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Transcriptional Control of Peptidoglycan Precursor Synthesis During Sporulation in *Bacillus sphaericus*

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Synthesis of enzymes functional in the synthesis of nucleotide precursors of peptidoglycan ceases upon initiation of sporulation in *Bacillus sphaericus*. During sporulation, two periods of synthesis of these enzymes occur. The first starts at spore septum formation and is coincident with forespore engulfment; it involves the synthesis of those enzymes required for making the precursor of vegetative-type peptidoglycan, including L-lysyl ligase but not *meso*-diaminopimelyl ligase. The second period occurs shortly before the appearance of cortex. It involves the synthesis of diaminopimelyl ligase and the other enzymes needed for making the precursor of cortical peptidoglycan, but not lysyl ligase. Both events are a consequence of derepression at the level of transcription. Neither period of synthesis occurs in asporogenous mutants whose morphological block is at the point of spore septum formation.

The cortical peptidoglycans of bacterial spores of all species investigated (28, 29) are basically similar to the more familiar peptidoglycans of bacterial cell walls. However, they contain some unique elements, notably muramic lactam, which should allow biosynthesis of this major spore component to be distinguished from that of the host cell vegetative cell wall. The appearance of muramic lactam has been correlated with cortex synthesis in sporulating cells of *Bacillus megaterium* (30).

Two peptidoglycan components, cortex and primordial cell wall, can be morphologically distinguished in most bacterial spores (cf. 14). The primordial cell wall is that layer of the spore integuments lying between the inner forespore membrane and the cortex which, unlike the cortex, persists during germination and develops into the vegetative cell wall during outgrowth (14). Its properties suggest that it is composed largely of vegetative cell wall peptidoglycan (27), so its synthesis should also be distinguishable from that of cortex. Development of the primordial cell wall and cortical layers of *Bacillus* spores becomes apparent at stage IV of sporulation, after engulfment of the forespore (6, 14, 23). The control of cortex synthesis is thus a model for understanding the regulation of events occuring in mid to late sporulation which are unique to that process. These include dipicolinate, spore coat, and exosporium synthesis (3, 23, 26), and sulfolactate synthesis in *B. subtilis* (31).

B. sphaericus is particularly well suited to studies of cortex synthesis. Its vegetative cell walls contain a peptidoglycan cross-linked between its L-lysine residues by D-alanyl-D-isoasparaginyl residues (7). This polymer is devoid of diaminopimelic acid (Dpm) and is only slowly hydrolyzed by lysozyme. However, the spore cortex peptidoglycan of this organism contains *meso*-Dpm, is devoid of lysine and aspartic acid (D. J. Tipper, Bacteriol. Proc., p. 24, 1969), and is highly susceptible to lysozyme. It has a structure indistinguishable from that of the cortical peptidoglycan of *B. subtilis* (L. Landbeck and D. J. Tipper, unpublished data). *B. sphaericus* spores also contain a more lysozyme-resistant peptidoglycan fraction that contains lysine and D-aspartate. It is probably primordial cell wall, resembling vegetative cell wall peptidoglycan (D. J. Tipper, unpublished data). Thus, any enzymes in *B. sphaericus* involved in the metabolism of Dpm-containing peptidoglycan but not of lysine-containing peptidoglycan must be specific for cortex synthesis or modification and without function in vegetative growth. The appearance of two such activities in synchronously sporulating cells of *B. sphaericus* has been demonstrated. The first is UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm ligase (Dpm ligase, EC 6.3.2.13) (24), which incorporates *meso*-Dpm into the UDP-MurNAc-pentapeptide precursor of cortex, and the second is an endopeptidase, which hydrolyzes cortical

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peptidoglycan between D-glutamate and *meso*-Dpm residues (5).

Just as Dpm ligase is specific for cortex synthesis in *B. sphaericus*, UDP-MurNAc-L-Ala-D-Glu:L-Lys ligase (Lys ligase, EC 6.3.2.7) is specific for synthesis of vegetative-type peptidoglycan. All of the other enzymes involved in synthesis of the nucleotide precursors of cortical and vegetative cell wall peptidoglycans are common to the two pathways (9). In particular, a single enzyme is probably responsible for both UDP-MurNAc-L-Ala-D-Glu-L-Lys:D-Ala-D-Ala ligase (EC 6.3.2.10) activity and for 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 (9). This activity is henceforth referred to as D-Ala-D-Ala ligase.

With the exception of alanine racemase, the enzymes common to the synthesis of cortical and vegetative cell wall peptidoglycans follow a common pattern of variation in specific activity during sporulation. This involves a decrease in specific activity of about 50% during the first 2 h of sporulation. This is followed, 1 to 2 h later, by a rapid increase in specific activity, in parallel with the appearance of the Dpm ligase activity (9).

In this paper we demonstrate that synthesis of all ligases involved in synthesis of the precursor of vegetative peptidoglycan ceases at the start of sporulation (T_0). Dilution causes the 50% drop in specific activities between T_0 and T_0 plus 2 h (T_2). Between T_2 and T_4, increase in total ligase activities occurs without much alteration in specific activities. A second period of synthesis of ligase activities between $T_{4.5}$ and $T_{6.5}$ corresponds to the period of marked increase in specific activities previously identified (9).

