

# Ratio of Teichoic Acid and Peptidoglycan in Cell Walls of *Bacillus subtilis* Following Spore Germination and During Vegetative Growth

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Cell walls were isolated from cells of *Bacillus subtilis* strain Marburg during synchronous outgrowth of spores, during the two synchronous cell divisions which followed, and at various times during exponential and early stationary growth. The amounts of teichoic acid and peptidoglycan components were determined in each cell wall preparation. The peptidoglycan is composed of hexosamine, alanine, diaminopimelic acid, and glutamic acid. The ratio of these was relatively constant in the cell walls at each stage of growth. The teichoic acid is composed of glycerol, phosphate, glucose, and ester-linked alanine. With the exception of glucose and ester-linked alanine, the ratios of these components were relatively constant throughout the growth cycle. There was a slight increase in the glucose content of the teichoic acid as the cells aged. There was no correlation between the amount of ester-linked alanine and the stage of growth. The ratio of teichoic acid (based upon phosphate content) to peptidoglycan (based upon diaminopimelic acid content) remained at nearly a constant level throughout the growth cycle. The conclusion is presented that these two cell wall polymers are coordinately synthesized during spore outgrowth and throughout the vegetative growth cycle.

The most abundant polymers comprising the cell walls of gram-positive bacteria are peptidoglycan, teichoic acid, and polysaccharide (18). The relative quantities of each vary greatly with different species and often among strains of the same species (2, 22). The mechanisms of biosynthesis of each of these polymers have been investigated in a number of laboratories. However, none of these studies has approached the question of coordination and integration of synthesis of the various polymers into the finished cell wall.

This investigation was begun with the goal of determining the extent of coordination of synthesis of cell wall teichoic acid and peptidoglycan by *Bacillus subtilis*. The rationale of choosing a sporeforming bacterium for the study was based upon the large amount of cell wall material synthesized in the absence of cell division during

outgrowth of the germinating spore into the vegetative cell. It proved possible to obtain synchronous spore germination, outgrowth, and several cycles of vegetative cell division. We were thus able to quantitate and compare the amount of peptidoglycan and teichoic acid in the cell walls of organisms just emerging from the dormant state, after one and two cell divisions, at various times during exponential growth, and finally as the cells entered the stationary phase of growth and began to sporulate.

## MATERIALS AND METHODS

**Organism and cultivation.** *B. subtilis* Marburg 168, obtained from R. S. Hanson, was grown in 0.8% Difco Nutrient Broth supplemented with (w/v): 0.076% CaCl<sub>2</sub>·H<sub>2</sub>O and 0.026% FeCl<sub>3</sub> (NBS medium). The medium was adjusted to pH 7.0 before autoclaving; the minerals were sterilized separately in concentrated solutions and added aseptically to the Nutrient Broth.

**Preparation of spore stock.** Cells were grown in carboys containing 10 liters of NBS medium. These were inoculated with 500 ml from shake-flask cultures which in turn had been inoculated from agar slants.

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The carboys were aerated vigorously with air sterilized by passage through a membrane filter (Millipore Corp., Bedford, Mass.). Foaming was minimized by addition of one drop of Antifoam A (Dow Corning Corp., Midland, Mich.) per liter. The carboys were placed in an incubator at 37 C; the temperature of the medium was lowered to approximately 30 C by the forced aeration. After 36 hr, the cells were almost entirely converted into spores; these were harvested with a Sharples Super-centrifuge. The spore paste was washed several times with water at 4 C. To remove the sporangia and intact cells, the paste was suspended in 120 ml of 0.02 M tris(hydroxymethyl)-aminomethane (Tris) buffer (pH 9.0) containing  $4 \times 10^{-4}$  M ethylenediaminetetraacetic acid, a crystal of deoxyribonuclease, 1.0 mg of ribonuclease, and 0.05 ml of myxobacter AL-1 enzyme (8). Complete lysis of sporangia was evident after 90 min at 37 C. The mixture was centrifuged at  $10,000 \times g$  for 10 min at 4 C. The pelleted spores were washed 5 times with water, 10 times with 1.0 M NaCl, and finally with 10 more water washes. The washed spores were lyophilized and stored at  $-16$  C.

