

## Distribution of Peptidoglycan Synthetase Activities Between Sporangia and Forespores in Sporulating Cells of *Bacillus sphaericus*

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Sporulating cells of *Bacillus sphaericus* 9602 containing fully engulfed forespores at different stages of maturity were broken by ultrasonic disruption, followed by grinding with alumina. In this way soluble enzymes derived mainly from the sporangial or from the forespore cytoplasm were obtained. Diaminopimelate ligase activity is required exclusively for cortical peptidoglycan synthesis, is absent during vegetative growth, and is synthesized during forespore maturation. It is found exclusively in the sporangial cytoplasm. L-lysine ligase is required for vegetative cell wall peptidoglycan synthesis but not for cortex synthesis. It is found in both fractions, but it has a fourfold higher specific activity in the forespore cytoplasm. Other enzymes that are required for synthesis of the nucleotide-pentapeptide precursors of both cortical and vegetative cell wall peptidoglycans are found in similar specific activities in both compartments. Mature spores, free of any residual sporangial material, have specific activities of all of these enzymes and of L-lysine ligase similar to those in forespores and in vegetative cells and are devoid of diaminopimelate ligase activity. Thus, the differential expression of at least one gene required for spore cortex synthesis in *B. sphaericus* occurs exclusively in the sporangial cytoplasm.

During sporulation in *Bacillus sphaericus*, as in other bacilli and clostridia (cf. 4, 14, 15), the first clearly unique morphological event is the formation of the sporulation septum near one-cell terminus, producing an asymmetric, two-compartment system. Time during sporulation in *B. sphaericus* is represented as  $T_n$ , meaning  $n$  hours after the end of exponential growth. The septum appears at  $T_2$  (6). Since the smaller compartment develops into the spore and the larger compartment can revert to vegetative growth at this stage (4, 22), each must contain at least one complete copy of the genome (cf. 1).

The further development of the spore (6) resembles that seen in *B. subtilis* (17) and other bacilli (4, 14, 15). Immediately after formation of the sporulation septum, engulfment of the *B. sphaericus* forespore commences by proliferation of the septal membrane around the smaller cell compartment. The surrounding cell terminus swells at the same time, and this is clearly visible by phase-contrast microscopy by  $T_{2.5}$ . Engulfment is complete at  $T_4$  and results in the formation of a forespore entirely surrounded by

two concentric membranes, the inner and outer forespore membranes (6). Topologically, the "inner" surfaces of these membranes face the forespore and sporangial cytoplasm, respectively, whereas the "outer" surfaces face one another. The forespore and its membranes are also surrounded by sporangial cytoplasm, membrane, and cell wall.

Shortly after engulfment is complete, between  $T_4$  and  $T_5$ , midcoat development commences and a primordial cell wall layer (PCW) is formed between the inner and outer forespore membranes, adjacent to the inner membrane (6). In *B. sphaericus*, as in other bacilli (4, 14), the PCW apparently develops into the cell wall of the outgrowing cell after spore germination. The forespore becomes semirefractile with a fully condensed nucleoid at  $T_5$ , before significant accumulation of cortex occurs. The cortex, which consistently stains less densely than the PCW, is then formed between the PCW and the outer forespore membrane. Synthesis of coats and exosporium continues during this period ( $T_5$  to  $T_7$ ); dipicolinate accumulates at the same time (21), and a mature, dormant, heat-resistant spore is formed after 8 to 9 h of sporulation (6).

Vegetative cell walls of *B. sphaericus* contain a peptidoglycan cross-linked between L-lysine

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residues by D-alanyl-D-isoasparaginyl residues (8). This polymer is devoid of diaminopimelic acid (Dpm) and is only slowly hydrolyzed by lysozyme. In *B. spaeiricus* as in other bacilli (15, 21), the spore cortex is a peptidoglycan that is highly susceptible to lysozyme and that is solubilized during spore germination. It contains *meso*-Dpm, is devoid of lysine and aspartic acid, and has a structure indistinguishable from that of *B. subtilis* spore cortex (24; D. J. Tipper, *Bacteriol. Proc.*, p. 120; 1964; L. Landbeck and D. J. Tipper, unpublished data). The properties of the PCW seen in spores of other species (23) suggest that it is composed largely of peptidoglycan with a structure similar to that of vegetative cell wall peptidoglycan. Thoroughly cleaned spores of *B. spaeiricus* contain a lysozyme-resistant peptidoglycan fraction that contains L-lysine, D-aspartate, and little, if any, Dpm. It is presumably PCW (D. J. Tipper, unpublished data).

Any enzyme in *B. spaeiricus* involved in the synthesis or degradation of Dpm-containing peptidoglycan, but not of lysine-containing peptidoglycan, must be specific for cortex synthesis or modification. Such an enzyme would be without function in vegetative growth and would be sporulation specific. The appearance of two such activities in synchronously sporulating cells of *B. spaeiricus* has been demonstrated. The first is an endopeptidase that hydrolyzes cortical peptidoglycan between its D-glutamate and *meso*-Dpm residues (5), and the second is involved in the synthesis of the nucleotide-pentapeptide precursor of cortex peptidoglycan (22).

