, since the proximal acceptor amino acid for cortex synthesis contains the additional carboxyl group of meso-diaminopimelate. As will be shown, this surmise is probably incorrect.

A preliminary report of these data was presented at the annual meeting of the American Society for Microbiology, 1970 (P. E. Linnett and D. G. Tipper, Bacteriol. Proc., 1970).

MATERIALS AND METHODS

Chemicals. D-Alanyl-D-alanine was obtained from Cyclo Chemicals, Los Angeles, Calif. D-Cycloserine was obtained from "Seromycin" capsules, Eli Lilly &

Co., Indianapolis, Ind., and was stored frozen as a 2 mg/ml solution in 10 mM tris(hydroxymethyl)amino-methane (Tris)-hydrochloride, pH 9. All other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo.

Radiochemicals. L-[U- ^{14}C]alanine (162 mCi/mmol) and DL-[1- ^{14}C]glutamic acid (20 mCi/mmol) were obtained from Amersham/Searle Corp., Des Plaines, Ill; D-[1- ^{14}C]alanine (9 mCi/mmol) was obtained from Calbiochem., Los Angeles, Calif., and L-[U- ^{14}C]lysine (248 mCi/mmol) from New England Nuclear Corp., Boston, Mass.

Enzymes. D-Amino acid oxidase was obtained from Worthington Biochemical Corp., Freehold, N.J. and was stored at $-20^{\circ}C$ as a 5 mg/ml solution in 0.1 M Na pyrophosphate buffer, pH 8.3. Catalase (Sigma Chemicals Co.) was a twice-crystallized suspension (from beef liver) stored in water with 0.1% thymol.

Preparation of D-[U- ^{14}C]alanyl-D-alanine. A mixture of 1.6 mM L-[U- ^{14}C]alanine (50 μ Ci), 160 mM Tris-hydrochloride, pH 8.5, 14 mM adenosine-5'-triphosphate (ATP), 5 mM $MnCl_2$, 2.4 mM reduced glutathione, 0.4 mM pyridoxal-5'-phosphate, and 50 μ liters of an enzyme preparation from *Streptococcus faecalis* R (a crude enzyme precipitated by 55% saturated ammonium sulfate from a cell extract, kindly supplied by F. C. Neuhaus; both alanine racemase and D-alanine D-alanine ligase activities were present) in a total volume of 250 μ liters were incubated for 3 h at $37^{\circ}C$ (27). The mixture was heated for 2 min at $100^{\circ}C$, cooled, centrifuged, and the supernatant fluid was fractionated by electrophoresis on 3 MM paper (Whatman) at 26 V/cm, pH 2.7, for 2 h. Autoradiography with Kodak RB-54 medical X-ray film showed complete separation of D-Ala-D-Ala from alanine (relative mobilities 46 and 100, respectively). The D-Ala-D-Ala containing band was eluted with 1% acetic acid. After lyophilization, the residue was dissolved in water to give about 2×10^4 counts per min per μ liter. Radiochemical yield was about 55%. After hydrolysis (6 N HCl, $105^{\circ}C$, overnight) and electrophoresis at pH 1.9, all radioactivity was found in the alanine region.

Preparation of UDP-MurNAc-peptide nucleotides. The preparation of UDP-MurNAc-L-Ala-D-Glu and UDP-MurNAc-L-Ala-D-Glu-L-Lys have been described (41). UDP-MurNAc-L-Ala-D-Glu-meso-Dpm was a gift from Roland Plapp.

An unresolved mixture of 6 mM UDP-MurNAc and 2 mM UDP-MurNAc-L-Ala was prepared from *S. aureus* Copenhagen cells. Penicillin G (10 μ g/ml) was added to a culture (3 liters) growing in SA broth (8) at one-third maximum growth (Klett = 100, red filter). After 90 min at $37^{\circ}C$, the cells were collected by centrifugation (10 min at $10,000 \times g$) and boiled in water (100 ml) for 10 min. After centrifugation, the supernatant fluid at $0^{\circ}C$ was mixed with an equal volume of 10% trichloroacetic acid also at $0^{\circ}C$. After 30 min the mixture was centrifuged and the supernatant fluid was extracted three times with an equal volume of diethyl ether. The resultant aqueous nucleotide mixture was fractionated on AG-X2 (Bio-Rad) and Biogel P2 (Bio-Rad) columns (8, 41). UDP-MurNAc and UDP-MurNAc-L-Ala were eluted

together. Amino-acid analysis of a hydrolyzed sample (6 N HCl, 105 C, overnight) and ultraviolet absorption at 260 nm established the purity and relative concentrations of the components of this mixture.

Electrophoresis and buffers. A Gilson Model D solvent-cooled apparatus was used with Whatman 3MM paper. The pH 2.7 buffer was pyridine-formic acid-water (4.5:25:1,000, by volume). The pH 3.9 buffer was pyridine-acetic acid-water (7.5:25:1,000, by volume). The pH 1.9 buffer contained 20 ml of concentrated formic acid per liter.

Radioactive counting. Samples of ^{14}C -labeled compounds on Whatman 3MM paper were counted by scintillation counting in 15 ml of toluene scintillator containing 4 g of 2,5-diphenyloxazole per liter and 0.3 g of dimethyl-1,4-bis-2-(5-phenyloxazolyl) benzene per liter. Counting efficiency for ^{14}C was 60 to 70%.

Culture conditions. Cell culture conditions and the method for determination of cell mass by turbidity measurements have been previously described (12, 41). A 5-ml amount of an exponential culture of *B. sphaericus* at a turbidity of 0.2 (0.54 mg dry wt of cells per ml) was used to inoculate 1-liter cultures in 2-liter Erlenmeyer flasks. The flasks had ridged bases to improve aeration. Vigorous shaking (250 rpm) at 33 C resulted in a doubling time in vegetative growth of about 37 min, and synchronous, 95% complete sporulation. Sporulation synchrony was estimated by dark-phase microscope observation of the percentage of terminally swollen cells and cells containing semirefractile pre-spores (cf 41).

Preparation of soluble enzyme extracts. Samples of culture were removed at intervals, chilled in ice, and centrifuged (10 min 12,000 $\times g$). The volume of each sample, in milliliters, was 25 (turbidity) $^{-1}$ in order to ensure similar yields of soluble protein. All subsequent operations were performed at 0 to 5 C. Buffer A (20 mM KPO₄, pH 7.8) was used for preparations assayed for D-Ala-D-Ala, L-lysine, and meso-diaminopimelate ligase activities. These activities were stable for several weeks when stored in this buffer at -20 C. Buffer B (50 mM Tris-hydrochloride, pH 8.0, at 25 C, 10 mM MgCl₂ and 4 mM dithiothreitol [DTT]) was used for preparations assayed for other activities, all of which were stable for several weeks when stored in small samples in this buffer at -80 C.

The cell pellets were washed with buffer, suspended in 2 ml of the same buffer and disrupted in 15-ml Corex tubes (immersed in an alcohol bath at -15 C) for 10 min by using the microtip of a Branson J 17V sonifier (Heat Systems, Inc.). The final temperature was 2 to 8 C. After centrifugation at 48,000 $\times g$ for 10 min, the supernatant was treated with 6 ml of saturated ammonium sulfate containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5. After 30 min at 0 C, the protein was collected by centrifugation at 12,000 $\times g$ for 10 min and dissolved in 2 ml of buffer to give an enzyme preparation containing about 5 mg of protein per ml. Protein concentrations were determined by the method of Lowry et al. (21) with bovine serum albumin as standard.

