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Insertion and Fate of the Cell Wall in *Bacillus subtilis*

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Cell wall assembly was studied in autolysin-deficient and -sufficient strains of *Bacillus subtilis*. Two independent probes, one for peptidoglycan and the other for surface-accessible teichoic acid, were employed to monitor cell surface changes during growth. Cell walls were specifically labeled with *N*-acetyl-D-[^{3}H]glucosamine, and after growth, autoradiographs were prepared for both cell types. The locations of silver grains revealed that label was progressively lost from numerous sites on the cell cylinders, whereas label was retained on the cell poles, even after several generations. In the autolysin-deficient and chain-forming strain, it was found that the distance between densely labeled poles approximately doubled after each generation of growth. In the autolysin-sufficient strain, it was found that the numbers of labeled cell poles remained nearly constant for several generations, supporting the premise that completed septa and poles are largely conserved during growth. Fluorescein-conjugated concanavalin A was also used to determine the distribution of α-D-glucosylated teichoic acid on the surfaces of growing cells. Strains with temperature-sensitive phosphoglucomutase were used because in these mutants, glycosylation of cell wall teichoic acids can be controlled by temperature shifts. When the bacteria were grown at 45°C, which stops the glucosylation of teichoic acid, the cells gradually lost their ability to bind concanavalin A on their cylindrical surfaces, but they retained concanavalin A-reactive sites on their poles. Discrete areas on the cylinder, defined by the binding of fluorescent concanavalin A, were absent when the synthesis of glucosylated teichoic acid was inhibited during growth for several generations at the nonpermissive temperature. When the mutant was shifted from a nonpermissive to a permissive temperature, all areas of the cylinder became able to bind the labeled concanavalin A after about one-half generation. Old cell poles were able to bind the lectin after nearly one generation at the permissive temperature, showing that new wall synthesis does occur in the cell poles, although it occurs slowly. These data, based on both qualitative and quantitative experiments, support a model for cell wall assembly in *B. subtilis*, in which cylinders elongate by inside-to-outside growth, with degradation of the stress-bearing old wall in wild-type organisms. Loss of wall material, by turnover, from many sites on the cylinder may be necessary for intercalation of new wall and normal length extension. Poles tend to retain their wall components during division and are turned over much more slowly.

It is now clear that some bacteria, such as *Streptococcus faecalis* (*faecium*), exhibit cell wall growth from discrete and well-defined zones. Each unit cell splits its wall band as it initiates cell division, forming two new bands of wall material which accompany and delineate cell enlargement. The bands are formed at the beginning of a growth cycle and can be used as morphological markers to monitor cell cycle-related events (14). Bacilli, particularly *Bacillus subtilis*, appear to have more complex modes of wall replication. There are conflicting reports with respect to how walls are assembled in bacilli (2, 11–13, 16, 30, 44). Some reports suggest a totally diffuse mode, in which many nascent wall insertion sites are involved. Still others, including the recent work of Schlaeppi and colleagues (52, 57), propose that wall synthesis in *B. subtilis* occurs from several sites in the cylinder. Wall synthesis occurs in a radial direction (new wall is deposited inside and old wall only is found outside) during growth (2, 21, 50, 51). Wall bands also appear to be present when examined carefully with several fixatives (1, 9). These wall bands, however, do not migrate into the cylindrical regions of the cell, and they tend to disappear as elongation occurs. Another important complication in assessing the patterns of wall growth in *B. subtilis* is the distinction of cell surface turnover from new growth. New wall is required to replace wall that has been turned over, as well as to provide surface components for new growth. A recent hypothesis suggests that this "turnover" is necessary for the process of surface extension in gram-positive rods (36–38). In assessing where bacilli insert new surface polymers laterally along the wall, it is necessary to determine whether there is a difference between sites of turnover and other sites of elongation, or whether there is only one kind of turnover as suggested by the inside-to-outside growth hypothesis. Furthermore, it is necessary to determine whether turnover occurs at distinct rates on completed poles and cylinders.

In a preliminary communication, we have proposed several experiments to provide a basis for establishing the mode of wall replication in *B. subtilis* (22). The basic experiment involves the following considerations: (i) concanavalin A (ConA) binds to α-D-glucosylated teichoic acids on the surfaces of bacilli (18, 43, 60), (ii) newly synthesized peptidoglycan is attached only to newly synthesized teichoic acid (43), and (iii) temperature-sensitive *B. subtilis gtaC* mutants (phosphoglucomutase deficient) insert glucose into their teichoic acids at permissive (42, 61) but not nonpermissive temperatures. Therefore, fluorescein-labeled ConA (Fl-ConA) can be used as a convenient probe to monitor the insertion and fate of cell wall material. Similar arguments

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have been made more recently by others (2, 3, 59), who have utilized phosphate limitation to replace teichoic acid with teichuronic acid in *B. subtilis* W23. In this report, we employ ConA and tritium-labeled *N*-acetylglucosamine as fully independent probes to study cell wall assembly and turnover in autolysin-deficient and -sufficient strains of *B. subtilis*. The results suggest that cell wall assembly occurs diffusely in both cell cylinders and completed septa (poles) but that turnover in the polar wall occurs much more slowly than in the cylindrical wall.