Synthesis of all bacterial cell wall peptidoglycans involves a soluble, cytoplasmically synthesized precursor, uridine-di-phospho-N-acetylmuramyl-pentapeptide (UDP-MurNac-pentapeptide), and it is probable that this is also true of the cortical peptidoglycan (21, 24). The pathways for the synthesis of the nucleotide pentapeptide precursors of cortical and vegetative cell wall peptidoglycans in *B. spaeiricus* are shown in Fig. 1. The two nucleotide-pentapeptides have a common nucleotide-dipeptide precursor, and the two pathways differ only in the enzymes for conversion of this nucleotide-dipeptide to the nucleotide-tripeptides containing L-lysine or *meso*-Dpm. Vegetative cells of *B. spaeiricus* contain L-Lys ligase, which is incapable of ligating Dpm, and Dpm ligase activity appears between  $T_{4.5}$  and  $T_6$  and is incapable of ligating L-Lys (22). A single enzyme is probably responsible for both UDP-MurNac-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala ligase activity and UDP-MurNac-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala

ligase activity, since these activities are present at a constant ratio throughout vegetative growth and sporulation (10). This activity is henceforth referred to as D-Ala-D-Ala ligase.

We have recently demonstrated (11) that synthesis of all of the enzymes involved in the synthesis of the nucleotide-pentapeptide precursor of vegetative cell wall peptidoglycan (cf Fig. 1) ceases at  $T_0$ , the initiation of sporulation in *B. spaeiricus*. Synthesis of all these enzymes, including L-Lys ligase (but not Dpm ligase), resumes immediately after spore septum formation at  $T_2$  and continues until fore-spore engulfment is complete at  $T_4$ . Synthesis of D-Ala-D-Ala synthetase, D-Ala-D-Ala ligase, and Lys ligase during this period is a consequence of derepression at the level of transcription (11, 22). Shortly after the termination of this period of enzyme synthesis, at  $T_{4.5}$ , synthesis of all of the enzymes involved in making the

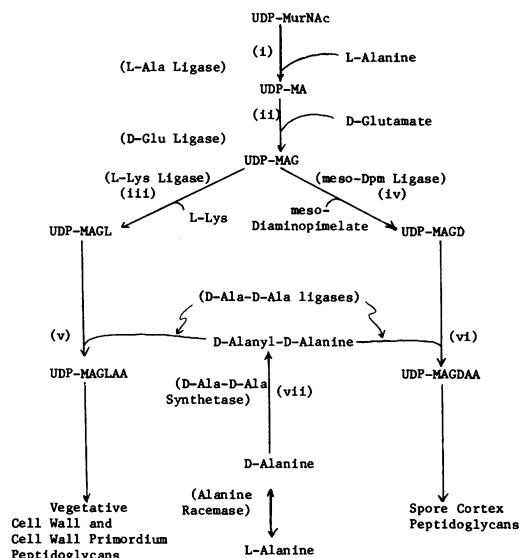


FIG. 1. Enzymatic reactions that have been demonstrated in *B. spaeiricus* 9602 and that are involved in the synthesis of the nucleotide-pentapeptide precursors of its vegetative cell wall and spore cortex peptidoglycans. Abbreviations: uridine-diphospho-N-acetyl-muramic acid, UDP-MurNac, UDP-M; alanine, Ala, A; D-glutamate, Glu, G; L-lysine, Lys, L; *meso*- $\alpha$ -diaminopimelic acid, Dpm, D. The enzymes are UDP-MurNac-L-Ala ligase (EC 6.3.2.8, L-Ala ligase); UDP-MurNac-L-Ala-D-Glu ligase (EC 6.3.2.9, D-Glu ligase); UDP-MurNac-L-Ala-D-Glu-L-Lys ligase (EC 6.3.2.7, L-Lys ligase); UDP-MurNac-L-Ala-D-Glu-*meso*-Dpm ligase (EC 6.3.2.13, Dpm ligase); UDP-MurNac-L-Ala-D-Glu-L-Lys-D-Ala-D-Ala ligase (EC 6.3.2.10, D-Ala-D-Ala ligase), alanine racemase (EC 5.1.1.1), and D-Ala-D-Ala synthetase (EC 6.3.2.4).

precursor of cortex peptidoglycan (cf. Fig. 1) commences and continues to  $T_6$ . This period starts while PCW is being synthesized and before the appearance of cortex. During this period, Dpm ligase is synthesized, but Lys ligase is not. Synthesis of Dpm ligase, D-Ala-D-Ala synthetase, and D-Ala-D-Ala ligase during this period is also a consequence of derepression at the level of transcription (11, 22). It is the purpose of this paper to demonstrate that the two temporally distinct transcription events occurring at  $T_2$  to  $T_4$  and  $T_{4.5}$  to  $T_{6.5}$  are also physically separate and functionally distinct. The first appears to involve the forespore genome and the second the mother cell genome. Apparently, their purpose is to prime the cell for synthesis of PCW and cortex, respectively.