General assay procedure. After incubation of ^{14}C -labeled substrates with enzyme fractions, the substrates were separated from products by electrophoresis. Electrophoresis at pH 3.9 rapidly and completely separated basic (L-lysine), neutral (alanine, alanyl alanine) and even acidic amino acids (D-glutamic acid) from the highly anionic nucleotide products (see Table 1). Assays for the synthesis of D-alanyl-D-alanine involved a similar procedure, but using electrophoresis at a lower pH (2.7) which better separated alanine from the dipeptide product (relative mobilities 50 and 100). Assay of alanine racemase depended upon the use of D-amino acid oxidase to oxidize the D-alanine product of racemization of L- ^{14}C alanine to pyruvate. Pyruvate and the residual L-alanine could be easily separated by electrophoresis at pH 3.9 (Table 1).

Incubations were performed at 37 C and reactions were terminated by heating at 100 C for 2 min. After precipitation of denatured protein by centrifugation for 5 min at 10,000 $\times g$, a sample of the supernatant was spotted on the origin of a sheet of Whatman 3MM paper for electrophoresis. Boiled enzyme controls employed enzyme that had been inactivated by heating for 2 min at 100 C prior to addition of substrates. Products were located by the use of appropriate standards detected with ninhydrin (amino acids and peptides) by ultraviolet absorbance (nucleotides) or with toluidine blue (pyruvate). Areas of the electrophoresis paper corresponding to the positions of substrate and products were excised and counted. Adjacent areas and areas intervening between substrates and products were also counted in order to detect unexpected products. The total recovery of counts in the test strips was compared with that in the substrate area of the boiled enzyme control. Total recovery was always 100 \pm 10%.

The time of incubation was chosen to allow no more than 10% conversion of substrate to product and no more than 5% racemization of alanine in the assay for alanine racemase. This insured a linear relationship between the amount of product formed and the time of incubation or the amount of enzyme added. Activi-

TABLE 1. Relative migration distances on paper electrophoresis at pH 3.9, 37 V/cm

Compound	Distance
Lysine	-72
D-Alanyl-D-alanine	-16
Alanine	-7
Diaminopimelate	-7
Glutamine	-7
Glutamate	21
UDPMAGLAA	35
UDPMAGL	41
Aspartate	50
UDPMAGDapAA	52
UDPMAGDap	58
UDPMAG	60
UDPMA	74
Cysteic acid	97
Pyruvate	135

ties are expressed in micromoles of substrate converted to product, per milligram of protein, per minute at 37 C.

Optimal metal ion concentrations were found by assaying for enzyme activities with $MgCl_2$ (1 to 150 mM), $MnCl_2$ (0.8 to 30 mM: above 30 mM, precipitation occurred) or KCl (3 to 200 mM) in the assay, keeping other components constant. For optimal pH determination, 50 mM Tris-hydrochloride buffers were employed in the pH range 7.5 to 9.5 at 37 C (not corrected for variations in ionic strength).

Standard assay conditions.

UDP-MurNAc:L-Ala ligase (EC 6.3.2.8). The assay mixture contained 50 mM Tris-hydrochloride, pH 8.5, 10 mM ATP, 25 mM $MgCl_2$, 4 mM DTT, 1 mM D-cycloserine, 0.5 mM UDP-MurNAc (containing 0.17 mM UDP-MurNAc-L-Ala), 0.5 mM L-[^{14}C]alanine (about 8×10^4 counts/min), and enzyme (about 25 μg of protein) in a total volume of 40 μ liters. After incubation for 10 min at 37 C, the products were fractionated by electrophoresis at 37 V/cm, pH 3.9 for 45 min.

UDP-MurNAc-L-Ala:D-Glu ligase (EC 6.3.2.9). The assay mixture contained 50 mM Tris-hydrochloride, pH 8.5, 10 mM ATP, 9 mM $MgCl_2$, 50 mM L-glutamine, 0.5 mM UDP-MurNAc-L-Ala (containing 1.5 mM UDP-MurNAc), 0.5 mM D-glutamic acid, 0.06 mM DL-[^{14}C]glutamic acid (about 8×10^4 counts/min), and enzyme (about 25 μg of protein) in a total volume of 40 μ liters. After incubation for 10 min at 37 C, the products were fractionated by electrophoresis at 37 V/cm, pH 3.9 for 1 h.

UDP-MurNAc-L-Ala-D-Glu:L-lys ligase (EC 6.3.2.7). Activity was assayed as previously described (41) except for an increase in the $MgCl_2$ concentration and a slight increase in pH. This activity and that of the diaminopimelate ligase were stable in extracts prepared by the KPO₄ buffer procedure. The assay mixture contained 50 mM Tris-hydrochloride, pH 8.7, 10 mM ATP, 25 mM $MgCl_2$, 0.5 mM UDP-MurNAc-L-Ala-D-Glu, 0.5 mM L-[^{14}C]lysine (about 8×10^4 counts/min), and enzyme (about 30 μg of protein) in a total volume of 4 μ liters. After incubation for 45 min at 37 C, products were fractionated by electrophoresis at 37 V/cm, pH 3.9, for 45 min.

UDP-MurNAc-L-Ala-D-Glu:meso-diaminopimelate ligase (EC 6.3.2.13) was assayed as described by Tipper and Pratt (41).

UDP-MurNAc-L-Ala-D-Glu-L-Lys:D-Ala-D-Ala ligase (EC 6.3.2.10). The assay mixture contained 60 mM Tris-hydrochloride, pH 8.5, 12 mM ATP, 76 mM $MgCl_2$, 0.6 mM UDP-MurNAc-L-Ala-D-Glu-L-Lys, 0.6 mM D-[^{14}C]Ala-D-Ala (about 3×10^4 counts/min) and enzyme (about 8 μg of protein) in a total volume of 33 μ liters. After incubation for 10 min at 37 C, products were fractionated by electrophoresis at 37 V/cm, pH 3.9, for 1 h. Under these conditions, specific activities were high (11 to 33 munits per mg of protein) and there was no difference in enzyme prepared by the KPO₄ buffer or the Tris-hydrochloride, $MgCl_2$, DTT buffer procedures.

UDP-MurNAc-L-Ala-D-Glu-meso-Dpm:D-Ala-D-Ala ligase. The assay mixture contained 60 mM Tris-hydrochloride, pH 8.4, 12 mM ATP, 76 mM

$MgCl_2$, 0.6 mM UDP-MurNAc-L-Ala-D-Glu-L-Lys, 0.6 mM [^{14}C]D-Ala-D-Ala (about 3×10^4 counts/min) and enzyme (about 8 μg of protein) in a total volume of 33 μ liters. After incubation for 10 min at 37 C, products were fractionated by electrophoresis at 37 V/cm, pH 3.9, for 1 h. Essentially the same specific activity and stability was found with this acceptor as with the lysine-containing acceptor (above).

Alanine racemase (EC 5.1.1.1). The assay mixture contained 25 mM KPO₄ buffer, pH 8.0, 20 mM pyridoxal 5'-phosphate, 5 mM L-[^{14}C]alanine (about 3×10^4 counts/min), and enzyme (about 2 μg of protein) in a total volume of 20 μ liters. After 10 min at 37 C, the reaction was terminated by heating at 100 C for 2 min. To the cooled mixture was added 5 mM Na pyrophosphate buffer, pH 8.3, 2 μM flavin-adenine dinucleotide (FAD), D-amino acid oxidase (5 μg of protein), and catalase (25 μg of protein, 850 units) giving a total volume of 23 μ liters. This mixture was incubated for 1 h at 37 C and then fractionated by electrophoresis at 37 V/cm, pH 3.9, for 25 min.