(Preliminary results of this work have been presented previously [H. L. T. Mobley and R. J. Doyle, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980; K201, p. 160, Conference on Bacilli, American Society for Microbiology, Ithaca, N.Y., August 1975].)

MATERIALS AND METHODS

Bacterial strains. *B. subtilis gtaC33* [*trpC2 pgm*(Ts)] (hereafter referred to as strain C33) and FJ6 [*metC3 thr*(Ts) Lyt-2] were kindly provided by D. C. Birdsell, University of Washington, Seattle, and Howard Rogers (see reference 28), respectively. The stocks were maintained on tryptose blood agar base (Difco Laboratories, Detroit, Mich.) plates.

Chemicals. All salts were of reagent grade. Fluorescein isothiocyanate was purchased from Calbiochem (San Diego, Calif.). Radioactive *N*-acetyl-D-[1-^{3}H]glucosamine (GlcNAc) (specific activity, 2.94 Ci/mmol) was a product of Amersham Corp., Chicago, Ill. Enzymes and organic compounds used in the phosphoglucomutase assay were obtained from Sigma Chemical Co., St. Louis, Mo. ConA and Fl-ConA were prepared as described previously (18, 20).

Growth conditions. Cells were grown in either Penassay broth (Difco) or Sargent salts (54) supplemented with ammonium sulfate (2 mg/ml); glucose (0.5% [wt/vol]); and L-tryptophan, L-threonine, or L-methionine (10 μg/ml) as required. Cultures were shaken at 200 rpm in a gyratory shaker at the indicated temperatures.

Transformation. DNA was isolated from strain C33 by phenol extraction and was used to transform cultures of strain FJ6 grown to competence by the method described by Young and Wilson (62). Transformants were selected on the basis of sensitivity to bacteriophage ϕ25 by cells grown at a permissive temperature and resistance to ϕ25 by cells grown at a nonpermissive temperature.

TABLE 1. Lysis of exponential cultures induced by azide and autolysis of native cell walls of *B. subtilis* strains

Bacterial strain	Rate of azide-induced lysis (R[liter/h])[a]	Autolytic activity of native cell walls (unit per mg of cell wall)[b]
168	0.88	1.72
C33	0.66	3.87
FJ6	0.04	0.27
BUL96	0.04	0.25

[a] Sodium azide (75 mM final concentration) was added to exponential-phase cultures growing in Sargent medium. Loss of turbidity was followed on a Klett-Summerson colorimeter. Growth rates at 37°C were 40 to 45 min per generation. Rate constants (R) were calculated as described previously from the slopes of semilogarithmic plots (32).

[b] Native cell walls were suspended in minimal salts (pH 7.2) to a density of exactly 1.5 mg (dry weight)/ml and incubated at 37°C. Apparent absorbance was followed at 450 nm. One unit of autolytic activity is arbitrarily defined as a reduction of 0.001 absorbance units per min.

TABLE 2. Cell wall hexose composition of *B. subtilis* strains grown under permissive (35°C) and nonpermissive (45°C) conditions

Bacterial strain	μmol of hexose per mg of cell wall grown at[a]:	
	35°C	45°C
168	1.21	1.09
C33	0.55	0.03
FJ6	1.58	1.75
BUL96	0.35	0.05

[a] Values shown were determined from the anthrone reaction (49) on cell wall suspensions. D-Glucose served as the standard. Cell walls were obtained from cells in balanced growth.

Cell walls. Cell walls were prepared by disrupting the bacilli in a French press (American Instrument Co., Inc., Silver Spring, Md.) at 18,000 lb/in^2. The walls were isolated by differential centrifugation and washed 12 times in cold deionized water (5, 6, 48). The walls contained active autolysin(s) as evidenced by their dissolution in buffers. Upon treatment with detergents and denaturants (48), the walls were free of autolysins, loosely bound proteins, and nucleic acids.

Cell wall turnover. Cell cultures (20 ml), growing exponentially in Penassay broth, were labeled for 0.08 to 0.16 generations with 0.2 ml of GlcNAc (40 μCi), harvested by rapid filtration (0.45-μm nitrocellulose filters; Schleicher & Schuell, Inc., Keene, N.H.), washed twice with 5 ml of unlabeled growth medium, and suspended in 20 ml of fresh prewarmed medium (31). Samples (1 ml) were taken at regular intervals as described by Jolliffe et al. (31). Growth rates were 24 ± 3 min per generation.