It has previously been shown that the accumulation of Dpm ligase activity depends upon continued transcription and translation (24). It is now demonstrated that accumulation of the D-Ala-D-Ala synthetase and D-Ala-D-Ala ligase activities is similarly dependent on continued transcription and translation during both periods of total activity increase during sporulation. The significance of these events is discussed with respect to the morphogenetic events occurring during these same periods of sporulation, as demonstrated by electron microscopy of thin sections (6). This correlation with morphogenesis is also seen in asporogenous mutants of *B. sphaericus*, whose block appears to be roughly at the time of spore septum formation (T_2), just before the accumulation of D-Ala-D-Ala ligase activity normally resumes.

MATERIALS AND METHODS

Growth and sporulation conditions. *B. sphaericus* 9602 was grown in BS broth plus spore salts at 33 C with vigorous aeration, as previously described (6, 7, 9). Refrigerated spore stocks were heat-shocked (30 min, 80 C) and used to inoculate primary cultures, which were in turn used to inoculate secondary cultures (prewarmed to 33 C) to obtain cultures in balanced exponential growth for sporulation, as previously described (6). This results in relatively synchronous sporulation of at least 95% of the cells. This was estimated by dark-phase microscope observations of the percentage of terminally swollen cells and of cells containing semirefractile forespores (6, 24).

Isolation of sporulation-defective mutants. An exponential-phase culture of *B. sphaericus* 9602 containing approximately 2×10^8 cells/ml (10 ml) was filtered through a sterile membrane filter (Millipore Corp., 0.45-μm pore size), which was washed with 10 ml of the sterile basal medium of Singer et al. (22), after which the cells were suspended in 10 ml of this basal medium at 33 C. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to a final concentration of 0.1 mg/ml, and after shaking for 30 min (1) the cells were filtered, washed with basal medium (10 ml), and suspended in basal medium (10 ml) and plated at appropriate dilutions on BS agar. After incubation for 2 days at 33 C, several colonies lacking the dark pigmentation characteristic of the fully sporulated parental cells were picked for further investigation. Asporogenous mutants were maintained on slants of BS agar and stored at 4 C with monthly subculture. BS agar is BS broth plus spore salts containing 1.5% agar (Difco).

Preparation of soluble enzyme extracts. Enzyme extracts from vegetative and sporulating cells were prepared by ultrasonic disruption, as previously described (9, 25), using buffer B [15 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8 at 25 C, 10 mM MgCl, and 4 mM dithiothreitol]. After centrifugation to remove fragments of cells (48,000 $\times$ *g*, 10 min), soluble protein in the supernatant was precipitated with 75% saturated $(NH_4)_2SO_4$; the protein was precipitated by centrifugation at 12,000 $\times$ *g* for 10 min and dissolved in 2 ml of buffer B. Culture volumes were chosen to give enzyme preparations containing about 5 mg of protein per ml. These were stable for several weeks when stored at −80 C. Protein concentrations were determined by the method of Lowry et al. (10), using bovine serum albumin as standard. Cell disruption was complete, and yields of soluble protein from a given volume of culture were quite reproducible (±5%).

Preparation of soluble enzyme extracts from antibiotic-treated cells. Enzyme extracts from cells treated with either chloramphenicol (Cm) or streptolydigin (Sln) during sporulation were obtained as follows. A 1-ml portion of a culture of *B. sphaericus* 9602 growing in BS broth plus spore salts at 33 C and in mid-exponential phase (turbidity 40 to 50 as determined with a Klett-Summerson photoelectric colorimeter, red filter) was used to inoculate 600 ml of BS broth plus spore salts in 2-liter baffled flasks, pre-

warmed to 33 C. After 9 h of vigorous shaking, at $T_{2.5}$ (i.e., 2.5 h after T_0, see below), phase-contrast observations indicated that the cells were 40% terminally swollen. At this time aliquots (200 ml) of this culture were rapidly transferred to three 500-ml baffled flasks. These had been prewarmed to 33 C and contained, respectively, sterile water (10 ml, control) and 2 mg of Cm per ml (10 ml; final concentration, 100 μg/ml). Vigorous shaking at 33 C was commenced immediately, and samples (10 ml) were removed at intervals of 20 min for phase-contrast observations and for preparation of soluble enzyme extracts, as described above. A second, similarly prepared 600-ml culture was divided into three 200-ml cultures containing the same concentrations of antibiotics at T_0 plus 4.5 h, when most of the cells were terminally swollen, but none were yet refractile.

Assay of ligase activities. All enzymes were assayed as previously described (9). One unit of enzyme converts 1 μmol of substrate to product in 1 min at 37 C. Specific activities are represented as units per milligram of protein.

Measurement of dipicolinate content of sporulating cells. The dipicolinate content of sporulating cells was determined by the method of Lewis (8), as previously described (24). Under these conditions, a 1-ml culture sample containing 125 nmol of dipicolinate gives a differential absorbance of 1.0.

Production of gelatinase. Cells of *B. sphaericus* 9602 and of asporogenous mutants were streaked onto plates of BS medium containing 0.4% gelatin and 1.5% agar. After incubation for several days at 33 C, a solution of 0.15 g of $HgCl_2$ per ml in 2.3 N HCl (8 ml) was pipetted onto the plates, forming a milky precipitate with unhydrolyzed gelatin. After 2 h at room temperature, colonies surrounded by a clear zone within the milky background were scored as gelatinase positive.