*Germination and outgrowth.* Germination of spore suspensions was followed by measuring the change in optical density at  $625 m\mu$  with a Bausch & Lomb Spectronic-20 colorimeter. A Zeiss phase-contrast microscope was employed to observe germination, emergence, and cell division. Water suspensions of dormant spores containing  $2.0 \times 10^8$  to  $2.0 \times 10^9$  spores per ml were heat-shocked at 65 C for 2.5 hr while being shaken slowly in a water bath. After cooling to 37 C, the spore suspensions were adjusted to a volume of 125 ml with a solution of Tris buffer (pH 7.5) and L-alanine, giving final concentrations of 0.1 M and 0.01 M, respectively. Spores were allowed to germinate for 20 min at 37 C during which time they were shaken. They were then transferred to 500 ml of NBS medium and incubated at 30 C. At appropriate stages of outgrowth and cell division, the cultures were rapidly chilled by addition of chipped ice to the flasks, and sodium azide (0.04 M) was added to inhibit growth. The cells were then harvested by centrifugation at 4 C, washed with cold 0.03 M potassium phosphate buffer, pH 7.0, and stored frozen until used for cell wall preparations.

*Preparation of cell walls.* Cell pastes obtained from the germinated spores and from cells harvested during exponential growth, during the presporulation phase, and during the postsporulation phase were suspended in 0.01 M phosphate buffer, pH 7.0. Glass beads ( $5 \mu$  in diameter) were added to the cell suspensions to give a volume ratio of 1:3. This mixture was subjected to sonic oscillation at maximal power output for 4 min by use of a model S125 Sonifier (Branson Instruments, Inc., Danbury, Conn.). The temperature was maintained below 10 C by submerging the cells in a salt water-ice bath during sonic treatment. Afterward, the beads were allowed to settle, and the supernatant fluids were centrifuged at  $1,000 \times g$  for 10 min. The supernatant fluids were again centrifuged at  $14,500 \times g$  for 10 min which sedimented the cell walls as a white, fluffy layer. This layer was separated from the heavier residue of intact cells and debris.

After heating for 30 min in a boiling-water bath, the walls were washed several times in water. They were then resuspended in 100 ml of 0.01 M phosphate buffer (pH 7.0) and incubated for 2 hr at 37 C with 50 mg of trypsin, 5.0 mg of deoxyribonuclease, and 50 mg of ribonuclease. In early distilled-water washes, the cell walls overlaid a small dark-brown pellet of nondescript debris. The cell wall pellets were carefully separated with a curved spatula. The dark brown material was not present after the washing procedure had been repeated 20 times.

*Analytical procedures.* Free amino acids in acid hydrolysates of cell walls were assayed by thin-layer chromatography of their dinitrophenyl derivatives (9) or by use of an amino acid analyzer. In the latter case, at least 50 nanomoles of sample was hydrolyzed in sealed tubes with 100  $\mu$ liters of 4 N HCl at 105 C for 8 hr. The samples were diluted to 2.3 ml with 0.2 N citrate buffer (pH 2.2), and a 1.0-ml sample was applied to each column of a Beckman 120 C amino acid analyzer.

Paper chromatography (Whatman no. 1 paper, previously washed with 2 N acetic acid and water) was used to detect the products of teichoic acid hydrolysis. Two solvent systems were used (2). These were composed of propanol-ammonia-water (6:3:1) and *n*-butyl alcohol-ethyl alcohol-water-ammonia (40:10:49:1) (organic phase). Amino acids were detected with ninhydrin spray (7), phosphorylated compounds with molybdate spray (10), polyols with the periodate-Schiff reagent (3), and reducing sugars with aniline-phthalic acid spray (14).