Fl-ConA binding by strains of *B. subtilis*. Cells were suspended in 40 mM Tris-hydrochloride (pH 7.4) until the turbidity approximately equalled 0.3 on a Coleman model 6/20 spectrophotometer (450 nm, 1 cm). This turbidity corresponded to ca. 15 μg (dry weight)/ml and 2 × 10^7 CFU/ml. A 1-ml volume of cell suspension was mixed with 0.5 ml of Fl-ConA (ca. 1 mg), incubated for 30 min at 4°C, and washed three times with Tris-hydrochloride. The pellet was suspended in 1.0 ml of buffer, and several drops were air dried on a clean microscope slide. The slides were mounted with a solution containing nine parts glycerol and one part 0.25 M sodium carbonate (pH 9.0) and observed under a Zeiss standard RA microscope equipped with an Osram HBO 200-W mercury lamp, BG12 exciter filter, and barrier filter 50. Plus-X-Pan film (Eastman Kodak) was used to record fluorescence. To enhance contrast, positive slides were copied with Kodak Technical-Pan film and developed with Kodak-110 developer.

Agglutination by ConA. Cells were suspended in 40 mM Tris-hydrochloride (pH 7.4) until the turbidity equalled 0.3 absorbance units (450 nm, 1 cm). Dilutions of ConA (100 μl) were added to the wells of microtiter plates along with equal volumes of cell suspensions. The plates were incubated for 2 h at 4°C. Agglutination was observed with a microtiter viewer (Cooke Laboratories). Protein was determined by the method of Lowry et al. (41), with crystalline ConA as the standard.

Autoradiography. Cell cultures, growing exponentially in 20 ml of Penassay broth at 35°C, were labeled (21, 47) for 2.0 generations with 0.4 ml of GlcNAc (80 μCi). Cells were

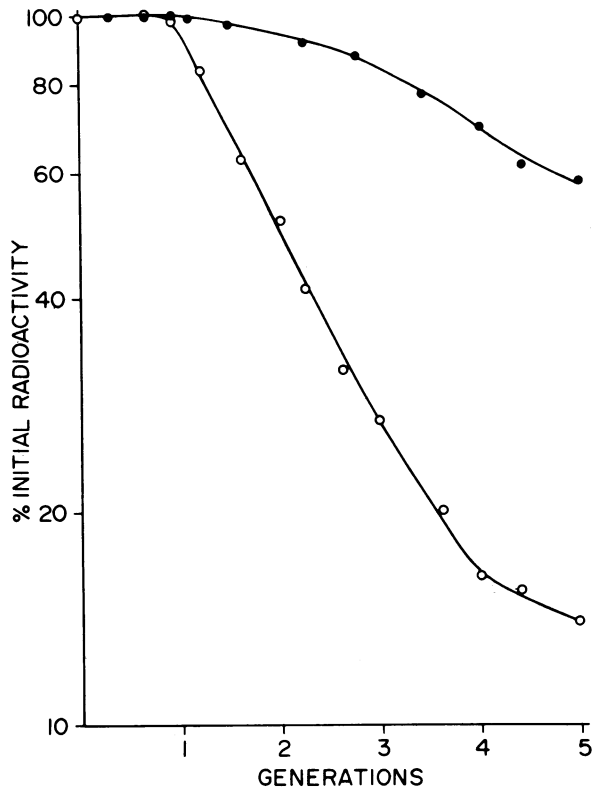

FIG. 1. Peptidoglycan turnover in *B. subtilis* strains. Exponential-phase cultures (20 ml) growing in Penassay medium were labeled for 0.10 to 0.16 generations with 40 μCi of GlcNAc, harvested by rapid filtration, washed, and suspended in fresh warm medium. At intervals, samples (1 ml) were filtered and washed with cold 5% trichloroacetic acid. Radioactivity was determined by liquid scintillation counting. Growth rate was 25 min per generation. Symbols: ●, strain BUL96; ○, strain C33.

harvested by rapid filtration (0.45-μm nitrocellulose filters; Schleicher & Schuell), washed with 10 ml of prewarmed medium, and suspended in 30 ml of fresh prewarmed medium. Exponential growth was maintained by periodic transfer to fresh medium. Expeditious handling of the suspensions and the use of prewarmed media, glassware, and pipettes prevented lag periods in growth upon transfer. At various intervals, 1-ml samples were removed, filtered, and suspended in 0.5 ml of medium. A few drops were spread on a microscope slide, air dried, and gently heat-fixed. The slide was dipped in nuclear emulsion NTB-2 (Eastman Kodak) as described by Kopriwa and Leblond (40). Slides were stored desiccated at 4°C for 7 days. The slides were developed with D-19 developer (Eastman Kodak) at 20°C.