## MATERIALS AND METHODS

**Organism and growth conditions.** *B. sphaericus* 9602 was grown in BS broth plus spore salts at 33 C with vigorous aeration as previously described (10, 22). Under these conditions, turbidity increases during vegetative growth, with a doubling time of 35 min.  $T_0$ , the initiation of sporulation, is taken as the point at which this doubling time abruptly increases to 80 min prior to a final round of postexponential vegetative cell division (6).

Phase-contrast microscope observations, quantitated as previously described (11, 22), enable the identification of two morphological stages in sporulation. The first is the appearance of terminal swelling at  $T_2$  to  $T_3$ , corresponding to the initiation of forespore engulfment, and the second is the appearance of semirefractility within the forespores at  $T_5$  to  $T_7$ , corresponding to the initiation of cortex development.

**Preparation of soluble enzyme extracts from sporulating cells. A. Ultrasonic disruption—normal procedure** (10, 22). Samples (50 ml) of a sporulating culture of *B. sphaericus* (250 ml) were removed at intervals and centrifuged at  $12,000 \times g$  for 10 min at 4 C. The cells were washed with 5 ml of buffer B [50 mM/tris(hydroxymethyl)aminomethane-hydrochloride, pH 8 at 25 C, 10 mM  $MgCl_2$ , and 4 mM dithiothreitol] at 4 C and were suspended in 1 ml of buffer B at 4 C in a 15-ml Corex test tube. These cells were disrupted for 10 min using the microtip of a Branson J17V sonifier (Heat Systems, Inc.) at a power setting that just failed to cause cavitation. The tip was precooled, and the tube was immersed in a bath at -10 C during disruption. Phase-contrast microscope observations indicated complete fragmentation of the sporangia at all stages of sporulation but persistence of forespores after  $T_5$ . The disrupted cell suspensions were centrifuged at  $48,000 \times g$  for 10 min. The supernatant fluid was mixed at 0 C with 3 ml of saturated  $(NH_4)_2SO_4$  containing 0.1 mM ethylenediaminetetraacetic acid, pH 7.5. After 30 min at 0 C, the protein precipitate was collected by centrifugation at  $12,000 \times g$  for 10 min, dissolved in 1 ml of buffer B, and stored at -80 C.

**B. Disruption of whole cells by grinding with alumina.** Cells from a sample (50 ml) of a sporulating culture of *B. sphaericus* at  $T_5$ , containing 40% prerefractile forespores, were isolated as described above. This pellet (300 mg, wet weight) was mixed with 900 mg of levigated alumina (Norton) in an ice-cooled mortar and ground by hand for a total of 5 min to give a thick paste. The paste was mixed by grinding with 2 ml of buffer B and was centrifuged at  $12,000 \times g$  for 10 min. The pellet was suspended in 2 ml of buffer B and centrifuged, and the combined supernatants were centrifuged at  $48,000 \times g$  for 10 min. The soluble proteins were isolated by  $(NH_4)_2SO_4$  precipitation, as described above, and dissolved in 1 ml of buffer B.

**C. Sequential disruption by normal ultrasonic treatment, followed by grinding with alumina.** Wet pellets (about 200 mg, wet weight) remaining after ultrasonic disruption by procedure A (above) were mixed with alumina (600 mg) and, after grinding for 5 min, the soluble proteins were isolated and precipitated with  $(NH_4)_2SO_4$ , as described above. They were dissolved in 0.5 ml of buffer B.

**D. Sequential disruption by brief and prolonged ultrasonic treatment, followed by grinding with alumina.** Samples (50 ml) of a sporulating culture of *B. sphaericus* (1 liter) were removed at intervals and centrifuged as described in procedure A. After washing with buffer B (5 ml), the cells were suspended in 2 ml of buffer B in a 30-ml Corex tube at 4 C. These cells were disrupted for 1 min using the 1.27-cm step horn of the J17V sonifier at about 15% of maximum power. Phase-contrast microscope observations indicated about 50% fragmentation of the sporangia. After centrifugation at  $48,000 \times g$  for 10 min, the soluble proteins were isolated from the supernatant fluid by precipitation with  $(NH_4)_2SO_4$ , as described above, and dissolved in 1 ml of buffer B, giving enzyme preparations S1.

The pellet of partially broken sporangia was resuspended in 2 ml of buffer B and disrupted for 10 min with the 1.27-cm step horn, as described above, resulting in 100% disruption. The soluble proteins were isolated by centrifugation and  $(NH_4)_2SO_4$  precipitation, as described above, and dissolved in 1 ml of buffer B, giving enzyme preparations S2.

The pellets from the second ultrasonic disruption (about 200 mg, wet weight) were ground with alumina (600 mg) as described above. The soluble proteins were extracted with 1 ml of buffer B, precipitated with  $(NH_4)_2SO_4$ , and dissolved in 0.5 ml of buffer B to give enzyme preparations G.