D-Ala-D-Ala synthetase (EC 6.3.2.4). The assay mixture contained 50 mM Tris-hydrochloride, pH 8.5, 12 mM ATP, 18 mM $MgCl_2$, 100 mM KCl, 2 mM glutathione (reduced), 1.6 mM D-[^{14}C]alanine (about 7×10^4 counts/min), and enzyme (about 7 μg of protein) in a total volume of 33 μ liters. After incubation for 30 min at 37 C, the mixture was fractionated by electrophoresis at 26 V/cm, pH 2.7, for 2 h.

RESULTS

Enzyme preparation. Breakage of mid log-phase vegetative *B. sphaericus* 9602 cells by ultrasonic disruption gave a 48,000 $\times g$ supernatant enzyme preparation which was precipitated with ammonium sulfate at 75% saturation to separate enzymes from low molecular weight substrates. This preparation was able to catalyze the stepwise addition of L-alanine, D-glutamic acid, L-lysine, and D-alanyl-D-alanine to UDP-MurNAc to yield the precursor for vegetative cell wall peptidoglycan, UDP-MurNAc-L-ala- γ -D-Glu-L-Lys-D-Ala-D-Ala. In addition, extracts made from synchronously sporulating cells, harvested after four or more hours post exponential growth at 33 C, were capable of adding meso-diaminopimelate to UDP-MurNAc-L-Ala-D-Glu to make the analogous nucleotide pentapeptide precursor of cortical peptidoglycan (cf 41). Both soluble enzyme preparations were also able to catalyze the formation of the necessary intermediate, D-alanyl-D-alanine from L-alanine in two steps. Enzyme activities demonstrated in this study are shown schematically in Fig. 1.

Cells harvested from different cultures at the same stage of growth yielded similar amounts of protein and reproducible specific activities for individual enzymes. The 48,000 $\times g$ pellets contained relatively little activity. The procedure described thus appears to extract a large

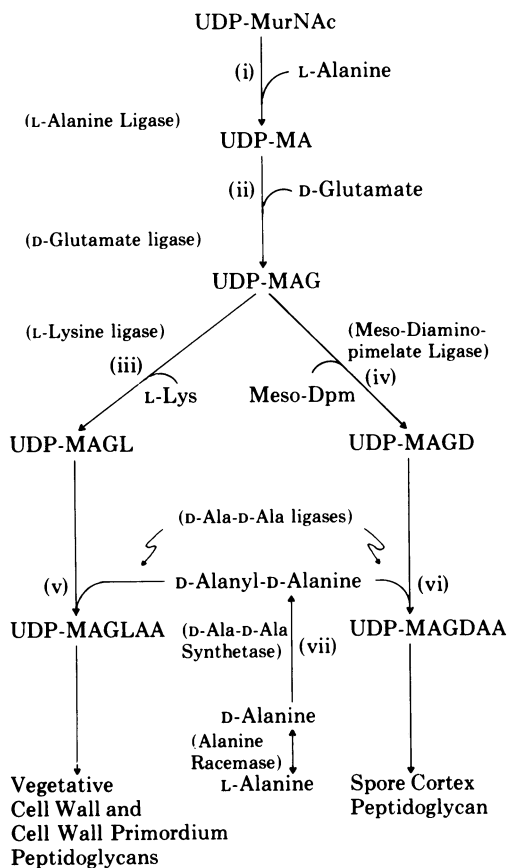


FIG. 1. Enzymatic reactions involved in synthesis of the nucleotide pentapeptide precursor of vegetative cell wall and spore cortex peptidoglycans which have been demonstrated in *B. sphaericus* 9602. Abbreviations: uridine-diphospho-*N*-acetyl-muramic acid, UDPM; alanine, A; D-glutamate, G; L-lysine, L; meso- α ϵ -diaminopimelic acid, D. Conventional amino acid abbreviations are used elsewhere.

and constant fraction of each of the enzymes assayed, so that the *in vitro* assays of enzyme activity are probably directly related to the levels of activity inside the cell at any stage.

Conditions for enzyme assays. ATP was included in the assays for all activities except alanine racemase, since it has been shown to be the obligate energy source for similar enzymes from other organisms (14, 15, 22, 27, cf 13). All the enzymes studied here, including the L-lysine- and meso-diaminopimelate ligases (25, 41), but not alanine racemase, showed broad pH optima between pH 8.0 and 9.0 using 50 mM Tris-hydrochloride buffers, as found for similar enzymes in other organisms (13). The specific activities decreased markedly below pH 8.0.

All of the enzymes except alanine racemase

were activated by Mg^{2+} or Mn^{2+} ions. The curves shown in Fig. 2 for UDP-MurNac-Ala-Glu-Lys:D-Ala-D-Ala ligase are typical. Optimal metal ion concentrations are given in Table 2. It can be seen that, for all the enzymes involved in amino acid addition except for the meso-diaminopimelate ligase (41), the specific activity at the optimal Mg^{2+} concentration was higher than that at the optimal Mn^{2+} concentration. The optimal Mg^{2+} concentrations found for the *B. sphaericus* enzymes were higher than those recorded for unfractionated *S. aureus* enzymes (8, 14, 15) where the optimal Mn^{2+} concentrations gave better enzyme activities than with Mg^{2+} . The higher optimal metal ion concentrations may be a consequence of the higher ATP concentrations used in this study (10 to 12 mM) compared with the 4 mM ATP used previously (14). The L-alanine and D-glutamate ligases and the D-Ala-D-Ala synthetase were tested for stimulation by K^+ ions. The only enzyme found to be stimulated was the D-Ala:D-Ala synthetase, whose specific activities at 10, 50, 100, and 200 mM KCl were 0.6, 2.1, 2.7, and 0.8 munits, respectively. This enzyme has been shown to be stimulated by K^+ or NH_4^+ ions when isolated from *S. faecalis* R (27) and from *S. aureus* (15). The assay mixture used for D-Ala:D-Ala synthetase contained 18 mM $MgCl_2$ (Table 2) and 100 mM KCl. Reduced glutathione was added to the assay mixture, as suggested by Neuhaus (27). The diaminopimelate ligase from *B. cereus* (25) and the UDP-GlcNAc:phosphoenolpyruvate transferase from *Staphylococcus epidermidis* (10) are also stimulated by K^+ and NH_4^+ ions. As the procedure

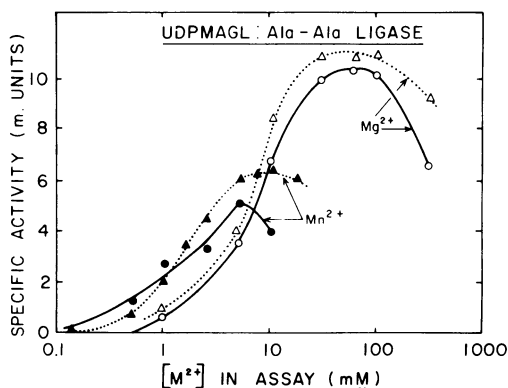


FIG. 2. Variation of the specific activity of UDP-MurNac-L-Ala-D-Glu-L-Lys:D-Ala-D-Ala ligase from *B. sphaericus* with divalent metal ion concentration. Enzyme from late exponential phase cells was used. Similar data were obtained with UDP-MurNac-L-Ala-D-Glu-meso-Dpm as acceptor (dotted curves).

TABLE 2. Optimal Mg^{2+} and Mn^{2+} concentrations for the soluble peptidoglycan precursor synthesizing enzymes of *B. sphaericus* 9602^a

Enzyme ^b	Vegetative enzyme ^c		Sporulation enzyme ^c		Ratio ^d
	Mg^{2+} (mM)	Mn^{2+} (mM)	Mg^{2+} (mM)	Mn^{2+} (mM)	
UDPM:L-Ala ligase (i)	— ^e	—	30	2	1.7
UDPMA:D-Glu ligase (ii)	—	—	9	2	
UDPMAG:L-lys ligase (iii)	20	2.5	40	4	1.2
UDPMAGL:Ala-ala ligase (iv)	60	6	—	—	2.0
UDPMAG:DAP ligase (v)	Not present	Not present	16 ^f	4 ^f	0.6
UDPMAGD-ala-ala ligase (vi)	—	—	80	10	1.3
D-Ala:D-Ala synthetase (vii)	20	5	—	—	2.2

^a The in vitro methods for enzyme assays are given in Materials and Methods.