RESULTS

Autolysin-deficient, phosphoglucomutase-deficient mutant of *B. subtilis*. Many bacteriophage particles capable of binding to *B. subtilis* cells require α-D-glucosylated teichoic acids for receptor site recognition (2, 5). Mutations leading to progeny deficient in the teichoic acid glucose are easily detected by phage resistance. Transformants of strain FJ6, with strain C33 DNA as the donor for the *gtaC* characteristics, were screened by incubating replicate plates at permissive and nonpermissive temperatures. Colonies were restreaked on plates to which a suspension of bacteriophage ϕ25 (2.5×10^5 phage per ml) were applied (5). After incubation at selective temperatures, several transformants demonstrated sensitivity to ϕ25 when grown at 35°C and resistance to ϕ25 when grown at 45°C. One isolate (designated BUL96) also retained the long-chain morphology of strain FJ6 and was further characterized. Strain BUL96 did not grow as bundled macrofibers as described by Fein (27) for strain FJ6 (see also references 45, 46). The transformant also did not retain the temperature-sensitive threonine marker characteristic of the parent FJ6 (J. Fein, personal communication). That strain BUL96 retained the autolysin-deficient allele of strain FJ6 was shown by several experiments. Table 1 shows rates of azide-induced lysis and rates of autolysis of native cell walls. We have observed in other studies that lysis induced by the azide anion is related to the endogenous autolysin level in *B. subtilis* (32). Strains FJ6 and BUL96 exhibit a similar slow rate of both azide-induced lysis and autolytic activity of isolated cell walls, whereas strain C33 shows significantly higher rates. Wild-type strain 168 gave lysis values similar to those reported previously (19, 32). In experiments related to the characterization of the mutants, we included data on strain 168 to provide a base for comparison.

That the cell wall teichoic acid was not being glucosylated at the higher temperature was confirmed directly by the data in Table 2 which show that although strain FJ6 glucosylated teichoic acid at both temperatures, cell walls from strains C33 and BUL96 were pauci-glucosylated after growth for several generations at 45°C, as determined by the anthrone reaction.

Cell wall turnover rates were determined as an independent characterization of the mutant strains. When the autolysin-deficient strains FJ6 and BUL96 were pulse-labeled with radioactive GlcNAc, it was observed that little of the radioactivity was lost from either strain for approximately two or three generations, after which exponential turnover of labeled peptidoglycan began (Fig. 1). The first-order constants for the exponential phase are summarized in Table 3. The results show that the autolysin-deficient strains FJ6 and BUL96 have markedly reduced rates of cell wall turnover compared with strain C33 and wild-type strain 168.

Insertion of glucosylated teichoic acid. Before any studies could be done on segregation of old and new cell wall in the temperature-sensitive phosphoglucomutase-deficient strain,

TABLE 3. Cell wall turnover in *B. subtilis* strains

Bacterial strain	Amt of autolysin	Rate of cell wall turnover (K[liter per generation])[a]
168	Sufficient	0.96
C33	Sufficient	0.70
FJ6	Deficient	0.17
BUL96	Deficient	0.21

[a] Exponential cultures (20 ml) in Penassay broth were labeled with 40 μCi of GlcNAc for 0.08 generations, filtered, washed, and suspended in fresh medium. Samples (1 ml) were taken at 20-min intervals, filtered, and washed with minimal salts. The filters were dissolved in a toluene-based solvent and counted by liquid scintillation. The turnover constant (K) is defined as $\ln(cpm_{initial}/cpm_{final})$ per generation (32). Data were derived only from linear portions of the semilogarithmic plots. Generation times were 21 to 24 min as determined by light-scattering measurements. All cultures were maintained at 35°C with shaking.

it had to be determined whether teichoic acid is glucosylated before, during, or after insertion of the polymer into the cell wall. Mauck and Glaser (43) showed that newly synthesized glucosylated teichoic acid polymers must be inserted into the cell wall concomitantly with newly synthesized peptidoglycan when the cells were shifted from a phosphate-poor to a phosphate-rich medium, but this does not answer the question. ConA should precipitate radioactivity if cell walls are labeled with GlcNAc under permissive conditions. If labeling of walls occurred during periods of chase at 45°C, little or no radioactivity should be bound by the lectin because the teichoic acids are nonglucosylated. Cell cultures of strain C33 were labeled with GlcNAc at 45 or 35°C and then chased at 45 or 35°C. Cell walls were isolated, digested with lysozyme, and precipitated with ConA (Table 4). The data show that when cells are labeled at the permissive temperature, the ConA precipitate of lysozyme-digested cell walls contains a significant percentage (16.1%) of the labeled peptidoglycan. This observation suggests that glucosylated teichoic acid is bound to the peptidoglycan synthesized near the time of pulse-labeling. However, when cultures are labeled at a nonpermissive temperature and chased at a permissive temperature, a much smaller percentage (2.7%) of the label is precipitated. A similar low percentage is obtained in cultures labeled at 45°C and chased at 45°C or labeled at 45°C, chased at 45°C, and then chased additionally at 35°C. Under these conditions, little glucosylated teichoic acid is bound to labeled peptidoglycan. These data suggest that glucosylation occurs before or during insertion of teichoic acid into the cell wall of exponential-phase cultures. It does not appear that glucosylation can take place after teichoic acid has been inserted into insoluble cell walls.

In earlier studies, we developed the use of ConA as a marker for surface-exposed teichoic acid in *B. subtilis* (5, 18, 20). Although glucosylated lipoteichoic acids interact with ConA, they do not appear to penetrate the cell wall of *B. subtilis* (60). According to theory (42, 61), phosphoglucomutase-deficient strains grown at nonpermissive temperatures should not be readily agglutinated by the lectin. In contrast, phosphoglucomutase-sufficient cells should be agglutinated by ConA, regardless of their growth temperatures. Results are presented which show that cells of all the strains can be agglutinated by low concentrations of ConA (Table 5). When strains C33 and BUL96 were grown at 45°C and then interacted with lectin, it was found that concentrations of the lectin of at least 500 μg/ml were needed to effect agglutination.