Chemicals. Sln was a gift from B. Weisblum; Cm was obtained from Calbiochem.

RESULTS

Variation in specific activity and total activity of UDP-MurNAc-L-Ala-D-Glu-meso-Dpm:D-Ala-D-Ala ligase during sporulation. The kinetics of increase in turbidity, terminally swollen sporangia, and semirefractile forespores during sporulation in *B. sphaericus* are shown in Fig. 1A. T_0, the nominal time of initiation of sporulation, is defined by the time at which there is an abrupt decrease in the exponential rate of growth (6), as indicated by the upper scale (hours of sporulation). Turbidity continues to increase from T_0 to $T_{2.5}$ and then remains constant to $T_{3.5}$, before increasing again. The plateau in turbidity around T_3 corresponds to the time at which terminal swelling becomes visible in the light microscope (Fig. 1A), and this is followed 3.5 h later by the appearance of refractility (Fig. 1A). The specific activity of D-Ala-D-Ala ligase starts to decrease shortly before T_0 and drops to 40% of its initial value by T_3 and then returns almost to its initial value during the subsequent 3-h period. These data are very similar to those previously published (9). In Fig. 1B, the protein content of the supernatant from ultrasonic disruption of the cells is plotted on a linear scale and closely parallels the increase in turbidity, until T_4. Both increase approximately linearly between T_0 and T_3, after which the total protein content remains constant for 2 h and then declines as the forespores become refractile. Since semirefractile forespores are not disrupted by ultrasonic treatment, their appearance probably accounts for the initial decrease in soluble protein released by this procedure. The total activity of the ligase increases exponentially until shortly before T_0, remains constant until T_2, increases markedly between $T_{2.5}$ and $T_{3.5}$, and increases again between $T_{4.5}$ and $T_{6.5}$. The constant total activity present from T_0 to $T_{2.5}$, a period during which the total soluble protein increases 2.5-fold, accounts for the 60% decrease in specific activity during this same period. The period from $T_{2.5}$ to $T_{3.5}$, during which total protein and ligase activity are both increasing, does not result in a marked increase in ligase specific activity. The second period of synthesis, however, during which total protein is constant or decreasing while total ligase activity is increasing, corresponds to the period of marked increase in specific activity previously noted (9).

Variation in total activity during sporulation of other enzymes involved in the synthesis of peptidoglycan precursors. Variation during sporulation of *B. sphaericus* in the specific activities of the L-lysyl, *meso*-diaminopimelyl, L-alanyl, and D-glutamyl ligases, and of D-alanyl-D-alanine synthetase and alanine racemase, has been described previously (9). When variations in the total activities of these enzymes are plotted, the L-alanyl ligase and D-alanyl-D-alanine synthetase activities show the same pattern as the D-alanyl-D-alanine ligase (Fig. 2). Accumulation of activities ceases shortly before T_0, remains constant until about T_2, increases until $T_{3.5}$ and, after a second plateau of about 1 h, increases again from about $T_{4.5}$ to $T_{6.5}$.

As previously demonstrated (24), the *meso*-Dpm ligase is undetectable before $T_{4.5}$ and appears between $T_{4.5}$ and $T_{6.5}$, in parallel with the second period of increase in activity of the L-alanyl and D-alanyl-D-alanine ligases and of D-alanyl-D-alanine synthetase (Fig. 2). The exact timing varies up to 1 h in different experiments, probably as a consequence of minor variations in the medium and the efficiency of aeration. However, when the kinetic