^b Abbreviations and roman numerals in parentheses are from Fig. 1.

^c The enzyme preparations were made from cells in the late log phase, 3 h after inoculation of culture (vegetative enzymes) or from cells when 10 to 20% refractile (sporulation enzyme).

^d Rate of specific activity at optimal Mg^{2+} concentration to that at optimal Mn^{2+} concentration.

^e Dash indicates not measured.

^f Taken from ref 41.

used here for enzyme preparation included ammonium sulfate precipitation without subsequent dialysis, the assay mixtures would be expected to include up to 10 mM NH_4^+ from the enzyme solution, as well as up to 5 mM K^+ from the 20 mM KPO_4 buffer used to dissolve the protein (where used). These levels of monovalent cations may be sufficient to allow full in vitro activities of the other enzymes studied here.

UDP-MurNAc:L-Ala ligase. D-Cycloserine (1 mM) was added to the assay mixture to inhibit racemization of the labeled L-alanine substrate and its subsequent dimerization to D-Ala:D-Ala (cf 24, 29, 38). Enzymes prepared using the 20 mM KPO_4 buffer, pH 7.8, gave only very low activities. When the enzymes were prepared using the 50 mM Tris-hydrochloride, 10 mM $MgCl_2$, 4 mM DTT buffer, pH 8.5, consistently higher levels of activity could be obtained, even after thawing and refreezing several times at $-90^\circ C$. DTT was added to the assay mixture to ensure a 4 mM level throughout the reaction.

UDP-MurNAc-L-Ala:D-Glu ligase. Preliminary attempts to assay for the addition of glutamate using DL-[1- ^{14}C]glutamic acid resulted in low activity for the desired enzyme but good conversion of the L-[^{14}C]glutamate to L-glutamine by glutamine synthetase (L-glutamate, ammonia ligase (ADP), EC 6.3.1.2). Glutamine was neutral at pH 3.9 and so was easily separated by electrophoresis from glutamate and the desired nucleotide dipeptide product. No glutamate racemase (EC 5.1.1.3) activity (cf 6) was detected in these *B. sphaericus* enzyme preparations. L-Glutamine has been shown to act as a product inhibitor for glutamine synthe-

tase in *B. subtilis* (5) and in *B. megaterium* (7). A high level of L-glutamine (50 mM) in the assay mixture used here was found to give little inhibition of glutamine synthetase activity with Mn^{2+} , but complete inhibition with Mg^{2+} , as found for *B. subtilis* (5). Enzymes produced with 20 mM KPO_4 buffer, pH 7.8, gave specific activities for the D-glutamate ligase of 0.8 to 1.6 munits, whereas enzymes produced in the Tris-hydrochloride, $MgCl_2$, DTT buffer gave increased specific activities (3.3 to 8.4 munits) without added DTT in the assay mixture.

Alanine racemase. A technique in which D-alanine was converted to pyruvate by D-amino acid oxidase (EC 1.4.3.3) was preferred to the conversion of L-alanine to pyruvate by glutamate pyruvate transaminase (EC 2.6.1.2) with an excess of 2-oxoglutarate (17) because the [^{14}C]pyruvate was formed in the first reaction in better yield (96%) and was more clearly localized on electrophoresis at pH 3.9 (see Table 1). Pyridoxal-5'-phosphate at 20 μM was found to stimulate the racemase 10-fold, as would be expected from studies with the enzyme from *L. fermenti* (17). Conditions were chosen to allow only about 5% conversion of L-alanine to D-alanine to minimize the reverse reaction.

Morphology and synchrony of sporulation. Under the culture conditions described, symmetric, vegetative cell division in *B. sphaericus* ceases after about 4 to 5 h (although some residual division occurs up to 5.5 h). During the subsequent sporulation, two distinct sequential morphological changes can be observed by phase contrast microscopy. About 3 h after the end of vegetative growth, the individual cells become visibly swollen at one terminus. The

final degree of swelling varies from culture to culture, but all of the cells become fully swollen over a period of about 2 h. About 6 h after the end of vegetative growth, 2.5 to 3 h after the cells become swollen, the forespore within the swollen terminus of the cells becomes sufficiently refractile to be visible in the phase contrast microscope (cf Fig. 3). The forespore achieves the hard, bright refractility of the mature spore after about two more hours of incubation. The occurrence of these visible events, swelling and the acquirement of refractility, are thus markers of the progress of sporulation and observation of them indicates the degree of synchrony and completeness of sporulation within a given culture (cf 41).

Formation of the asymmetrical spore septum appears to occur coincident with the initiation of swelling, which thus would be called stage II in the conventional terminology of the sequence of sporulation morphology (cf 18, 35). By the time the cells are fully swollen, the forespore has been engulfed and sporulation has reached stage III. These events have been observed both by staining of septa by the method of Gordon and Murrell (9) and also by electron microscopy (S. Holt, J. Gauthier, and D. Tipper, unpublished observations). Electron microscopy has suggested a similar correlation of swelling and spore formation in *Clostridium cochlearium* (33).

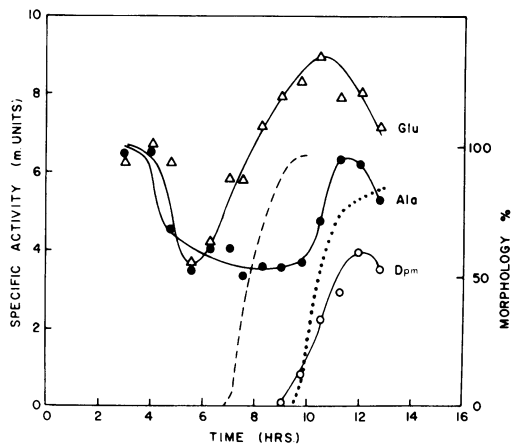


FIG. 3. Variation in activity of the L-alanine and D-glutamate ligases from *B. sphaericus* during vegetative growth and sporulation. Assays were performed with soluble enzymes prepared using the Tris-hydrochloride, $MgCl_2$, DTT buffer (see Materials and Methods). UDP-MurNac: L-Ala ligase (●); UDP-MurNac-L-Ala; D-Glu ligase (Δ); UDP-MurNac-L-Ala-D-Glu: meso Dpm ligase (○); percentage of terminally swollen cells (-----); percentage of cells containing visibly refractile fore-spores (●●●●).

The appearance of refractility closely follows the appearance of the diaminopimelate ligase which renders the cells competent for cortex precursor synthesis (41, see below). Electron microscope observations (S. Holt, J. Gauthier, and D. Tipper, unpublished observations) demonstrate that the appearance of refractility does indeed correspond to the period during which cortex and progenitors of the cells coats are accumulating and also that this process is preceded by the synthesis of the cell wall primordium (see Discussion).

Under the culture conditions described, cells routinely achieved 95% swelling and 90% refractility within 1.5 h periods during a sporulation process of about 10 h at 32 C, showing that satisfactory synchronization was obtained (41). *B. sphaericus* is very slow to release its spores from the residual sporangia unless cells are harvested after 10 h of sporulation and re-suspended in a smaller volume of an appropriate buffer (e.g., 0.1 M KPO₄, pH 6.8), so spore release cannot be used as a morphological criterion of the completion of sporulation.