When strain C33 was grown for several generations at 45°C, it was found that Fl-ConA would not bind to the cells. When an exponential-phase culture was shifted to 35°C, samples removed at intervals, and the samples mixed with Fl-ConA, areas of fluorescence were observed only after 0.6 to 1.0 generation. The cell cylinders and septa were stained with conjugated lectin (Fig. 2), but the poles were hardly

TABLE 4. Precipitation of ConA by lysozyme-digested, GlcNAC-labeled strain C33 cell walls

Growth conditions			cpm/mg of cell wall[d]	cpm precipitated by ConA per mg of cell wall	Cell wall precipitated (% of total counts in sample)
Growth temp (°C)[a]	Chased (°C)[b]	Additional chase (°C)[c]			
45 (1.2)	45 (0.6)		30,990	560	1.8
45 (0.7)	45 (0.7)	35 (0.6)	16,808	735	4.4
45 (0.6)	35 (0.4)	35 (0.4)	25,316	672	2.7
35 (0.9)	35 (0.5)		49,804	8,020	16.1

[a] Exponential cultures (1,400 ml) of strain C33, in minimal salts as described by Spizizen (58) and supplemented with D-glucose (0.5%), casein hydrolysate (0.22%), and tryptophan (50 μg/ml), were grown in the presence of 400 μCi of GlcNAc (2.94 Ci/mmol) at the indicated temperature. Length of growth time in presence of label is shown in parentheses as the number of generations. Growth rate at 35°C was 40 min per generation. At 45°C, the growth rate was 33 min per generation.

[b] Cultures were chased (by the addition of 10 mmol of unlabeled *N*-acetyl-D-glucosamine) at the indicated temperature for the number of generations, which are shown in parentheses. Because the K_m for GlcNAc uptake is 3.7 μM (47), the chase should be effective.

[c] Cultures were allowed to grow an additional number of generations (shown in parentheses) at the indicated temperature.

[d] Cell walls were isolated, digested with lysozyme, and precipitated with ConA. Precipitates were washed and then dissolved with α-methyl-D-mannoside, and samples were assayed for radioactivity.

TABLE 5. ConA agglutination of *B. subtilis* strains grown at permissive (35°C) and nonpermissive (45°C) temperatures

Bacterial strain	Minimal concn (μg/ml) of ConA required for agglutination at the following temp[a]:	
	35°C	45°C
168	7.8	7.8
FJ6	15.6	15.6
C33	7.8	>500[b]
BUL96	7.8	>500

[a] Cells were grown in Sargent salts supplemented with D-glucose (0.5%) and tryptophan or methionine (10 μg/ml) to late-exponential phase, harvested at 4°C, and washed three times in 50 mM Tris-hydrochloride (pH 7.4). Cell suspensions were adjusted to 0.45 absorbance units at 450 nm on a Coleman model 6/20 spectrophotometer. Cells (100 μl) were added to microtiter wells containing equal volumes of ConA in the wells. Agglutination patterns were determined with the aid of a stereomicroscope.

[b] No agglutination was observed at less than 250 μg.

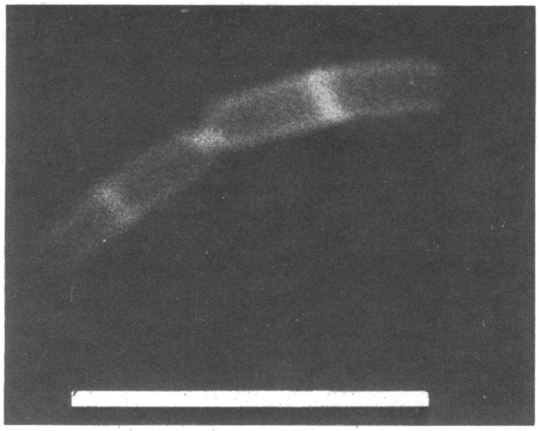

FIG. 2. Interaction between Fl-ConA and strain C33 after a shift from nonpermissive to permissive conditions. Cells were prepared for photography 0.8 generations after the temperature shift. Note that the cell poles are not as fluorescent as the cell cylinders or the septa. Growth rate in the minimal medium was 40 min per generation at the permissive temperature. Cells grown at the nonpermissive temperature were unable to bind the lectin. Bar, 15 μm.

stained at all (Fig. 2). After 2 to 3 generations of growth, Fl-ConA bound to all areas of the cell (data not shown). The results suggest that cell cylinders turn over rapidly and expose glucosylated teichoic acid to ConA after about one-half generation.