Total and inorganic phosphate was measured by the method of Lowry et al. (12). Glucose was assayed with the Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J.). Glycerol was determined by use of glycerol dehydrogenase coupled to nicotinamide adenine dinucleotide reduction (4). The phenol-sulfuric acid method of Hodge and Hofreiter (11) was employed to measure total carbohydrate, and total hexosamines were measured by the modified Morgan-Elson procedure of Reissig et al. (16).

## RESULTS

*Germination and division synchrony.* It was desirable to have a synchronously growing population of cells so that the cell wall composition could be examined at early and defined stages during the morphogenetic and growth cycles of the cells. Synchronous outgrowth of spores was achieved by inducing simultaneous germination. Poor germination was obtained with freshly harvested spores, as has been previously reported by others (15). More rapid and quantitative germination occurred after storage of the spores for 8 months at  $-16$  C. The optimal conditions for germination of the aged spores were determined. Maximal germination resulted when the spores were heat-shocked for 2.5 hr at 65 C and then incubated in a germination medium containing 0.01 M L-alanine in 0.1 M Tris buffer, pH 7.5. The progress of germination was moni-

tored by observing the change in turbidity of the spore suspension at 625  $m\mu$ . Turbidity decreased linearly for 8 min to a level approximately 50% of that at the beginning; a maximal decrease of 60% was reached at 20 min. Microscopic examination revealed that nearly 95% of the spores had germinated within this time.

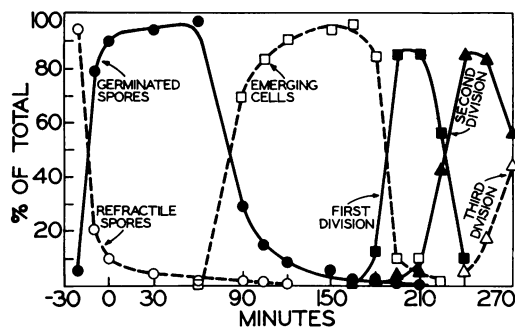


FIG. 1. Germination of *Bacillus subtilis* spores followed by synchronous division of vegetative cells. Heat-shocked and germinated spores were suspended in NBS medium and incubated with shaking at 30 C. Samples were removed at various times for observation with a phase-contrast microscope. Loss of refractility was used as an index of germination. Outgrowth and cell division were estimated by determining the numbers of single rod-shaped cells (emerging cells), chains of two cells (first division), and chains of four cells (second division). Longer chains of cells fragmented; thus, it was impossible to determine subsequent division times. Each determination represents an average of at least 200 cells counted.

Synchronous outgrowth was achieved by shaking the germinating spores in NBS medium at 30 C. At various time intervals, the cells were examined microscopically. It proved relatively easy to identify and count the cells representing the different stages of morphology and growth. The results of a typical experiment are presented in Fig. 1. The spores grew out essentially quantitatively into vegetative cells by 120 min. After emerging from the spore, the cells divided with good synchrony for two cell divisions. It was possible to determine visually which cells had divided since the daughters remained connected in chains of two cells (first division) or four cells (second). By the third division, it became impossible to determine the stage of individual cells because of fragmentation of chains.

*Cell wall analyses.* To obtain cell walls from cells at carefully prescribed early stages of growth, we took advantage of the synchrony of spore germination, outgrowth, and the first two cell divisions. Walls were also prepared from cells harvested after the time when synchrony was lost. A growth curve, illustrating the times and stages of cell morphology at which cell wall preparations were made, is presented in Fig. 2. At the pre-sporulation stage, indicated by the letter H in the figure, dense forespores were readily visible with phase microscopy. Also present were some refractile endospores and a few free spores. Post-sporulation cells contained mature, refractile spores still encased in the sporangium.