Variations in activities of enzymes involved in the synthesis of the precursors of vegetative cell wall and cortical peptidoglycans during the end of vegetative growth and sporulation are shown in Fig. 3, 4 and 5. In each case, the percentage of swollen and refractile cells are included in the figures as morphological markers of the sporulation process. The variations of enzyme activities represented in these figures were reproducible for each enzyme in different sporulating cultures.

UDP-MurNac-Ala-Glu:Lys and diaminopimelate ligases. The variations in the activities of these two enzymes during sporulation in *B. sphaericus* has previously been described (41). The diaminopimelate ligase is a sporulation specific enzyme whose activity is absent from cells harvested before 4 h of sporulation. It appears shortly before the cultures begin to exhibit refractility. The activity of this enzyme is indicated in Fig. 3, 4 and 5 as a further marker of the sporulation process. The activity of the L-lysine ligase, which is required only for the synthesis of vegetative peptidoglycan, decays after the end of vegetative growth, except for a small but reproducible increase in activity just prior to the onset of refractility. This increase is followed by a further decline as the forespores become refractile (Fig. 5).

UDP-MurNac:L-Ala and UDP-MurNac-Ala:D-Glu ligases. L-Alanine and D-glutamate ligases, the enzymes which convert UDP-MurNac to UDP-MurNac-L-Ala-D-Glu, the common precursor for both cell wall and spore cortical peptidoglycan synthesis, are present both

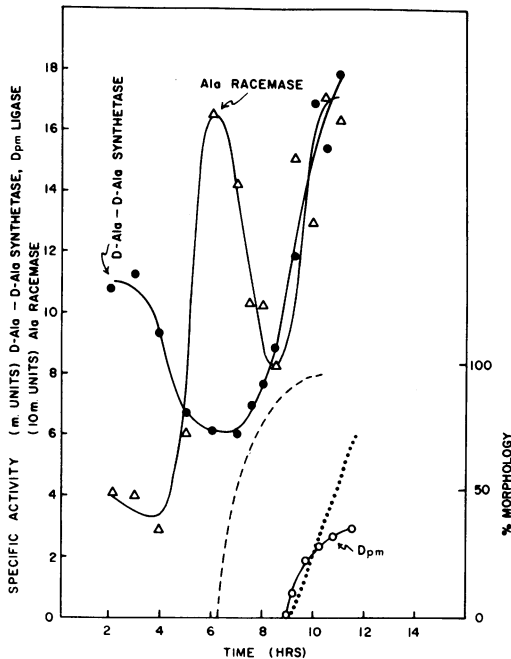


FIG. 4. Variation of activity of alanine racemase and *D*-alanyl-*D*-alanine synthetase from *B. sphaericus* during vegetative growth and sporulation. The variation of UDPMAG: *meso*-Dpm ligase (O) and percentage of morphology (see Fig. 3) are included as markers of the sporulation process. Assays were performed with soluble enzymes prepared using the 0.02 M KPO_4 buffer (see Materials and Methods). Ala racemase (Δ); *D*-Ala-*D*-Ala synthetase (\bullet).

during vegetative growth and sporulation as expected (Fig. 3). The activity of both enzymes, like that of the *L*-lysine ligase, decays at the end of vegetative growth. The activity of the *L*-alanine ligase increases again in parallel with the appearance of the diaminopimelate ligase. The *D*-glutamate ligase also increases in activity as sporulation progresses, but this increase starts earlier, after about 2 h of sporulation (when cells are beginning to swell) and continues for some time, ceasing somewhat before the activities of the diaminopimelate and *L*-alanine ligases are maximal. In this particular experiment, the activities were followed to 13 h, at which time all three were decreasing, probably as a consequence of autolysis of the sporangia surrounding mature spores.

***D*-Alanyl-*D*-alanine synthetase and alanine racemase.** Alanine racemase and *D*-alanyl-*D*-alanine synthetase are required for the synthesis of *D*-alanyl-*D*-alanine from *L*-alanine, both for vegetative cell wall and cortical peptidoglycan synthesis. As expected, both enzymes are present throughout vegetative growth and sporula-

tion (Fig. 4). The activity of the *D*-alanyl-*D*-alanine synthetase, like that of the *L*-alanine ligase, decays at the end of vegetative growth and increases again later in the sporulation process, most of the increase being concomitant with the appearance of the diaminopimelate ligase.

Alanine racemase has the highest specific activity of those enzymes assayed in this study, and by contrast with all of the others, this activity does not decay, but increases fourfold between the end of vegetative growth and the time when the cells begin to swell terminally. During swelling, the activity decays again to half of this maximal activity and then increases once again, roughly in parallel with the appearance of the diaminopimelate ligase (Fig. 4).

***D*-Alanyl-*D*-alanine ligases.** Synthesis of vegetative cell wall peptidoglycan requires the addition of *D*-alanyl-*D*-alanine to UDPMurNAc-*L*-Ala-*D*-Glu-*L*-Lys and synthesis of the cortical peptidoglycan requires addition of *D*-alanyl-*D*-alanine to the analogous nucleotide tripeptide

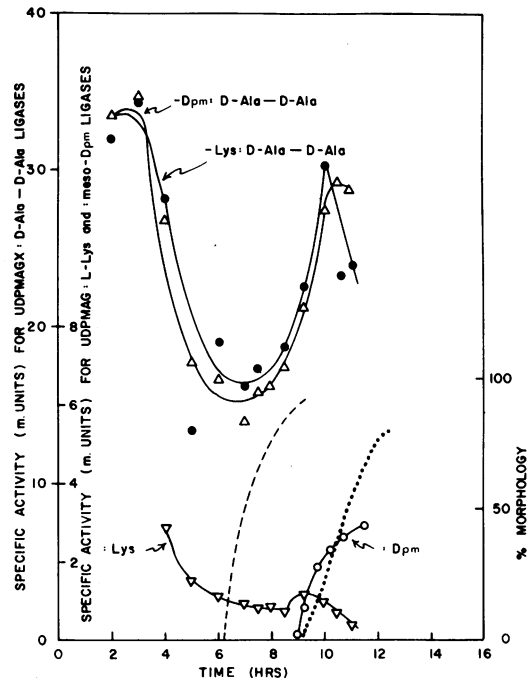


FIG. 5. Variation in activity of the *D*-alanyl-*D*-alanine ligases from *B. sphaericus* during vegetative growth and sporulation. The variation of UDMAG: Lys ligase (∇), UDPMAG: Dpm ligase (O), and percentage of morphology data (see Fig. 3) are included as markers of the sporulation process. Assays were performed with soluble enzymes prepared using the 0.02 M KPO_4 buffer (see Materials and Methods). UDP-MurNAc-*L*-Ala-*D*-Glu-*L*-Lys: *D*-Ala-*D*-Ala ligase (\bullet); UDP-MurNAc-*L*-Ala-*D*-Glu-*meso*-Dpm; *D*-Ala-*D*-Ala ligase (Δ).

containing diaminopimelate. It was anticipated that two different enzymes might be required for these processes and that the appearance of activity for cortical peptidoglycan synthesis would, like that of the diaminopimelate ligase, be a sporulation specific event. This is not so. D-Ala-D-Ala ligase activity for either acceptor is present in vegetative cells and throughout sporulation (Fig. 5). Both activities decay in parallel after the end of vegetative growth and increase again later in sporulation, in parallel with the appearance of the diaminopimelate ligase.