Binding of Fl-ConA and fate of the old cell wall. Having obtained results that suggest that glucosylation of teichoic acid occurs during insertion of the polymer into the cell wall and that new wall requires ca. 0.5 generations (2, 3, 21, 51, 59) to traverse the thickness of the wall during growth in strain C33, the fate of old cell wall could be monitored with the Fl-ConA-glucosylated teichoic acid interaction. Strain C33 was grown at the permissive temperature and then shifted to a nonpermissive temperature. Samples were taken periodically, stained with Fl-ConA, and observed under a fluorescence microscope. Cells grown at permissive temperatures stained evenly along the cell cylinder and intensely at the point of septation (Fig. 3A). As growth continued at the nonpermissive temperature, the cell cylinder began to lose the ability to bind Fl-ConA (Fig. 3B). After 2.8 generations, much cylindrical fluorescence was lost, leaving only the poles (once septa) fluorescing intensely. After 3.2 generations, as division continued, many fields revealed bacteria with only one pole fluorescing (Fig. 3D).

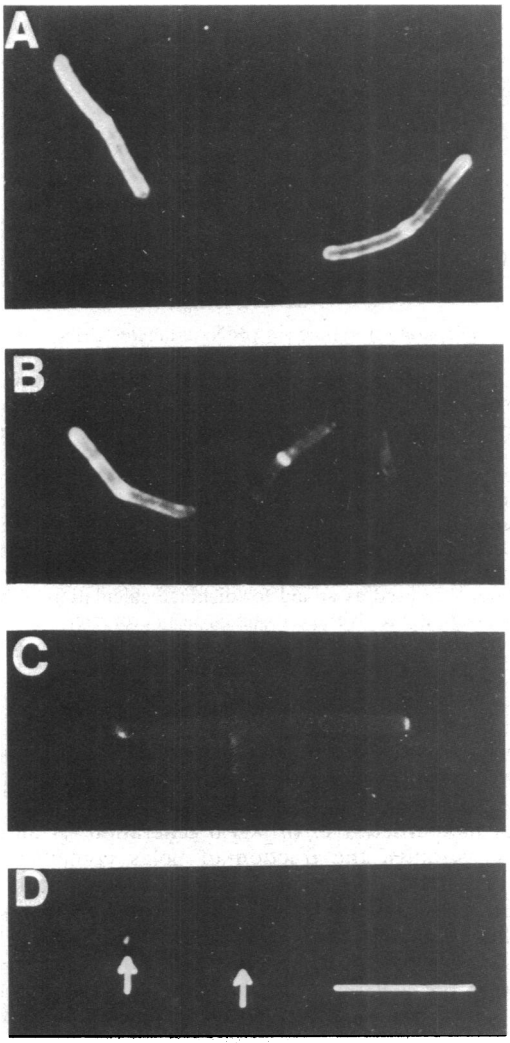

FIG. 3. Binding of Fl-ConA to strain C33 after a shift from permissive (35°C) to nonpermissive (45°C) conditions. Intervals (generations) after the shift: 0 (A), 1.6 (B), 2.8 (C), and 3.2 (D). Growth rates were 33 min per generation at the nonpermissive temperature. The arrows in (D) represent the distance between two completed cell poles. One pole does not fluoresce, Bar, 7 μm.

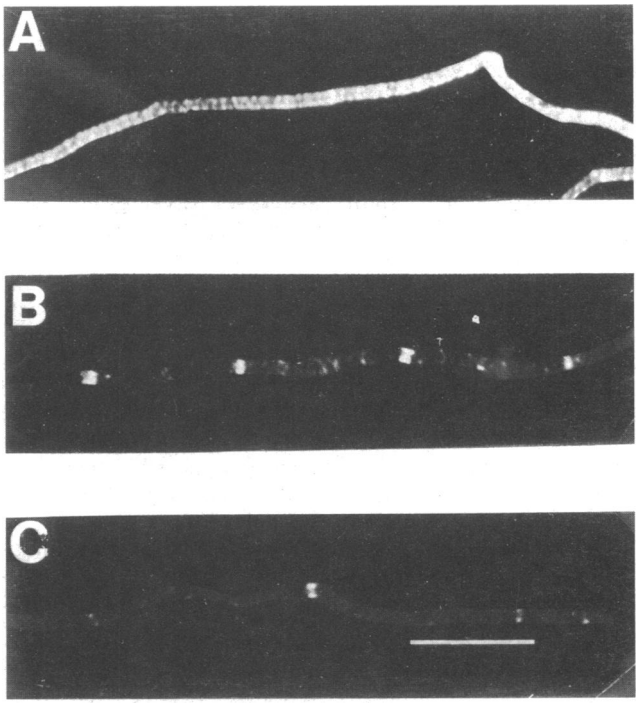

FIG. 4. Binding of Fl-ConA to *B. subtilis* BUL96 after a shift from permissive to nonpermissive conditions. Growth rates were as described in the legends to Fig. 2 and 3. Intervals (generations) after the shift: 0 (A), 2.5 (B), and 3.3 (C). Bar, 10 μm.