**E. Preparation of clean, dry spores and preparation of soluble enzymes from them.** A culture (750 ml) of *B. sphaericus* was shaken in a 2-liter baffled flask for 15 h at 33 C. At this time, 97% of the cells contained mature, phase-bright spores, but only 10% of these spores were free from sporangia. After storage without shaking at 4 C for 2 days, 60% of the spores were free, and they were harvested by centrifugation at  $12,000 \times g$  for 10 min and washed with 100 ml of 20 mM  $KH_2PO_4$ , pH 7.8 (buffer A). The spores were suspended in 16 ml of buffer A and subjected to ultrasonic disruption with the microtip

of a Branson J17V sonifier for 30 min while immersed in a bath at  $-8^{\circ}\text{C}$ . This resulted in complete disruption of the remaining sporangia but had no apparent effect on the free spores, which were isolated by centrifugation at  $12,000 \times g$  for 10 min. The pellet was washed twice by resuspension in buffer A (using brief ultrasonic treatment) and centrifugation. The final pellet contained 1 or 2% of phase-dark spores and small amounts of cellular debris but no intact vegetative cells or sporangia. It was washed once with water, suspended in about 10 ml of water, and lyophilized to give clean, dry spores (230 mg).

Spores (100 mg) were suspended in 15 ml of buffer B, mixed with 20 ml of clean glass beads (0.15 to 0.25 mm), and broken in a Braun cell homogenizer (VWR Scientific, Bronwill Division). After two periods of breakage of 1 min at maximum speed with continuous cooling with liquid  $\text{CO}_2$ , phase-contrast microscopy indicated complete disruption of spores. The suspension was centrifuged at  $35,000 \times g$  for 30 min, and the protein was precipitated from the clear supernatant fluid with  $(\text{NH}_4)_2\text{SO}_4$ , as described above. The protein precipitate was dissolved in 2 ml of buffer B.

**F. Preparation of heat-shocked spores and soluble proteins.** A second culture (750 ml) of *B. sphaericus* was harvested after 15 h at  $33^{\circ}\text{C}$  (98% phase-bright spores, 10% free) and suspended in buffer A (80 ml). After incubation at  $37^{\circ}\text{C}$  for 2 h, all of the spores were free, but much cell debris remained. The spores were suspended in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5 (at  $25^{\circ}\text{C}$ )–0.01 M  $\text{MgCl}_2$  (20 ml) containing 0.25 mg of lysozyme (Sigma Chemical Co.) per ml and incubated at  $37^{\circ}\text{C}$  with gentle shaking for 1 h. *B. sphaericus* vegetative cell wall peptidoglycan is slowly hydrolyzed by lysozyme (8). Ten percent sodium dodecyl sulfate (5 ml) was then added and, after further incubation for 0.5 h at  $37^{\circ}\text{C}$ , the spores were washed 10 times with water and lyophilized. They then appeared to be entirely free of contamination by fragments of sporangial cells and were about 2% phase dark.

Dry spores (65 mg) were suspended in water (20 ml) and incubated at  $65^{\circ}\text{C}$  for 1 h. This heat activation results in rapid germination on suspension in appropriate media. After centrifugation, the pellet of heat-activated spores was lyophilized and mixed with 200  $\mu\text{l}$  of buffer B and 600 mg of alumina and ground for 8 min. Soluble proteins were extracted with 1 ml of buffer B and precipitated with  $(\text{NH}_4)_2\text{SO}_4$ , as described above. The precipitated proteins were dissolved in 0.5 ml of buffer B.

**Enzyme assays.** All enzyme assays were performed as previously described (10, 22). One unit of enzyme will convert one micromole of substrate to product in 1 min under these standard assay conditions. Specific activity is presented as milliunits per milligram of protein. Protein was determined by the Lowry procedure (12).

## RESULTS

**Development of resistance to ultrasonic disruption in forespores.** In all of our previous

publications concerning the soluble enzymes involved in the synthesis of the nucleotide-pentapeptide precursors of peptidoglycans in *B. sphaericus* (10, 11, 22), these enzymes were obtained by ultrasonic disruption and precipitation with  $(\text{NH}_4)_2\text{SO}_4$ , as described in procedure A above. After  $T_6$ , when 60% of the cells contained semirefractile forespores, both phase-dark and semirefractile forespores were resistant to even 10 min of ultrasonic disruption, according to procedure A. At least 75% of the forespores were recovered in the pellet of residual particulate material, and the supernatant fluid from this procedure represented largely the sporangial cytoplasmic contents.