There are two simple explanations of these data. A single enzyme may be responsible for both activities, or two distinct enzymes may be synthesized and present during both vegetative growth and sporulation. Since there is no apparent function for a specific Dpm:D-Ala-D-Ala ligase during vegetative growth, the former explanation seems more likely. The existence of both activities during vegetative growth was further investigated and, as shown in Fig. 6,

both enzyme activities could be demonstrated in cultures as early as 2 h after inoculation. Variation in the relative specific activities with the two tripeptide acceptors suggests that activity with the lysine acceptor is somewhat more labile than activity with the diaminopimelate acceptor. This may account for the differential loss of activity with the lysine acceptor late in sporulation (Fig. 5). Enzymes isolated at the end of vegetative growth or towards the end of the sporulation process had identical pH optima of about 8.5 with either the lysine or diaminopimelate acceptors (Fig. 7), and the optimal divalent metal ion concentrations were the same within experimental error with either acceptor (Fig. 2, Table 2). No attempt has been made to separate the two activities. We have recently found that soluble enzyme prepared from *E. coli* K-12 by the same procedure described here for *B. sphaericus* will add D-alanyl-D-alanine as efficiently to the unnatural L-lysine containing acceptor as to the natural diamino-

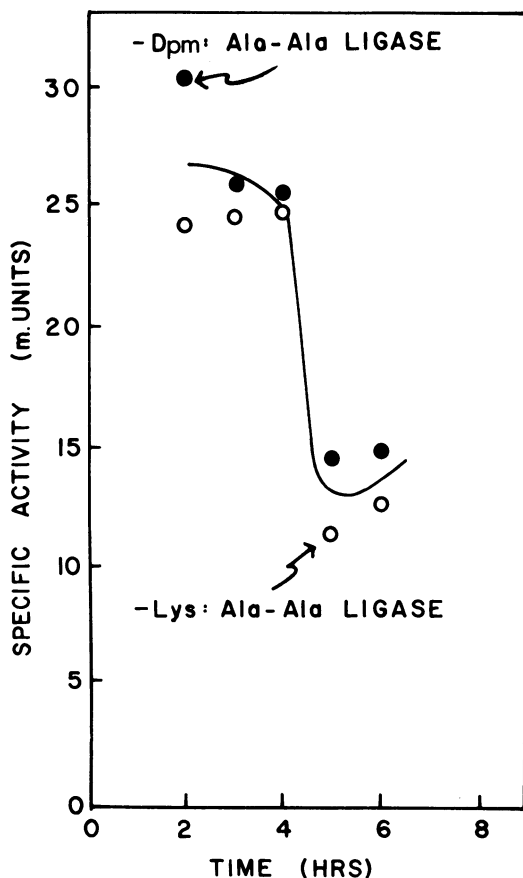


FIG. 6. Variation of activity of the D-alanyl-D-alanine ligase(s) from *B. sphaericus* during vegetative growth. Assays were performed on two sets of enzymes

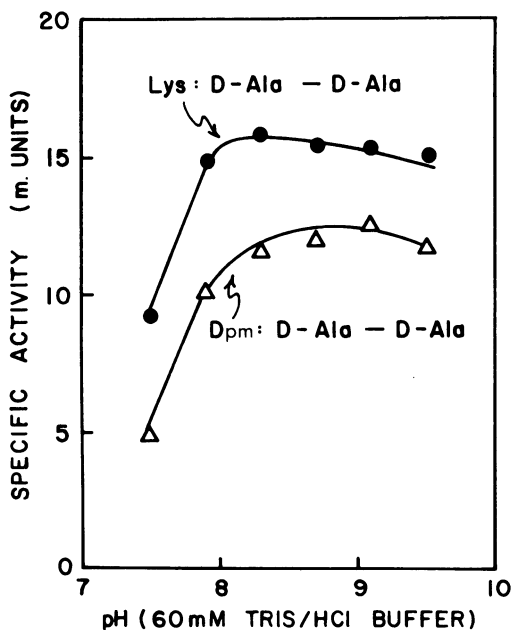


FIG. 7. Effect of pH on the specific activities of D-alanyl-D-alanine ligases from *B. sphaericus*. The assays with the lysine-containing acceptor (○) and with the diaminopimelate-containing acceptor (▲) were carried out with enzymes from log phase and from 10% refractile sporulating cells, respectively. Abbreviations as in Fig. 1.

prepared using 0.02 M KPO_4 buffer from two separate cultures (see Materials and Methods). UDP-MurNAc-L-Ala-D-Glu-L-Lys: D-Ala-D-Ala ligase (○); UDP-MurNAc-L-Ala-D-Glu-meso-Dpm: D-Ala-D-Ala ligase (●).

pimelate containing acceptor (P. Linnett, unpublished observations), and it is probable that a single enzyme also is responsible for both activities in *B. sphaericus*.

All of the data presented are summarized in Fig. 8 in which the specific activities are plotted on a log scale so that specific activities and temporal variations in these activities can be compared for all of the enzymes described.

DISCUSSION

The currently available data on the activities of enzymes involved in the synthesis of the precursors of vegetative cell wall and cortical peptidoglycans in *B. sphaericus* (Fig. 8) indicate that only diaminopimelate ligase activity is specific for the synthesis of the cortex. There is little evidence to suggest that the enzyme responsible for the addition of D-alanyl-D-alanine to the nucleotide-tripeptide product of diaminopimelate addition is also specific for cortex synthesis, and the best interpretation of the current data is that a single D-Ala-D-Ala ligase is responsible for synthesis of both vegetative cell wall and cortical peptidoglycans. It has also been demonstrated (M. Guinand and D. Tipper, unpublished observations) that particulate membrane preparations from *B. sphaericus* vegetative cells and from cells at various stages of sporulation are capable of polymerizing both the lysine and diaminopimelate containing nucleotide pentapeptides, together with UDP-GlcNAc, to form peptidoglycan. As originally observed in *S. aureus* (1a), the peptidoglycan polymerase activity does not require the addition of the cross bridge amino acid (a β -D-asparaginyl residue) in order to function, and as observed previously in *E. coli* (16), a peptidoglycan polymerase whose normal substrate contains meso diaminopimelate will also polymerize the nucleotide containing lysine.

Synthesis of the cortical peptidoglycan probably involves the activity of enzymes unique to this process whose function is to modify the product of peptidoglycan polymerase, either by removal of D-Glu-meso-Dpm-D-Ala-D-Ala to give those MurNAc residues substituted by single L-alanine residues, or by removal of the entire pentapeptide with formation of muramic lactam, perhaps in a process concomitant with de-N-acetylation of the MurNAc residues. To date, all attempts at demonstrating the presence of such activities in particulate preparations from *B. sphaericus*, *B. subtilis*, or *B. megaterium*, have been unsuccessful (M. Guinand and D. Tipper, unpublished observations). Thus, synthesis of spore coat (cf. 40), dihydrodipicolinate dehydrogenase (4), and

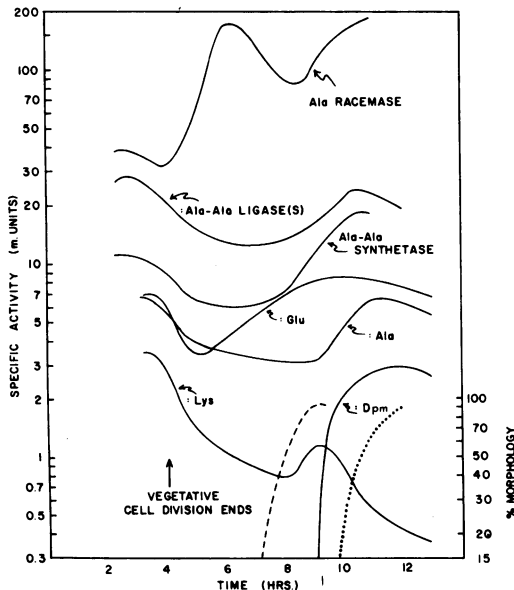


FIG. 8. Variation in specific activity of all enzymes studied. Activity is plotted on a log scale. Data for D-Ala-D-Ala ligase activity with the alternative Dpm- and Lys-containing nucleotide acceptors combined.

the diaminopimelate ligase in *B. sphaericus* remain the only demonstrated sporulation specific events required for the synthesis of unique components of bacterial spores (cf. 11), although several enzyme activities clearly relevant to sporulation, including the diaminopimelate-sensitive aspartokinase (19) also increase in activity at or near the onset of sporulation. A unique γ -D-Glu-meso-Dpm endopeptidase, capable of hydrolysing cortical but not vegetative peptidoglycan, does appear during sporulation of *B. sphaericus* (M. Guinand, G. Michel, and D. Tipper, in press). It may be involved in production of the C-terminal L-alanine residues of the cortical peptidoglycan.