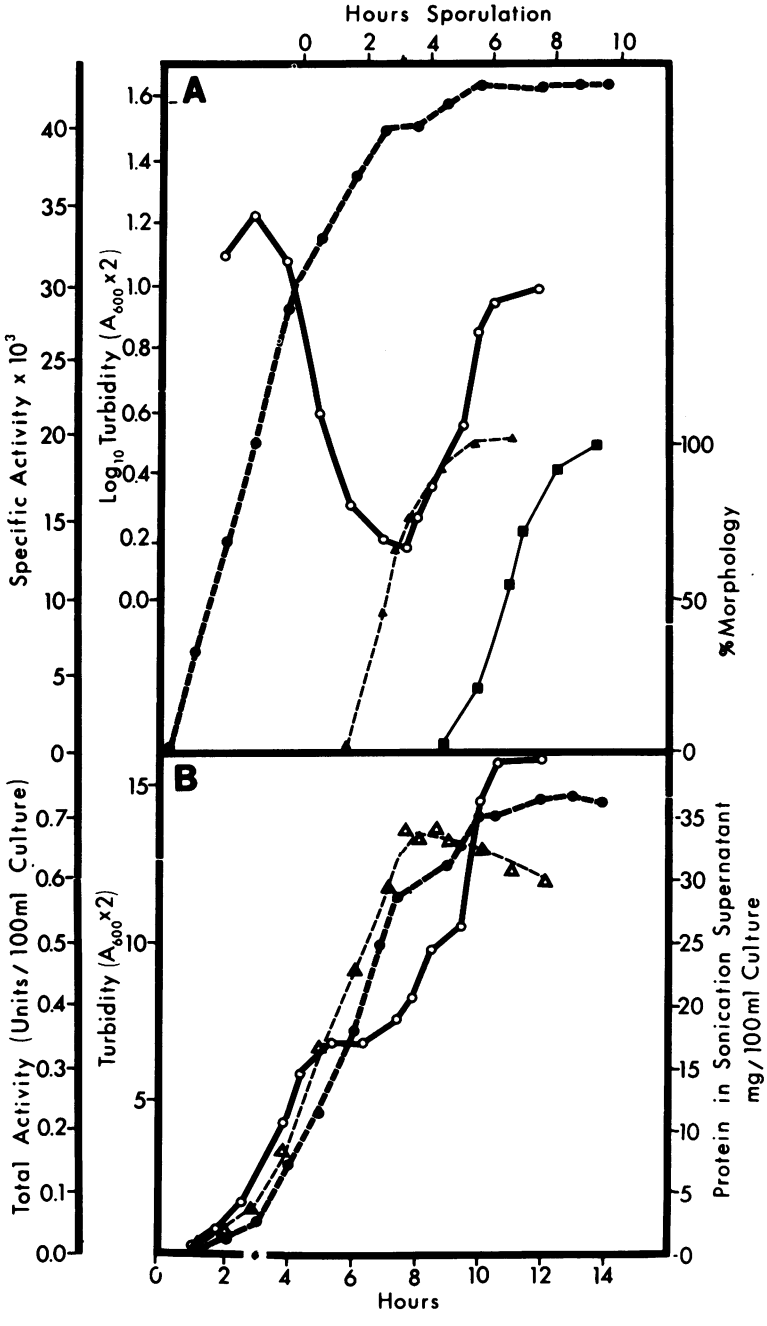

FIG. 1. *(A) Variation in the specific activity of UDP-murNAc-L-Ala-D-Glu-meso-Dpm:D-Ala-D-Ala ligase during sporulation. Symbols: ○, ligase activity; ●, turbidity (log scale); ▲, percentage of terminally swollen cells; ■, percentage of semirefractile forespores. (B) Variation in the total activity of D-Ala-D-Ala ligase during sporulation. Symbols: ○, ligase activity; ●, turbidity (linear scale); △, total protein content of supernatant from ultrasonic disruption. T_0, the time of initiation of sporulation, is defined by the abrupt increase in turbidity doubling time at 4.5 h (upper scale).*