**Recovery of soluble enzymes from sporangia and forespores by grinding with alumina.** Membrane fragments have been prepared from sporulating *B. sphaericus* cells by grinding with glass beads in a colloid mill (5), and it was observed that this procedure did disrupt semirefractile forespores. However, this procedure was not applicable to small quantities of cells or forespores. Cells at 4, 5, and 6 h of sporulation, containing 0, 40, and 80% semirefractile forespores, were disrupted by ultrasonic treatment (procedure A, 10 min), resulting in complete disruption of sporangia and production of soluble enzyme fractions S (Table 1). The pellets from centrifugation, containing forespores, were ground with alumina. After subsequent addition of buffer and centrifugation, the residual insoluble cell fragments formed a pigmented layer on top of the alumina, and microscopic examination of this layer enabled the degree of disruption to be assessed. This was somewhat variable but was complete in those preparations for which the data is presented in Table 1. Although the 4-h sample contained fully engulfed forespores, the pellet after ultrasonic disruption was largely debris, and grinding of this residual particulate material released only 3.4% of the total soluble protein. This is apparently a nonspecific fraction, since it had a low specific activity of the Lys ligase, which is enriched in the forespore fraction (see below). Maximal yields of soluble protein in the fraction released by grinding were already achieved in the second sample, which had only 40% refractility, and the properties of this fraction and that released from the third sample were similar. These fractions contain a very low specific activity of the Dpm ligase and less than 2% of the total Dpm ligase activity, whereas they contained a relatively high specific activity of the Lys ligase and between 17 and 26% of the total activity of the cells. For each cell preparation, mixing of the soluble



TABLE 1. Sequential disruption of sporulating *B. sphaericus* cells: distribution of Lys and Dpm ligase activities

Sporulation (h)	% Refractility <sup>a</sup>	Enzyme Prepn	Soluble protein (mg)	Ligase sp act (mU/mg)		% of total		
				Dpm	Lys	Soluble protein	Ligase activity	
							Dpm	Lys
4	0	S	49.2	0.08	0.75	96.6		99.5
		G	1.68	0.00	0.10	3.4		0.5
5	40	S	45.6	0.65	0.62	91.4	98.4	83
		G	4.28	0.12	1.37	8.6	1.6	17
		TG	40.2	0.66	0.86	100		
6	80	S	44.6	1.35	0.40	92.2	99.5	74
		G	3.98	0.08	1.65	7.8	0.5	26

<sup>a</sup> Cells, all of which were terminally swollen, contained the indicated proportion of semirefractile forespores. Soluble enzyme preparations (S) were prepared by prolonged ultrasonic disruption according to procedure A in the text. Soluble enzyme preparations (G) were obtained from the residual insoluble material by grinding with alumina (procedure C). Preparation TG was from cells ground without prior ultrasonic treatment (procedure B). Protein is given as milligrams per 100 ml of culture.

enzyme fractions from ultrasonic and grinding treatments gave specific activities approximately the average of those found with the individual enzyme fractions. Thus, neither fraction contained an inhibitor or activator not also found in the other fraction. Moreover, when a sample of the 40% refractile cells was ground without prior ultrasonic treatment, the specific activities of the Dpm and Lys ligases were approximately those expected from the combination of the total material isolated previously by sequential ultrasonic and grinding treatments; therefore, both treatments give similar recoveries of enzyme activities (Table 1).

Equivalent activities of enzymes involved in the synthesis of the nucleotide-pentapeptide precursor of vegetative cell wall peptidoglycan could be obtained from exponential phase vegetative cells, either by grinding with alumina or by ultrasonic disruption (Table 2). It is concluded that these two treatments are equally effective in preserving the activity of these enzymes but that grinding with alumina is capable of releasing soluble enzyme from cell components that resist ultrasonic treatment.

The Dpm ligase released from the sporulating cells by ultrasonic treatment shows the previously described (10, 22) increase in specific activity between 4 and 6 h of sporulation (Table 1), whereas the Lys ligase activity in the same fraction shows the previously described (10, 22) decrease in specific activity.

**Peptidoglycan precursor synthesizing activities in mature spores.** Mature, dry spores that had been freed of residual sporangial fragments by prolonged ultrasonic treatment and washing could be broken by shaking with glass beads. The resultant soluble enzyme fraction

contains specific activities of the L-lysyl and D-alanyl-D-alanine ligases, D-alanyl-D-alanine synthetase, and alanine racemase within a factor of two of those found in vegetative cells (Table 2). Like vegetative cells, they were devoid of detectable Dpm ligase activity (Table 2). Mature spores of *B. sphaericus*, which have been freed from residual sporangial fragments by autolysis and treatment with lysozyme, followed by washing with 2% sodium dodecyl sulfate and heat activation for 1 h at 65°C, were capable of germinating with high efficiency and synchrony (D. J. Tipper, unpublished data). These sodium dodecyl sulfate-washed, heat-shocked spores should be free of any surface-bound enzyme activities, yet they still contained all of the enzyme activities needed for synthesis of the precursor of vegetative cell

TABLE 2. Enzyme activities in mature spores and vegetative cells<sup>a</sup>

Activity	Sp act (mU/mg)			
	Spore prep		Vegetative prep	
	Alu-mina	Beads	Alu-mina	Ultra-sonic
L-Ala ligase	11	ND	12	6.5
D-Glu ligase	5.1	ND	3.8	6.9
L-Lys ligase	5.2	3.56	4.5	2.84
Meso-Dpm ligase	0	0	0	0
Dpm:Ala-Ala ligase	ND	30.1	ND	28.1
Lys:Ala-Ala ligase	62	32.0	52	26.8
Ala-Ala synthetase	8.0	9.65	10	10.5
Ala racemase	ND	89	ND	44