Studies with a meso-diaminopimelate and lysine-requiring auxotroph of *B. megaterium* have shown that peptidoglycan synthesis in this species stops for a period of about 3 h after the end of exponential growth, as demonstrated by the lack of incorporation of labelled diaminopimelate (31, 32). The spore septum is formed during this period, and morphological studies indicate that, in contrast to the normal cell division process, this occurs without insertion of substantial quantities of cell wall material between the membranes, even when septation is complete (13, 35). In contrast, the sporulation septum in *B. sphaericus* appears to consist of two unit membranes separated by a thin layer

of unknown character, contiguous with part of the cell wall (S. Holt, J. Gauthier, and D. Tipper, manuscript in preparation) and the process of sporangial swelling may involve some peptidoglycan biosynthesis also. However, these events involve only a fraction of the first 3 h of sporulation and it is probable that a hiatus in peptidoglycan synthesis occurs in *B. sphaericus* as in *B. megaterium*. This hiatus is consistent with the decay in specific activity of the precursor synthesizing enzymes (other than alanine racemase) during this period of sporulation in *B. sphaericus* (Fig. 8). We have no direct information on the half-life of these enzyme activities. The turnover of protein that occurs during sporulation as a consequence of the activation of intra-cellular proteolytic enzymes is well documented in other bacilli (1, 2, 3, 20, 34) and almost certainly occurs in *B. sphaericus*. However, the activities of the diaminopimelate, lysine and D-Ala-D-Ala ligases and of the D-Ala-D-Ala synthetase in sporulating cells are stable in the presence of chloramphenicol (41; P. Linnett, unpublished observations) and in crude homogenates and these activities may not turn over significantly during the first few hours of sporulation. Moreover, the protein content of the enzyme preparations, representing that fraction of the total cell soluble protein released by ultrasonic oscillation, doubles between the time of the end of vegetative division and the appearance of 10% swelling (4.5 to 7 h) and then remains constant (this is also true of the turbidity of the cultures). Thus the initial decrease seen in the specific activities of the L-alanine, D-glutamate, and D-Ala-D-Ala ligases and the D-Ala-D-Ala synthetase may simply represent dilution of stable, existing enzyme by newly synthesized protein. This decrease is, in fact, approximately 50% in each case. The 50% decay in specific activity also occurs in stage III asporogenous mutants which do not demonstrate the subsequent increases in specific activities (unpublished observations).

In apparent contrast, the lysine ligase activity in sonically prepared extracts of the parent continuously decays throughout sporulation (41). With the exception of alanine racemase and the D-glutamate ligase, enzyme activities do not increase again until hour four of sporulation when a transient increase in the activity of the lysine ligase occurs (41).

It should be emphasized that the enzyme preparations assayed were prepared by sonication, a procedure which does not break the semi-refractile forespores of *B. sphaericus* and which, therefore, represents the contents of both

sporangia and forespores only until about 9 h, and reflects only the contents of the sporangia after about 10 h. This also explains why the specific activities of the sonically prepared extracts decay as sporulation is completed and the sporangia begin to lyse after 12 to 13 h. As will be demonstrated in a subsequent publication, the transient increase in the lysine ligase activity is probably due to exclusive synthesis of this enzyme within the developing forespores, a process which is probably required for synthesis of the cell wall primordium, the inner layer of the spore integuments which develops into vegetative cell wall during germination (cf 40).

L-Alanine, D-glutamate and D-alanyl-D-alanine ligases, together with D-alanyl-D-alanine synthetase and alanine racemase, are required for the synthesis of the precursors of both cortex and cell wall primordium peptidoglycans. Most of the increase in activity of L-alanine and D-alanyl-D-alanine ligases, together with the increase in activity of D-alanyl-D-alanine synthetase and of alanine racemase (towards the end of sporulation), is parallel with the appearance of diaminopimelate ligase. This is consistent with coordinate derepression of these enzyme activities for synthesis of spore cortex. An initial slower rate of increase in the activity of these enzymes, before appearance of diaminopimelate ligase activity, may also be consistent with the derepression of the synthesis of these enzymes, together with lysine ligase, within the developing forespore (detected while it is still sensitive to ultrasonic oscillation) for cell wall primordium synthesis.

The enzymes studied catalyze sequential steps in the unique pathway for synthesis of peptidoglycan precursor nucleotides. Coordinate control of the synthesis of these enzymes, in response to changing demands for peptidoglycan synthesis, would seem to be advantageous to the cell. Evidence for a putative operon containing the genes for synthesis of the L-alanine, meso-diaminopimelate and D-Ala-D-Ala ligases together with D-Ala-D-Ala synthetase has been found in *Escherichia coli* (23). The D-glutamate ligase has not been mapped, but its synthesis and that of alanine racemase seems to be under separate control in *B. sphaericus*. The alanine racemase gene in *E. coli* is well separated from the genes for the ligases mentioned (cf 23). Alanine racemase activity displays an unusual rise in activity at the beginning of sporulation shortly followed by an anomalous increase in the activity of the D-glutamate ligase. It is possible that this burst in synthesis of alanine racemase activity is due to derepression of synthesis of this enzyme for a

function not directly related to peptidoglycan synthesis. For example, several bacillus species are known to produce peptide antibiotics early in sporulation and these peptide antibiotics contain D-amino acids (cf 36). Perhaps the alanine racemase activity is required for synthesis of such antibiotics. We currently have no explanation for the unusual increase in the D-glutamate ligase at a similar time, although bacitracin does contain D-glutamate.

All enzymes were assayed in vitro under conditions giving maximal specific activity. Recovery of enzyme activities was constant and probably high. However, the specific activity of these different enzymes under in vivo conditions is unknown. Thus, unless there are gross differences between relative activities in vivo and in vitro, it appears that the maximal rate of synthesis of the pentapeptide nucleotide precursors of peptidoglycan may be limited by the activities of the diaminopimelate and lysine ligases, since their specific activities are considerably less than those of any of the other enzyme activities determined. By contrast, the activities of enzymes involved in the synthesis and addition of D-alanyl-D-alanine are in apparent excess. As suggested by Neuhaus et al. (28), therefore, feedback inhibition of D-alanyl-D-alanine synthetase by its product may be necessary to prevent trapping of the L-alanine of the cell in this product.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grants HD 029572 and AI 10806 from the National Institute of Child Health and Development and the National Institute of Allergy and Infectious Diseases, respectively.