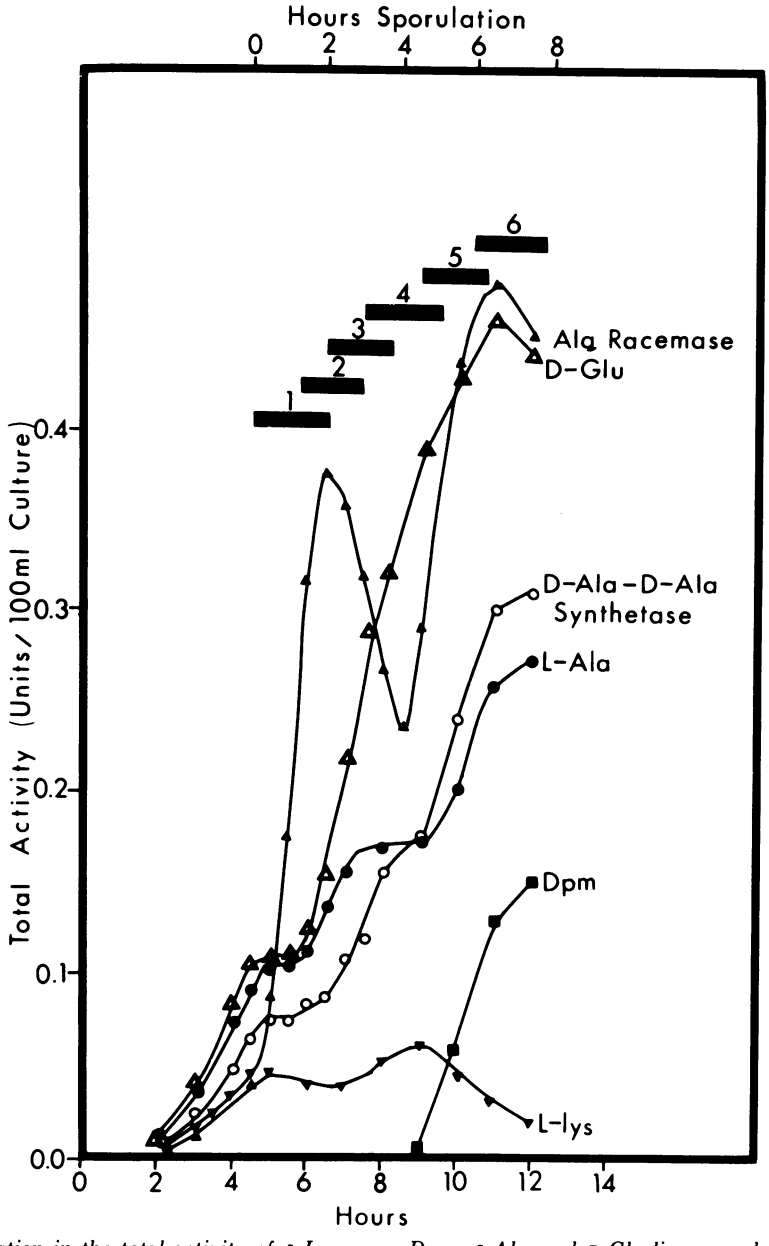

FIG. 2. *Variation in the total activity of* L-*Lys, meso-Dpm,* L-*Ala, and* D-*Glu ligases and of* D-*Ala-*D-*Ala synthetase and alanine racemase during sporulation. The kinetics of turbidity increase and the appearance of swollen and refractile cells were those shown in Fig. 1A. Symbols:* ▼, L-*Lys ligase;* ■, *meso-Dpm ligase;* ●, L-*Ala ligase;* Δ, D-*Glu ligase;* ○, D-*Ala-*D-*Ala synthetase (activity* × *0.5);* ▲, *alanine racemase (activity* × *0.05). The bars on this figure represent the periods during sporulation when the following morphological events occur (6): 1, postexponential symmetric vegetative cell division; 2, asymmetric spore septum formation; 3, initial terminal swelling (phase-contrast observations) and commencement of engulfment; 4, late stages of engulfment; 5, primordial cell wall synthesis; 6, cortex synthesis (electron micrographic observations) and the acquisition of semirefractility (phase-contrast observations) by the forespores.*

data are normalized to the appearance of refractility, coincidence is always found between the appearance of Dpm ligase activity (Fig. 2) and the second period of increase in D-Ala-D-Ala ligase (Fig. 1) and D-Ala-D-Ala synthetase activities (Fig. 2). The data in Fig. 1 and 2 were obtained in a single experiment.

The L-lysine ligase activity behaves similarly during early sporulation, increasing in activity from T_2 to T_4. However, a second period of accumulation of activity does not occur and, in fact, the total detectable L-lysine ligase activity decreases as the Dpm ligase activity increases (Fig. 2). The increase in D-glutamate ligase activity starting at T_2 is much more marked than that of L-Lys ligase and continues to T_6. However, there is no clear delineation of two periods. In contrast, the variation in activity of alanine racemase follows an almost reciprocal pattern. Activity increases eightfold between T_0 and T_2 and then decays by 35% between T_2 and T_4, before increasing to 130% of its T_2 value between T_4 and T_6. This second increase is only partially coincident with the appearance of Dpm ligase activity, which occurs about 1 h later.

Effect of antibiotic inhibitors of ribonucleic acid and protein synthesis on the accumulation of D-Ala-D-Ala synthetase and D-Ala-D-Ala ligase activities. In two separate experiments, cultures of sporulating *B. sphaericus* containing, respectively, 40% terminally swollen and almost completely swollen cells, were treated with either Cm or Sln (100 μg/ml). The specific activities of D-Ala-D-Ala ligase in these antibiotic-containing cultures were compared with those in untreated controls (Fig. 3). Since the total soluble protein released from these cultures by sonic oscillation was almost constant for the duration of both experiments, except for a 10% increase between $T_{2.5}$ and T_3, the curves for specific activity also reflect total activity. In cultures treated with either Cm or Sln, no further increase in swelling occurred, nor did any semirefractile spores appear upon prolonged incubation, although severe clumping of cells in the presence of Sln made quantitation difficult. Cm is an inhibitor of bacterial protein synthesis that interacts reversibly with the larger ribosomal subunit, inhibiting peptide chain elongation (16), and Sln is an inhibitor of bacterial ribonucleic acid synthesis that interacts reversibly with the β subunit of ribonucleic acid polymerase, inhibiting ribonucleic acid chain elongation (2, 20, 21). This concentration of Cm was found to rapidly inhibit (96%) the incorporation of labeled leucine into trichloroacetic acid-precipitable protein, and this concentration of Sln was found to inhibit (87%) the incorporation of labeled uridine into acid-precipitable material in vegetative cells or in cells at 4 h of sporulation (P. Linnett, unpublished data). The 2-h period commencing at $T_{2.5}$, at about 40% swelling, corresponds to the middle of the first phase of accumulation of total ligase activity, whereas the period from $T_{4.5}$ to $T_{6.5}$ corresponds to the second period of increase in activity (Fig. 1 and 2). The increase in activity of the D-Ala-D-Ala ligase seen in the controls in both of these periods was completely prevented by the presence of Cm (Fig. 3). Sln also prevented the accumulation of activity during these periods, although the effects of Sln were more delayed and less complete than those of Cm. The delay is too marked to reflect the half-life of bacterial messengers within the normal 1- to 3-min range and more likely reflects a longer delay in the accumulation of intracellular Sln.

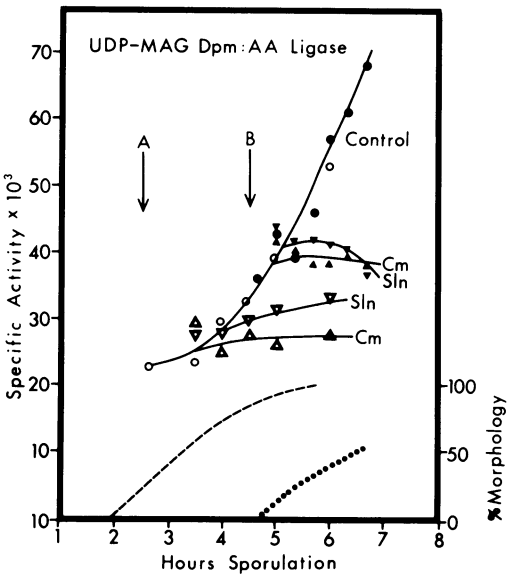

FIG. 3. *Effects of Cm and Sln on the increase in activity of D-Ala-D-Ala ligase during sporulation. The data for two separate experiments are presented: A (open symbols), antibiotics added at $T_{2.5}$ (arrow), and B (solid symbols), antibiotics added at $T_{4.5}$ (arrow). Symbols: ○, ●, ligase activity in control without drug; △, ▲, ligase activity in cultures containing 100 μg of Cm per ml; ▽, ▼, ligase activity in cultures containing 100 μg of Sln per ml. Morphology in the control culture is shown: – – –, percent swelling; · · ·, percent refractility.*