<sup>a</sup> ND, Not determined. Procedures for preparation of soluble enzymes are as follows (see text). Vegetative preparations: alumina, procedure B; ultrasonic, procedure A. Spore preparations: alumina, procedure F; beads, procedure E.

wall peptidoglycan from UDP-MurNAc (Table 2). Again, this preparation was devoid of detectable Dpm ligase activity. As previously described (10), the enzyme preparations from vegetative cells do not distinguish between the alternate UDP-MurNAc-tripeptide acceptors for D-alanyl-D-alanine, which contain either Dpm or Lys in the C-terminal position. This is also true for the enzyme released from mature spores (Table 2).

**Distribution of Dpm and Lys ligase activities during forespore maturation.** Samples of cells from a synchronously sporulating culture of *B. sphaericus* were disrupted by two sequential ultrasonic treatments, followed by grinding with alumina (procedure D above). The first ultrasonic treatment was brief and used a 1.27-cm step horn with a lower power output density than the microtip previously used. It was hoped to achieve more uniform degradation of the cells and the isolation of intact forespores at an early stage. The initial ultrasonic treatment resulted in about 50% cell breakage. The second, prolonged treatment resulted in complete breakage of sporangia and left the majority of forespores intact only when the sporangia contained 50% or more prerefractile forespores, as in the normal procedure. The yield of soluble protein released by the final grinding procedure (Fig. 2) was maximal from this time (5.5 h, sixth sample) and was similar to the 8% found

previously (Table 1). No significant difference was seen between the supernatant enzymes from the two ultrasonic treatments, and only in the fifth sample (at 5.2 h) and in subsequent samples does the soluble protein released by grinding represent a specific forespore fraction of the cells. Thus, in the first four samples, all of the ligase activities of both sporangial and forespore fractions are found in the supernatants from ultrasonic disruption, and this fraction of the fifth sample also contains considerable forespore material. However, this fraction from the sixth and subsequent fractions is relatively free of forespore components.

The data for Dpm ligase show the expected appearance of activity at about 4 h of sporulation, increasing over the next 2-h period in parallel with the appearance of semirefractile forespores. The activity of Dpm ligase in the fraction released by grinding is negligible, even in the later fractions in which the recovery of forespores was high. Since these fractions were not washed, even the small amount of activity found probably represents contaminating sporangial material.

The Lys ligase activity in the supernatant fluids from ultrasonic disruption shows the previously described (22) fall after a transient rise in the second sample (about 3.5 h). In contrast, the fractions released by alumina grinding from forespores from 5.2 h on (fifth and subse-

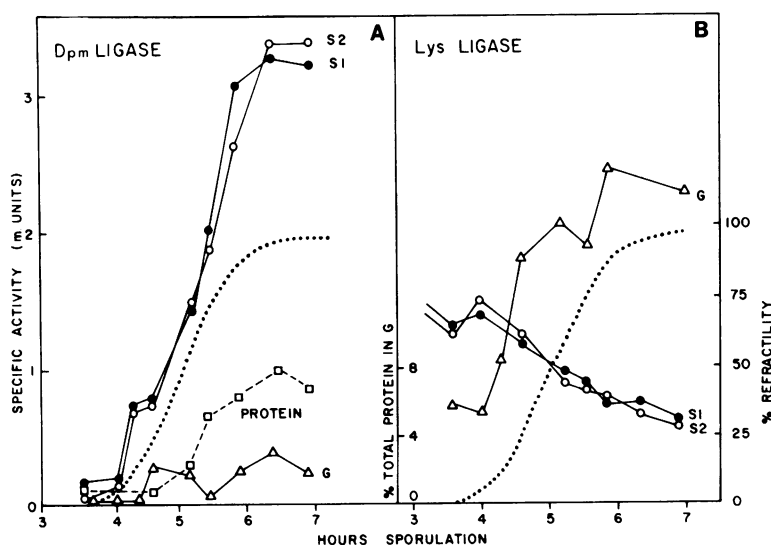


FIG. 2. Distribution of Dpm and Lys ligase activities between sporangia and forespores during forespore development. Cells at the indicated stage of sporulation were sequentially disrupted (procedure D, text) by two ultrasonic treatments (preparations S1 and S2) and by grinding with alumina (preparations G). Specific activities of ligases in the supernatant fractions from these procedures are presented. (A) Percentage of the total soluble protein (S1 + S2 + G) in preparations G (□). Specific activities of Dpm ligase in preparations S1 (○), S2 (●), and G (△). (B) Percentage of semirefractile forespores (dotted line). Specific activities of Lys ligase in preparations S1 (○), S2 (●), and G (△).

quent samples) show relatively high and constant specific activities of Lys ligase. When the distribution of total activity of the Dpm and Lys ligases is calculated between the sporangial and forespore fractions, again it is found that the forespore fraction contains less than 2% of the Dpm ligase activity but 20 to 25% of the Lys ligase activity.