LITERATURE CITED

- Aubert, J. P., and J. Millet. 1965. Etude d'une L-leucyl- β -naphthylamide hydrolase en relation avec la sporulation chez *Bacillus megaterium*. C.R. Acad. Sci. **261**:4274-4277.
- Anderson, J. S., P. M. Meadow, M. A., Haskin, and J. L. Strominger. 1966. Biosynthesis of the peptidoglycan of bacterial cell walls. I. Arch. Biochem. Biophys. **116**:487-515.
- Bernlohr, R. W., and B. H. Gray. 1969. Enzyme inactivation during initiation of sporulation, p. 186. In L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
- Bernlohr, R. W., and C. Leitzmann. 1969. Control of sporulation, p. 192. In G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., London.
- Chasin, L. A., and J. Szulmajster. 1969. Enzymes of dipicolinic acid biosynthesis in *Bacillus subtilis*, p. 133. In L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
- Deuel, T. F., and E. R. Stadtman. 1970. Some kinetic properties of *Bacillus subtilis* glutamine synthetase. J. Biol. Chem. **245**:5206-5213.
- Diven, W. F. 1969. Studies on amino acid racemases. II. Purification and properties of the glutamate racemase for *Lactobacillus fermenti*. Biochim. Biophys. Acta **191**:702-706.
- Elmerich, C. 1972. Le cycle du glutamate, point de depart du metabolisme de l'azote, chez *Bacillus megaterium*. Eur. J. Biochem. **27**:216-224.
- Good, C. M., and D. J. Tipper. 1972. Conditional mutants of *Staphylococcus aureus* defective in cell wall precursor synthesis. J. Bacteriol. **111**:231-241.
- Gordon, R. A., and W. G. Murrell. 1967. Simple method of detecting spore septum formation and synchrony of sporulation. J. Bacteriol. **93**:495-496.
- Gunetileke, K. G., and R. A. Anwar. 1966. Biosynthesis of uridine diphospho-N-acetylmuramic acid. J. Biol. Chem. **241**:5740-5743.
- Hammes, W. P., and F. C. Neuhaus. 1974. On the specificity of phospho-N-acetylmuramyl-pentapeptide translocase. The peptide subunit of uridine diphosphate-N-acetylmuramyl-pentapeptide. J. Biol. Chem. **249**:3140-3150.
- Hanson, R. S., J. A. Peterson, and A. A. Youste. 1970. Unique biochemical events in bacterial sporulation. Annu. Rev. Microbiol. **24**:53-90.
- 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. A. Nathenson, D. N. Dietzler, J. S. Anderson, and J. L. Strominger. 1966. Formation of UDP-acetylmuramyl peptides, p. 324-337. In E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
- Ito, E., and J. L. Strominger. 1962. Enzymatic synthesis of the peptide in bacterial uridine nucleotides. I. Enzymatic addition of L-alanine, D-glutamic acid and L-lysine. J. Biol. Chem. **237**:2689-2695.
- Ito, E., and J. L. Strominger. 1962. Enzymatic synthesis of the peptide in bacterial uridine nucleotides. II. Enzymatic synthesis and addition of D-alanyl-D-alanine. J. Biol. Chem. **237**:2696-2703.
- Izaki, K., M. Matsushashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell wall. XIII. J. Biol. Chem. **243**:3180-3192.
- Johnston, M. M., and W. F. Diven. 1969. Studies on amino acid racemase. I. Partial purification and properties of the alanine racemase from *Lactobacillus fermenti*. J. Biol. Chem. **244**:5414-5420.
- Kay, D., and S. C. Warren. 1968. Sporulation in *Bacillus subtilis*. Morphological changes. Biochem. J. **109**:819-824.
- Kuramitsu, H. K., and S. Yoshimura. 1972. Elevated diaminopimelate-sensitive aspartokinase activity during sporulation of *Bacillus stearothermophilus*. Biochim. Biophys. Acta **264**:152-164.
- Leighton, T. J., P. K. Freese, R. H. Doi, R. A. J. Warren, and R. A. Kelln. 1972. Initiation of sporulation in *Bacillus subtilis*: requirement for serine protease activity and ribonucleic acid polymerase modification, p. 238. In H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
- 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.
- Lugtenberg, E. J. J. 1972. Studies on *Escherichia coli* enzymes involved in the synthesis of uridine diphosphate-N-acetyl-uramyl-pentapeptide. J. Bacteriol. **110**:26-34.
- Lugtenberg, E. J. J., L. de Haas-Menger, and W. H. M. Ruyters. 1972. Murein synthesis and identification of cell wall precursors of temperature-sensitive lysis mutants of *Escherichia coli*. J. Bacteriol. **109**:326-335.

24. Lynch, J. L., and F. C. Neuhaus. 1966. On the mechanism of action of the antibiotic O-carbamyl-D-serine in *Streptococcus faecalis*. *J. Bacteriol.* **91**:449-460.
25. Mizuyo, Y., and E. Ito. 1968. Purification and properties of uridine diphosphate N-acetylmuramyl-L-alanyl-D-glutamate:meso-2,6-diaminopimelate ligase. *J. Biol. Chem.* **243**:2665-2672.
26. 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.
27. Neuhaus, F. C. 1962. The enzymatic synthesis of D-alanyl-D-alanine. I. Purification and properties of D-alanyl-D-alanine synthetase. *J. Biol. Chem.* **237**:778-786.
28. Neuhaus, F. C., C. V. Carpenter, J. L. Miller, N. M. Lee, M. Gragg, and R. A. Stickgold. 1969. Enzymatic synthesis of D-alanyl-D-alanine. II. Control of D-alanyl-D-alanine ligase (ADP). *Biochemistry* **8**:5119-5124.
29. Neuhaus, F. C., and J. L. Lynch. 1964. The enzymatic synthesis of D-alanyl-D-alanine. III. On the inhibition of D-alanyl-D-alanine synthetase by the antibiotic D-cycloserine. *Biochemistry* **3**:471-480.
30. Osborn, M.-J. 1969. Structure and biosynthesis of the bacterial cell wall. *Annu. Rev. Biochem.* **38**:501-538.
31. Pitel, D. W., and C. Gilvarg. 1970. Mucopeptide metabolism during growth and sporulation in *Bacillus megaterium*. *J. Biol. Chem.* **245**:6711-6717.
32. Pitel, D. W., and C. Gilvarg. 1971. Timing of mucopeptide and phospholipid synthesis in sporulating *Bacillus megaterium*. *J. Biol. Chem.* **246**:3720-3724.
33. Pope, L., and L. J. Rode. 1969. Spore fine structure in *Clostridium cochlearium*. *J. Bacteriol.* **100**:994-1001.
34. Prestidge, L., V. Gage, and J. Spizizen. 1971. Protease activities during the course of sporulation in *Bacillus subtilis*. *J. Bacteriol.* **107**:815-823.
35. Ryter, A. 1965. Etude morphologique de la sporulation de *Bacillus subtilis*. *Annu. Gust. Pasteur* **108**:40-60.
36. Sadoff, H. L. 1972. Sporulation antibiotics of *Bacillus* species, p. 157. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), *Spores V*. American Society for Microbiology, Washington, D.C.
37. Schleifer, K. H., and O. Kandler. 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. *Bacteriol. Rev.* **36**:407-477.
38. Strominger, J. L., E. Ito, and R. H. Threnn. 1960. Competitive inhibition of enzymatic reactions by oxamycin. *J. Amer. Chem. Soc.* **82**:998-999.
39. Tipper, D. J. 1973. Bacterial cell walls. *In* G. Timasheff and S. Fasman (ed.), "Biological macromolecules", vol. 6B. Marcel Dekker.
40. Tipper, D. J., and J. J. Gauthier. 1972. Structure of the bacterial endospore, p. 3. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), *Spores V*. American Society for Microbiology, Washington, D.C.
41. Tipper, D. J., and I. Pratt. 1970. Cell wall polymers of *Bacillus sphaericus* 9602. II. Synthesis of the first enzyme unique to cortex synthesis during sporulation. *J. Bacteriol.* **103**:305-317.
42. 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.
43. Warth, A. D., and J. L. Strominger. 1972. Structure of the peptidoglycan from spores of *Bacillus subtilis*. *Biochemistry* **11**:1389-1395.