The effects of these antibiotics on the accumulation of D-alanyl-D-alanine synthetase activity in the same two periods of sporulation is shown in Fig. 4. Again, the increase in activity

seen in the controls during both periods is prevented by the presence of these concentrations of both Cm and Sln, and the effect of Sln seems to be somewhat delayed and somewhat less complete. Figures 3 and 4 also demonstrate that, in the presence of these concentrations of Cm and Sln, the activities of the D-Ala-D-Ala ligase and the D-Ala-D-Ala synthetase are relatively stable, having a half-life of at least 5 h. This is also true for the *meso*-Dpm ligase activity, as previously demonstrated (24), and for the L-Lys, L-Ala, and D-Glu ligase activities (unpublished data).

Properties of sporulation-defective mutants. Ten separately isolated mutants, all of which failed to form pigmented colonies on BS agar at 33 C, were tested for their ability to sporulate in BS broth. Three, designated Spo3, Spo4, and Spo7 following the recommendations of Young and Wilson (32), were almost completely asporogenous in broth and were chosen for further studies. None produced any swollen sporangia or refractile spores on plates of BS agar. Spo3 failed to give any normal sporangia during prolonged (30 h) incubation in BS broth at 33 C. Cell lysis was minimal, many cells became misshapen, and some terminal, spherical minicells were produced. Spo4 and Spo7 behaved similarly in BS broth, except that they also gave 1 or 2% refractile spores and should, therefore, be classified as oligosporogenous mutants. In contrast, the parent contained 95% sporangia with refractile forespores by 10 h of incubation and had 95% free, mature spores by 30 h. Samples of cultures of the parent and of the three mutants were assayed for dipicolinate 6, 8, and 32 h after the termination of exponential growth. The sporulating parent contained 30, 163, and 230 μm/liter of culture, respectively, at these three times. The culture of Spo3 contained no dipicolinate at any time, whereas the cultures of Spo4 and Spo7 contained no dipicolinate at the two earlier periods and 41 and 21 μm/liter of culture, respectively, at 32 h. This corresponds to 18 and 9% of the dipicolinate content of the parent. After 32 h of incubation, the activities of *meso*-Dpm ligase in Spo mutants 3, 4, and 7 were 0, 18, and 8%, respectively, of that in the parent. Thus, with respect to two biochemical markers of late-sporulation events, *meso*-Dpm ligase and dipicolinate synthesis, Spo3, Spo4, and Spo7 achieve 0, 18, and 8% of the parental level of expression. Much fewer refractile spores are seen in Spo4 and Spo7, probably a reflection of the delay in expression of these sporulation events in these mutants.

All three Spo mutants showed the marked

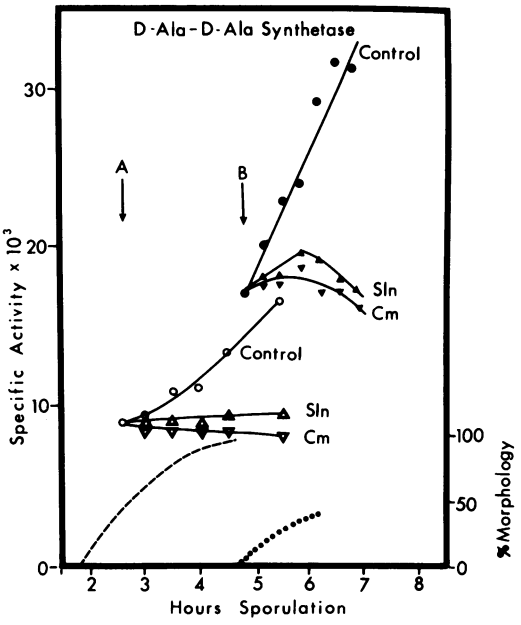

FIG. 4. *Effects of Cm and Sln on the increase in activity of D-Ala-D-Ala synthetase during sporulation. The data for the same two experiments depicted in Fig. 3 are given. Symbols:* ○, ●, *synthetase activity in controls without drug;* ▽, ▼, *synthetase activity in cultures containing 100 μg of Cm per ml;* △, ▲, *synthetase activity in cultures containing 100 μg of Sln per ml;* - - -, *percentage of terminally swollen cells in control culture;* · · · ·, *percentage of semirefractile forespores in control culture.*

rise in alanine racemase activity seen in the parent between T_0 and T_2, though not the subsequent fall. Staining with crystal violet (4) demonstrated that all of the mutants produced some asymmetric sporulation septa. Since they fail to show the terminal selling that is coincident with engulfment (6), they are blocked between stage 0 and stage 3 of sporulation, apparently at stage 2 (14).