Although strain C33 dramatically reveals the loss of Fl-ConA binding sites within the cylinder, the slow turnover rate and long-chain morphology of strain BUL96 make this organism a valuable complementary tool to study segregation of cell wall. Figure 4 shows fluorescence patterns obtained when exponential cultures were shifted from the permissive to a nonpermissive temperature. At the time of the temperature shift, the chain exhibited rather uniform fluorescence (Fig. 4A). Because of the length of the cell chain, it was difficult to focus evenly over the fluorescent area. There was a gradual loss of cylindrical fluorescence after about 2 to 5 generations of growth at 45°C (Fig. 4B). However, polar regions formed before the temperature shift remained fluorescent. As new septa were formed and cell elongation continued, old fluorescent poles were moved further apart (Fig. 4C). The positions of poles and septa were confirmed by bright-field illumination.

Autoradiography of labeled cell walls. The binding of Fl-ConA follows the fate of cell surface-glucosylated teichoic acid but does not reveal interior organization of cell wall polymers. Autoradiography was performed to determine whether the fate of peptidoglycan was similar to that of teichoic acid. Strain BUL96 was labeled for two generations with GlcNAc, washed, transferred to fresh medium, and periodically sampled, and autoradiographs were prepared. The results (Fig. 5) demonstrate the fate of labeled cell wall.

Cells appeared densely and uniformly labeled at the time of transfer to fresh medium. After 1.4 generations of growth, a pattern of cell wall segregation began to emerge. Silver grains remained tightly clustered at the points of completed poles. However, silver grains were sparse and dispersed during length extension and subsequently lost during inside-to-outside growth. As growth continued, labeled septa were moved further apart until clusters of silver grains were separated by long chains of unlabeled cells. To demonstrate that labeled cell wall was retained in the poles, autoradiographs were routinely observed under both bright-field and phase illumination. This procedure made it possible to locate the relative position of poles, septa, and grains. Dense clusters of silver grains were observed at the terminal poles and at the points of septation (complete but unseparated poles). Grains within the cylindrical regions were clearly differentiated from grains at the poles. Resolution was estimated to be ±1 μM, although it is possible that some errors may have arisen due to the differences in emulsion thickness in various parts of the cells (35).

The results suggest that the wall deposited on the cylindrical regions of autolysin-deficient cells mostly collects at sites of septation and in large part at sites that had already developed or were developing at the time of chase. If this interpretation is correct, then the following predictions can be made: (i) after enough time has passed so that some of the cylinder has turned over ($n > 1.0$ generation for autolytic-sufficient strains), the fraction of poles containing label should be $(1/2)^n$. In chains (autolytic-deficient cells), the distance in micrometers between dense grain clusters should approximately double every generation, and (ii) the percentage of silver grains overlaying the cell should increase with respect to the cell pole and decrease with respect to the cylindrical region. Both of the predictions can be tested by quantitative measurements of autoradiographs of GlcNAc-labeled strains C33 and BUL96.

Autoradiographs of strain BUL96 filaments were photographed under a light microscope (×400), and enlargements were made of GlcNAc-labeled cells that were allowed to grow in fresh medium for 1.4, 1.9, 2.4, and 2.9 generations.

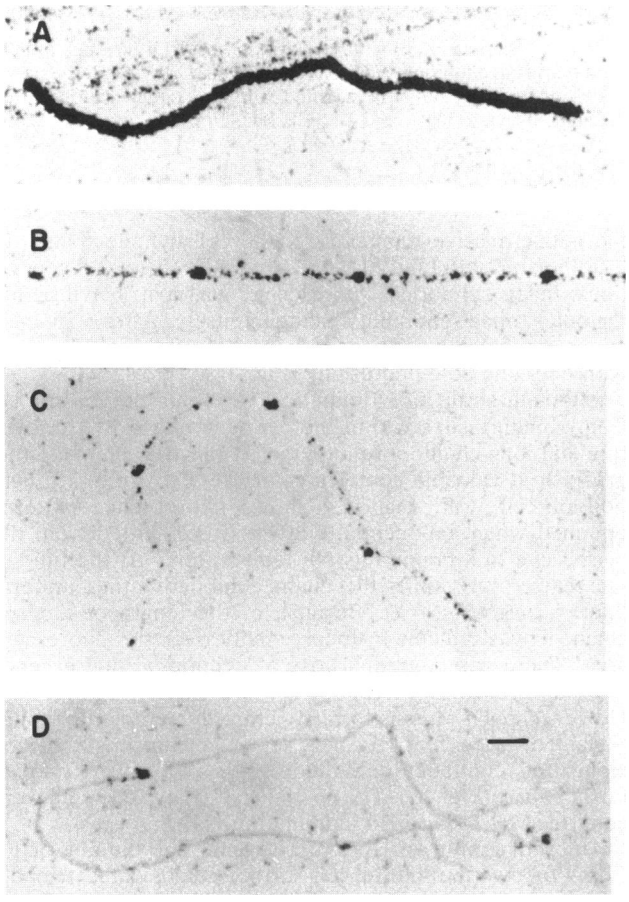

FIG. 5. Autoradiography of *B. subtilis* BUL96 labeled with GlcNAc. Cells labeled for two generations in Penassay (24 min per generation) were filtered, washed, and transferred to fresh medium. Samples were taken at intervals, fixed to slides, coated with NTB-2 nuclear track emulsion, maintained at refrigerated temperatures, and developed after 7 days. Interval (generations) after the transfer to fresh medium: 0 (A), 1.4 (B), 1.9 (C), and 2.9 (D). Bar, 5 μm.