**Distribution of other enzymes involved in peptidoglycan precursor synthesis during forespore maturation.** The soluble enzyme preparations obtained by ultrasonic disruption of sporulating *B. sphaericus* cells between  $T_{4.5}$  and  $T_7$  showed an approximately twofold increase in specific activity of D-Ala-D-Ala ligase, D-Ala-D-Ala synthetase, and D-Glu ligase during this period (Fig. 3), as previously described (10, 11). This corresponds to the second period of synthesis of these enzymes, which occurs coordinately with the appearance of Dpm ligase activity (11), as shown in Fig. 3. L-Ala ligase activities were not determined, and data for S1 (shown) and S2 were very similar. The specific activities found in the forespore-specific G fractions (from  $T_{5.2}$  on) were essentially constant and similar to those found in vegetative cells and mature spores (Table 2), although scatter in the data would obscure minor variations.

## DISCUSSION

The process of bacterial cell wall peptidoglycan biosynthesis is vectorial. The phospho-MurNAc-pentapeptide moiety of the UDP-MurNAc-pentapeptide precursor is transferred to a  $C_{55}$ -isoprenoid alcohol phosphate acceptor at the inner surface of the cytoplasmic membrane, converted to a complete disaccharide-peptide monomer of the completed peptidoglycan, and polymerized on the outer surface of the membrane. Topography (see Introduction) has led to the hypothesis (21) that PCW is synthesized from precursors made in the forespore by the inner forespore membrane, whereas cortex is synthesized from precursors made in the sporangial cytoplasm by enzymes in the outer forespore membrane. It is predicted that, in *B. sphaericus*, Dpm ligase, the single unique enzyme required for synthesis of the precursor of cortex peptidoglycan, will be found only in the sporangial cytoplasm.

Mature spores of *B. sphaericus*, like vegetative cells of the same organism, are found to contain all of the enzyme activities necessary for the conversion of UDP-MurNAc to the UDP-MurNAc-pentapeptide precursor of vegetative peptidoglycan, but they are found to lack Dpm ligase. After extensive ultrasonic disruption, forespores can be isolated from sporulating *B.*

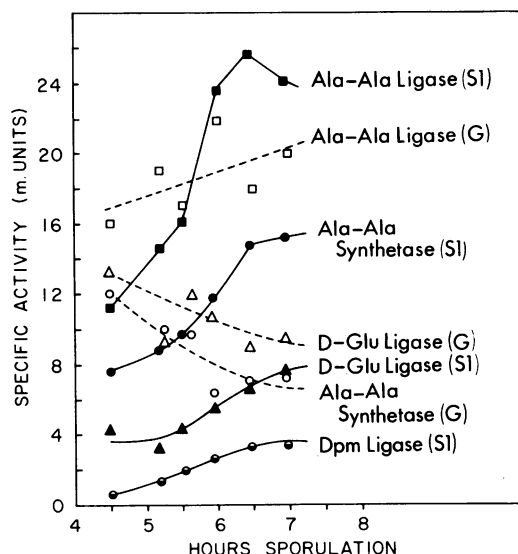


FIG. 3. Distribution of other peptidoglycan precursor-synthesizing activities between sporangia and forespores during forespore development. The same preparations for which data on Dpm and Lys ligase activities are presented in Fig. 2 were used, but only data for preparations S1 (full lines and solid symbols) and G (dashed lines and open symbols) are presented. Data for preparations S2 were similar to those for S1. Symbols: ■, □, UDP-MurNAc-L-Ala-D-Glu-meso-Dpm:D-Ala-D-Ala ligase activities; ●, ○, D-Ala-D-Ala synthetase activities; ▲, △, UDP-MurNAc-L-Ala:D-Glu ligase activities; ○, ○, UDP-MurNAc-L-Ala-D-Glu-meso-Dpm ligase activities. The sporangium contains 92% of the total soluble protein, even late in forespore maturation (Table 1, Fig. 1), and all sporangial soluble protein is found in preparations S1 + S2. Thus, variations in specific activities of preparations S1 and S2 reflect mostly variations in sporangial enzyme contents, independent of the degree of contamination by forespore contents.

*sphaericus* cells at  $T_5$  or later, essentially free from contaminating sporangial cytoplasm. At this stage, when PCW synthesis is apparent in thin sections but before significant cortex synthesis or spore coat assembly is visible, Dpm ligase is being actively synthesized in the sporangial cytoplasm and is excluded from the forespores, as predicted. The period from  $T_{4.5}$  to  $T_{6.5}$  is also the second period of accumulation during sporulation of all of the enzymes common to the synthesis of vegetative and cortical peptidoglycan precursor (11), and the data in Fig. 3 are consistent with the hypothesis that all of them are also synthesized in the sporangial cytoplasm as a consequence of coordinate derepression of their structural genes in this cell compartment.