Activity of D-Ala-D-Ala ligase in Spo mutants. Variation in total and specific activity of D-Ala-D-Ala ligase in Spo mutants 3, 4, and 7 during exponential and postexponential growth is shown in Fig. 5. All three mutants grew vegetatively at a rate similar to that of the parent, although they tended to cease exponential growth (see arrows in Fig. 5) at a lower turbidity. The vegetative specific activities of D-Ala-D-Ala ligase in the mutants and in the parent were similar, but ligase activity begins to decrease in the mutants somewhat earlier than in the parent, at about 1 h before the end of exponential growth. Decrease during the subsequent 2- to 3-h period results in a 50 to 75% loss in specific activity which, as can be seen from

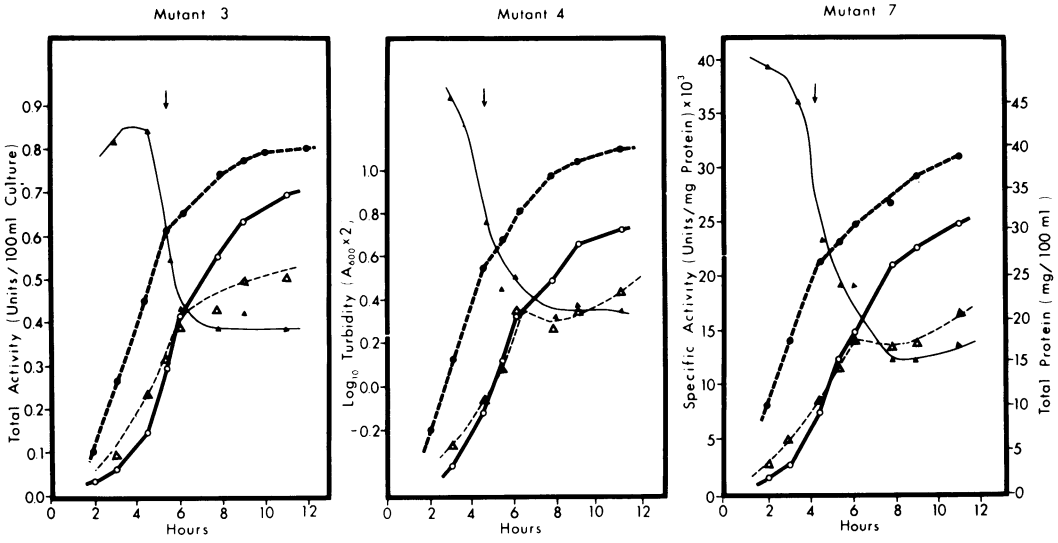

FIG. 5. *Variation in activity of UDP-MurNAc-L-Ala-D-Glu-meso-Dpm:D-Ala-D-Ala ligase activity during postexponential growth in Spo mutants 3, 4, and 7. In each case, the presumed end of exponential growth is indicated by the arrow. Symbols:* ●, *turbidity (log scale);* ○, *total protein in supernatants from ultrasonic disruption;* △, *ligase total activity;* ▲, *ligase specific activity.*

the curves for total activity and total protein content, is a consequence of continued protein synthesis in the presence of much slower accumulation of ligase activity. In mutants 4 and 7 the accumulation of ligase activity continues for 1 h after the end of exponential growth, and in mutant 3 some increase in activity occurs well after the end of exponential growth. Incubations were continued until 7 h after the end of exponential growth, at which time the parent ligase activity had returned to its vegetative level and parental cells had achieved 80% refractility.

DISCUSSION

It has previously been demonstrated (24) that the appearance of *meso*-Dpm ligase activity during sporulation in *B. sphaericus* is dependent upon continued transcription and translation and is presumably a consequence of control of transcription of the structural gene for the enzyme. It is also possible that the gene involved could have a product, such as a protease, which is very short-lived and which converts an inactive precursor of *meso*-Dpm ligase to active enzyme, but the simpler hypothesis is more attractive, even though functional protease modification of enzymes during sporulation has been demonstrated, for example in the case of aldolase (19).

With the exception of the L-Lys ligase, all of the other activities involved in the synthesis of the vegetative cell wall peptidoglycan precursor are also required for synthesis of the precursor of cortical peptidoglycan. It was previously noted (9) that the specific activity of these enzymes fell by about 50% during the first 2 h of sporulation and increased again in concert with the appearance of the Dpm ligase, the only exception being alanine racemase. We have now shown that the accumulation of the total activity of all of these enzymes (again with the exception of alanine racemase) ceases quite abruptly at T_0 and that the fall in specific activity between T_0 and T_2 is a consequence of dilution by newly synthesized protein. Examination of the kinetics of variation in cell volume and cell number in *B. sphaericus* during this period (G. Khachatourians and D. J. Tipper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I19, p. 120) demonstrates that the average cell volume halves while cell number doubles during a postexponential cell division occurring at about $T_{0.5}$. Thin sections of sporulation septa, which are formed at T_2, indicate that they, like vegetative septa, contain peptidoglycan (6). The synthesis of these two cross walls is sensitive to vancomycin (unpublished data) but occurs without increase in the total activity of the ligase enzymes.