Teichoic acids were removed from a prepara-

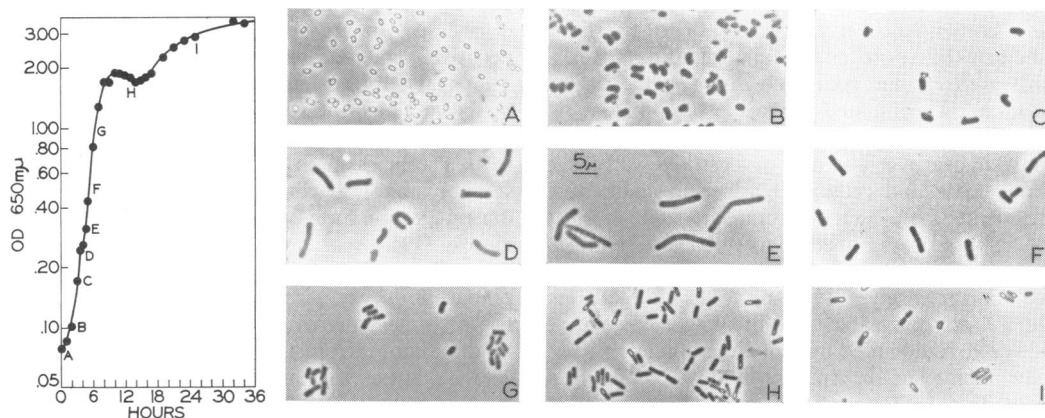


FIG. 2. Growth curve of *Bacillus subtilis* illustrating the morphology of the spore inoculum and of the vegetative cells at times when the cells were harvested for cell wall isolation. A suspension of heat-shocked and germinated spores was inoculated into NBS medium to give an initial count of  $2.5 \times 10^7$  spores per ml. The letters adjacent to points on the growth curve (left) correspond to the phase-contrast photomicrographs (right) and, except for point A (the spore inoculum), represent the times at which cells were harvested. These growth stages are referred to in the text as: (B) initially emerging cells; (C) middle emerging cells; (D) first division; (E) second division; (F) 1 to 2 hr after second division; (G) late exponential phase; (H) presporulation cells, forespores present; and (I) postsporulation cells, refractile endospores present.

tion of cell walls of exponential-phase cells by extraction with 10% trichloroacetic acid for 80 min at 60 C. After centrifugation, the teichoic acids present in the supernatant fraction were decanted and precipitated by the addition of 5 volumes of absolute ethyl alcohol. The precipitate was washed with ether and then acetone, and was dried in vacuo. The teichoic acid was hydrolyzed in 2 N HCl for 3 hr at 100 C and analyzed by paper chromatography. Glycerol, inorganic phosphate, glucose, alanine, and the mono- and diphosphate esters of glycerol were qualitatively identified by reference to known standards. Glucosamine was not found in the chromatogram of the teichoic acid hydrolysate. It has been shown that trichloroacetic acid extraction removes hexosamines from *B. subtilis* teichoic acid (24). With the exception of alanine, the teichoic acid contained no component found in peptidoglycan. It was thus possible to analyze quantitatively the components of the two polymers without first separating them. Alanine in the teichoic acid presented no problem since it is ester-linked and was easily removed with dilute alkali (2).

Analyses of cell wall preparations from early growth stages were complicated by the presence of spore coats. It proved impractical to completely free the cell walls of spore coats by repeated differential or sucrose gradient centrifugation. The spore coats were efficiently removed by completely dissolving the cell walls with lysozyme and then sedimenting the residual spore coats by centrifugation.