Like mature spores, forespore cytoplasm isolated after  $T_5$  contains vegetative levels of activity of all of the enzymes required for synthesis of the precursor of vegetative peptidoglycan (Fig. 3), including a high specific activity of Lys ligase (Fig. 2). In contrast, the sporangial cytoplasmic fraction has already lost 60% of its Lys ligase specific activity by  $T_2$  as a consequence of dilution (11), and preparations obtained after  $T_{5.5}$ , when they are essentially free of forespore material, have an even lower activity of Lys ligase, in spite of the period of accumulation of total activity between  $T_2$  and  $T_4$  (11). This increase occurs during the first period of accumulation of activity of all of the enzymes common to the synthesis of vegetative and cortical peptidoglycan precursors (11), and these data are consistent with the hypothesis that this first period of synthesis occurs (to a major extent) in the forespore, which is being engulfed at that time, and that these enzymes within the forespore remain stable during the rest of sporulation. Meanwhile, there is a gradual decay of the L-Lys ligase within the sporangial cytoplasm. The decrease seen in Lys ligase activity of sonication supernatant material between  $T_4$  and  $T_6$  (Fig. 3) is, at least in part, explained by the decreasing recovery during that period of that fraction of the enzyme activity (25% of the total) residing in the forespores. However, some enzyme degradation must also be taking place.

Outgrowth after spore germination must involve considerable synthesis of vegetative-type peptidoglycan, and the presence of the appropriate precursor-synthesizing activities within the dormant spore is to be expected. Their presence in the forespore is also consistent with the hypothesized role of the forespore (21; see above) in the synthesis of a PCW of vegetative-type peptidoglycan, and their synthesis during the earlier stages of sporulation is consistent with labeling studies on the time of synthesis of forespore core proteins in *B. subtilis* (9).

With respect to peptidoglycan-synthesizing enzymes, the forespore resembles a vegetative cell, whereas the sporangium expresses sporulation-specific activities. It has been hypothesized (5), on the basis of its substrate specificity, that the D-Glu:meso-Dpm endopeptidase activity that accumulates in the membranes of sporulating *B. sphaericus* between  $T_1$  and  $T_4$  (5) is located in the outer forespore membrane, where it could function in the post-polymerization steps of cortical peptidoglycan synthesis. This hypothesis also requires synthesis of this enzyme activity, which is unique to sporulation, within the sporangium. Spore coat and exosporium constituents are also assembled

and presumably synthesized in the sporangial cytoplasm, in which most late sporulation-specific events seem to take place. However, it should be emphasized that PCW synthesis is probably not equivalent to normal vegetative cell wall synthesis since, in *B. sphaericus*, it does not involve synthesis of the structured external T layer protein, which is found on the vegetative cell wall (7). There is no morphological evidence for its presence adjacent to the PCW and, in *B. polymyxa*, which has a very similarly structured protein layer (16), this is also absent from the spore and appears very early during spore germination and outgrowth, while the spore core is still entirely surrounded by ruptured spore coats (13). In *B. subtilis*, immunological evidence also indicates that the spores are entirely devoid of the teichoic acid component of the cell wall but that this appears immediately on germination (2). Thus, PCW synthesis appears to be a very limited expression of vegetative-type cell wall synthesis, involving only the peptidoglycan component. If this is the consequence of forespore metabolism, it is differentiated from normal vegetative cell functions.

It may be asked whether the forespore genome expresses any other sporulation-specific information or whether it merely behaves like a vegetative cell under conditions of nutritional starvation. Clearly, it may play more than a passive role in the acquisition of dormancy, heat resistance, ultraviolet resistance, and the accumulation of low-molecular-weight substances, such as dipicolinate and sulfolactic acid (24), which are unique to sporulation. However, recent data (D. Ellar, personal communication) suggest that active calcium accumulation during sporulation is a function of the sporangial cytoplasmic membrane and that the accumulated calcium passes through the two opposed forespore membranes by a more passive process. It is possible that dipicolinate and sulfolactic acid are synthesized in the sporangium and transported into the forespore, perhaps as calcium chelates. Mature spores also have vegetative levels of proteolytic enzymes (19), in contrast to the relatively high levels of protease activity found in sporulating cells, and these sporulation proteases are also probably produced within the sporangium. On the other hand, mature spores of *B. megaterium* (18) and *B. subtilis* (D. J. Tipper and P. Setlow, unpublished data) contain unique hydrophilic proteins of relatively small size, which comprise up to 30% of the soluble proteins of the spore. In *B. megaterium* these proteins are made in a discrete period between  $T_3$  and  $T_4$  of sporulation,



about 1 h before the acquisition of refractility (18). It seems probable that this sporulation-specific event occurs within the forespore, since transport of these proteins from the sporangium seems unlikely.

It is probable that quite distinct sporulation-specific events are expressed within the sporangium and forespore, and in *B. sphaericus* it has been demonstrated that the sporangium alone expresses the unique information required for making the precursor of cortex, illustrating the differential expression of genes within the two compartments of the sporulating cell.

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