The increase in activity of the L-Ala and D-Ala-D-Ala ligases and of D-Ala-D-Ala synthetase during sporulation occurs in two steps. The first, commencing at the time of spore septum formation, occurs before complete forespore

engulfment and well before the appearance of the Dpm ligase activity and includes all of those enzymes required for synthesis of the precursor of vegetative cell wall peptidoglycan, including L-Lys ligase. The second occurs in parallel with the appearance of Dpm ligase activity and includes all of the other enzymes needed for cortical peptidoglycan precursor synthesis, but not L-Lys ligase. These two steps have been consistently seen in repeated enzyme preparations. The data for D-Glu ligase activity are less repeatable, although increase in total activity does always occur between T_2 and T_6 (Fig. 2).

The susceptibility of both steps in the accumulation of D-Ala-D-Ala ligase and D-Ala-D-Ala synthetase activities to Cm and Sln demonstrates that, like the appearance of Dpm ligase activity, these events are the consequence of control at the level of transcription. The activity of both of these enzymes, like that of Dpm ligase, is relatively stable in cells in the presence of either Cm or Sln, and all activities involved in peptidoglycan precursor synthesis are relatively stable in crude, cell-free extracts. It is, therefore, probable that these enzyme activities are not subject to rapid turnover during sporulation and that variations in total activity accurately reflect variations in the rate of synthesis, i.e., in the rate of transcription of the appropriate structural genes.

Until about T_5, ultrasonic treatment causes disruption of both the mother cell and the forespore compartments of the sporulating cell. Thus, it cannot be determined by this procedure whether the first increase in ligase activity is a consequence of expression of the mother cell or of the forespore genome. If it occurs within the mother cell compartment, it could be functional in peptidoglycan synthesis involved in expansion of the cell terminus during engulfment, since during this period the average cell volume doubles (Khachatourians and Tipper, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I19, p. 120). However, it has not been demonstrated that peptidoglycan synthesis is involved in this process. It seems more probable that accumulation of the activities of the enzymes occurs in the putative forespore, so as to ready it for synthesis of primordial cell wall later in sporulation and for vegetative cell wall during outgrowth of the spores. In a forthcoming paper (D. J. Tipper and P. E. Linnett, submitted for publication), it is demonstrated that late forespores and mature spores have a relatively high specific activity of all of the enzymes required for synthesis of the vegetative peptidoglycan precursor, including L-Lys ligase, consistent with this hypothesis.

The second period of accumulation of activity of peptidoglycan precursor synthesizing enzymes includes only those required for synthesis of the precursor of cortex and occurs shortly before the appearance of refractility and of cortex itself. The specificity and timing of this enzyme synthesis indicates a functional relationship to cortex production, which is probably the responsibility of the mother cell cytoplasm and the outer forespore membrane (23; Tipper and Linnett, submitted for publication). This contention is supported by the appearance of the forespore cytoplasm, which at this time has already adopted a condensed appearance similar to that seen in mature spores (6), indicating that it may no longer be functional in gross macromolecular synthesis. In a forthcoming paper (Tipper and Linnett, submitted for publication), it is demonstrated that the Dpm ligase is located exclusively in the mother cell cytoplasm, as predicted.

Our data are reminiscent of the two periods of peak incorporation of labeled Dpm noted by Vinter in studies of sporulating *B. cereus* (25). His data indicated that the first period of incorporation resulted in formation of primordial cell wall, whereas the second period of incorporation resulted in synthesis of cortex. The cortexless mutant of *B. cereus* var. *aleste* apparently synthesizes primordial cell wall at a time coincident with this first peak of Dpm incorporation (15). Pitel and Gilvarg (17, 18), studying the incorporation of Dpm during sporulation in a Dpm^- Lys^- double auxotroph of *B. megaterium*, found it to commence after forespore engulfment, with no incorporation between T_0 and T_3 and continuous incorporation between T_3 and T_8. Electron micrographs of *B. sphaericus* (6) show that primordial cell wall synthesis precedes cortex synthesis, although by a relatively short period of about 0.5 h, and 1.5 to 2 h after the accumulation of L-Lys ligase activity (Fig. 2), so that the relationship between these events is less obvious than that between Dpm ligase synthesis and cortex synthesis.

Sporulation mutants are usually classified according to the morphological stage at which development is blocked, and in *B. subtilis* this has been correlated with the sequence of biochemical events that parallels the morphological events (26). In *B. sphaericus* it is now demonstrated that, in three mutants that are blocked morphologically between stages 1 and 2, the early program with respect to synthesis of peptidoglycan precursor synthesizing activities is followed reasonably faithfully. With the exception of alanine racemase, whose activity

accumulates at an accelerated rate, synthesis of these enzymes is turned off. However, the subsequent derepression of synthesis of these activities (other than alanine racemase) does not occur, consistent with the occurrence of these events in the parent only after stage 2. Thus, in *B. sphaericus*, as in *B. subtilis*, the expression of biochemical events related to sporulation is coupled to the normal program of morphogenesis.

The genes for L-Ala, *meso*-Dpm, and D-Ala–D-Ala ligases are clustered in *Escherichia coli* and may form part of an operon (11, 12, 13). The coordinate derepression of these enzymes together with D-Ala–D-Ala synthetases during midsporulation in *B. sphaericus* would also be consistent with operon-like organization of their structural genes, with expression determined by a single control element.

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