The cortex of spores is known to contain peptidoglycan which is solubilized by lysozyme (21). As a consequence, it was necessary to determine whether the spore coats contaminating the cell wall preparations contained any residual cortex material. Germinated spores were shaken in Nutrient Broth until it was evident that each spore had grown out into a vegetative cell. Free spore coats and cells with adhering coats were sedimented by centrifugation at  $5,000 \times g$  at 4 C. Spore coats adhering to cells were removed by low-amplitude sonic oscillation for 30 sec at 4 C. This released most of the coats while causing almost no detectable cell breakage. Vegetative cells were sedimented by centrifugation at  $1,000 \times g$  for 5 min. The supernatant fluid was centrifuged at  $3,000$  to  $5,000 \times g$  for 10 min. This resulted in deposition of a pellet containing mostly spore coats. By repeated slow-speed centrifugation in distilled water, a preparation of spore coats was obtained which was free from intact cells or cell walls, as judged by examination with a phase-contrast microscope. The spore coats were acid-hydrolyzed, and the amino acid-amino sugar content was determined with an amino acid

TABLE 1. *Chemical composition of Bacillus subtilis spore coats*

Component	Amt/mg of sample
	nanomoles
Diaminopimelic acid.....	0
Alanine.....	261
Glutamic acid.....	329
Glucosamine.....	trace
Muramic acid.....	0
Lysine.....	216
Histidine.....	120
Arginine.....	123
Aspartic acid.....	385
Threonine.....	118
Serine.....	312
Proline.....	162
Glycine.....	634
Half-cystine.....	142
Valine.....	trace
Methionine.....	trace
Isoleucine.....	68
Leucine.....	131
Tyrosine.....	293
Phenylalanine.....	159
Glycerol.....	381
Phosphate.....	305

analyzer. The results (Table 1) show that the spore coats contained no muramic acid or diaminopimelic acid and less than 0.2 nanomoles of glucosamine per mg of material. It is apparent from the results that no residual cortex peptidoglycan was present. The spore coat fraction also contained approximately 3% each of glycerol and organic phosphate. An electron micrograph of a negatively stained preparation is shown in Fig. 3. The coats appear empty and devoid of cortex material. Thus, the solubilization of cell walls by lysozyme to rid them of spore coats does not lead to inaccuracies in the subsequent analyses. Presumably, the spore cortex is completely solubilized during germination, as has been reported previously (13).

Samples of each of the different cell wall preparations were treated with 2 N  $\text{NH}_4\text{OH}$  at room temperature overnight to remove the ester-linked alanine from the teichoic acid (19). After centrifugation, the amount of liberated alanine in the supernatant fluids was determined by thin-layer chromatography of the dinitrophenyl derivative. The ratio of alkali-extractable alanine to phosphate in the cell walls fluctuated between 0.04 and 0.11 (Table 3). There was no correlation between the relative amount of ester-linked alanine and the cell walls prepared at the various growth stages.

The cell wall preparations from which ester-



FIG. 3. Electron micrograph of *Bacillus subtilis* spore coats. Purified spore coats were placed upon carbon-parlodion coated grids, stained with 2% phosphotungstic acid (pH 6), rinsed with water, and observed in a Zeiss-EM-9A electron microscope.

linked alanine had been removed were washed with water, suspended in 1.0 mM Tris buffer (pH 8.5), and then digested with 10  $\mu$ g of lysozyme per ml for 12 hr at 37 C. The digests were centrifuged at 15,000  $\times$  g for 15 min to sediment any residual spore coats. Analyses of the residual spore coats showed that the lysozyme digestion did not solubilize any of the glycerol or phosphate found to be present in these structures. The supernatant fluids containing only solubilized cell walls were acid-hydrolyzed and their composition was determined.

The results for the components of peptidoglycan and teichoic acid are presented in Tables 2 and 3, respectively. The molar ratios of the peptidoglycan components remained essentially constant in the cell walls isolated from cells during outgrowth from spores, during exponential cell division, and just before and after sporulation. The components of teichoic acid also remained essentially constant throughout the growth cycle. The relative amount of glucose in the teichoic acid increased slightly as the cells aged. Possible significance of this increase in glucose content is obscure because of the small overall change.

From the analytical results presented in Tables 2 and 3, the ratio of teichoic acid to peptidoglycan

TABLE 2. Peptidoglycan components in lysozyme digests of *Bacillus subtilis* cell walls isolated from different growth stages<sup>a</sup>

Growth stage	Total hexo-samine	Al-anine	Gluta-mic acid	DAP
Initially emerging cell.....	2.17	1.62	1.03	1.00
Middle emerging cell.....	2.46	1.78	1.06	1.00
First division.....	2.41	1.63	1.11	1.00
Second division.....	2.48	1.60	0.99	1.00
Early exponential growth..	2.44	1.55	1.02	1.00
Late exponential growth..	2.19	1.48	0.97	1.00
Presporulating cell.....	2.47	1.59	1.06	1.00
Postsporulating cell.....	2.41	1.60	0.93	1.00

<sup>a</sup> Numbers refer to molar ratios to diaminopimelic acid (DAP) and represent the average of at least three determinations.

TABLE 3. Teichoic acid composition of *Bacillus subtilis* cell walls isolated from different growth stages<sup>a</sup>

Growth stage	Phos-phate	Glyc-erol	Gluc-ose	Ester-linked alanine
Initially emerging cell.....	1.00	1.06	0.59	0.08
Middle emerging cell.....	1.00	1.13	0.57	0.11
First division.....	1.00	1.04	0.70	0.07
Second division.....	1.00	1.14	0.73	0.10
Exponential growth.....	1.00	1.03	0.76	0.11
Late exponential growth..	1.00	0.94	0.73	0.04
Presporulating cell.....	1.00	0.89	0.75	0.08
Postsporulating cell.....	1.00	1.10	0.80	0.03

<sup>a</sup> Numbers represent averages of at least three determinations and are expressed as molar ratios to phosphate. Phosphate, glycerol, and glucose analyses were performed with lysozyme digests of cell walls. Ester-linked alanine determinations were performed upon alkaline extracts of whole cell walls.

in the cell walls at each stage of growth can be calculated. These results, based upon the ratio of phosphate (teichoic acid) to diaminopimelic acid (peptidoglycan), are presented in Table 4. The molar ratios of phosphate to diaminopimelic acid remained at a fairly constant level in the cell walls from all growth stages with the exception of a significantly low ratio in walls of postsporulating cells. These results are interpreted as evidence that the two major polymeric constituents of *B. subtilis* cell walls, peptidoglycan and teichoic acid, are coordinately synthesized from the time of outgrowth of the spore to the time the cell once again sporulates.

TABLE 4. Ratio of teichoic acid to peptidoglycan in cell walls of *Bacillus subtilis* isolated from different growth stages

Growth stage	Amt ( $\mu$ moles) /mg of un- digested cell walls <sup>a</sup>		Phos- phate/ DAP
	Phos- phate	DAP	
Initially emerging cell . . . . .	0.89	0.26	3.46
Middle emerging cell . . . . .	0.57	0.16	3.67
First division . . . . .	0.76	0.20	3.89
Second division . . . . .	0.95	0.24	3.91
Early exponential growth . . . . .	1.10	0.28	3.94
Late exponential growth . . . . .	1.36	0.35	3.90
Presporulating cell . . . . .	1.48	0.38	3.94
Postsporulating cell . . . . .	0.84	0.29	2.84

<sup>a</sup> These values are based upon the weight of the cell wall samples before lysozyme digestion. The samples taken through the early exponential growth phase contained considerable amounts of residual spore coats. The variable contribution of the spore coats to the sample weights caused distorted fluctuations in the amount of phosphate and diaminopimelic acid (DAP) per milligram of cell wall. The actual determinations of phosphate and DAP were performed on lysozyme-solubilized walls from which the spore coats had been removed. Since the spore coats contained no lysozyme-soluble phosphate or DAP, the ratio of phosphate to DAP in the cell walls was not affected.

#### DISCUSSION

Conversion of the dormant bacterial spore into actively growing vegetative cells is accompanied by extensive changes in morphology. One would expect that alterations in the structure of the cell wall would accompany these changes in morphology. The results of this investigation show that the chemical composition of the two major cell wall polymers, peptidoglycan and teichoic acid, does not vary appreciably throughout the growth cycle from spore to vegetative cell to sporulating cell. Calculations based upon the molar concentrations of cell wall components indicate that approximately 62% of the wall is composed of teichoic acid and 37% is peptidoglycan. This ratio remains essentially constant throughout the growth and morphology cycle. This indicates that these wall polymers are coordinately synthesized during normal cell growth and division.

Young (23) reported that variations in the chemical composition of *B. subtilis* 168 I<sup>-</sup> C<sup>+</sup> cell walls accompanied growth for different lengths of time and in different media. The content of alanine increased during 10 hr of incubation.

Glutamate, diaminopimelic acid, and muramic acid were essentially constant over this time period. Galactosamine content increased twofold at the time of maximal competence, 5 hr, and then decreased. With the exception of galactosamine, which we did not analyze, our analyses of cell walls at different growth times and those of Young (23) do not differ significantly.

Rogers and Garrett (17) observed that the biosynthesis of both teichoic acid and peptidoglycan was inhibited in suspensions of two strains of *Staphylococcus aureus* by a deficiency of glycine, glutamic acid, or lysine, or by the presence of either cycloserine or 5-fluorouracil. These three amino acids are found in the organism's peptidoglycan but not in teichoic acid. Moreover, cycloserine and 5-fluorouracil are inhibitors of peptidoglycan synthesis. These results led the authors to postulate that the formation of teichoic acid may be dependent on the availability of binding sites in the peptidoglycan. Such a dependency would not be absolute since it was reported in the same paper that low levels of penicillin, a specific inhibitor of transpeptidation in peptidoglycan (20), inhibited peptidoglycan synthesis by 90 to 100% while only partially blocking teichoic acid synthesis.

Several lines of evidence indicate that synthesis of these two wall polymers is not always coordinate. Armstrong, Baddiley, and Buchanan (2) mentioned that growth of *B. subtilis* in a high concentration of glucose (2%) selectively suppressed teichoic acid synthesis. Wolin, Archibald, and Baddiley (22) reported variations in the amounts of teichoic acids (based on percentage of phosphate in the wall) in three mutants of *S. aureus* strain 8511. In two of these mutants, the ribitol teichoic acid of the wild type was replaced by a glycerol teichoic acid. Both peptidoglycan (1, 5) and teichoic acid (4) have been synthesized in vitro. There was no dependence for the synthesis of one polymer upon the presence of or simultaneous synthesis of the other.

Chin, Younger, and Glaser (6) studied the biosynthesis of teichoic acids during germination of *B. subtilis* W-23 and *B. licheniformis* spores. The *B. subtilis* strain which they used contains a polyribitolphosphate teichoic acid. They showed, by analyzing for the presence of ribitol, that the spores contained no teichoic acid and that the synthesis of this polymer began at the time of complete germination, approximately 20 to 30 min after heat-shocked spores were placed in a germination medium. The strain of *B. subtilis* studied in our investigation contains a polyglycerolphosphate teichoic acid. Because of large amounts of glycerol in spore components, we were unable to analyze for teichoic acid until the vegetative cells had emerged from the spore coats.

Thus, our first determinations were made 100 min after germination began. Our results cannot be extrapolated to the important period of cell wall synthesis between onset of germination and emergence from the spore coat. It would be interesting to determine whether the time at which peptidoglycan synthesis begins in strain W-23 coincides with that reported by Chin, Younger, and Glaser for teichoic acid synthesis and whether synthesis is coordinated thereafter.

Our results indicate that the synthesis of peptidoglycan and teichoic acid during outgrowth from germinated spores and during normal cell growth and division is a coordinated process. The mechanism by which nascent polymers of teichoic acid and peptidoglycan are knit into the fabric of the finished cell wall is not known. It seems evident from the in vitro synthesis and inhibitor studies of others that the formation of one polymer is not obligately dependent upon the other and that the mechanism of integration is easily disturbed.

#### ACKNOWLEDGMENT

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