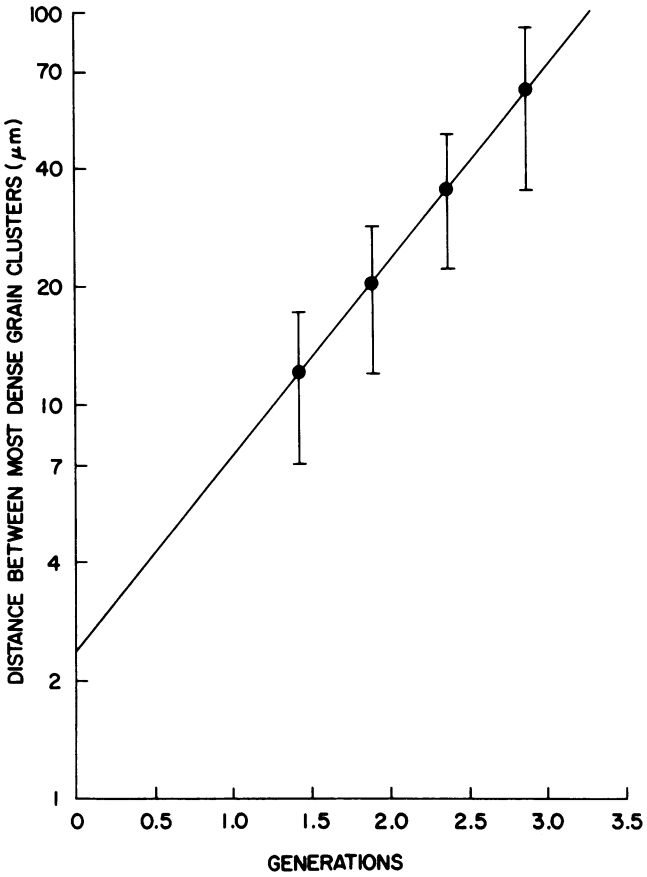

FIG. 6. Separation of poles in GlcNAc-labeled cells of *B. subtilis* BUL96. Exponential-phase cultures of *B. subtilis* BUL96 were grown in Penassay medium, labeled with GlcNAc for two generations, filtered, washed, and suspended in fresh prewarmed medium. Samples were taken approximately every 0.5 generations and autoradiographs were prepared. Photographs were taken under a light microscope (×400), and enlargements were made so that the final magnification was ×2,500. For each group, the distance between most dense grain clusters (for example, see Fig. 5) were measured and averages were calculated (40 to 185 separate measurements were performed within each sample group). A photograph of a stage micrometer was used as an aid for distance calculations. The data are expressed as the average distance in micrometers ± standard deviation.

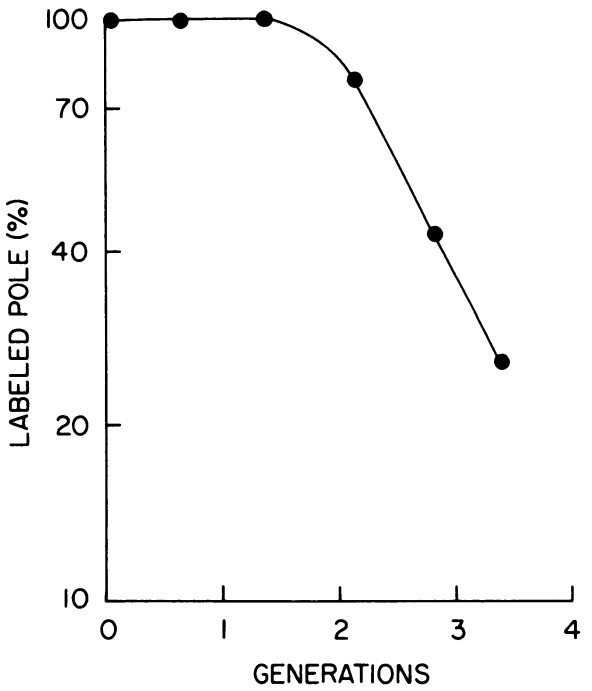

FIG. 7. Conservation of radioactivity in poles of GlcNAc-labeled cells of strain C33. Exponential-phase cultures of strain C33 were grown in Penassay medium, labeled with GlcNAc for two generations, filtered, washed, and suspended in fresh prewarmed medium. Samples were taken approximately every 0.7 generations, and autoradiographs were prepared for each sample group. Cells were observed under the light microscope (×1,000), and the percentage of cell poles that contained silver grains was calculated. Data represent the percent labeled poles versus the number of generations of exponential growth in fresh medium.

The distance between the densest grain clusters (shown previously to be associated with poles) was determined for each sample group. Averages were calculated and plotted against the number of generations (Fig. 6). Measurements were taken beginning with 1.4 generations of growth in fresh medium. At this point, label within the cylinder was "diluted" by elongation and, to a small degree, lost during turnover (Fig. 1), leaving silver grain clusters overlaying a subset of the poles easily visible. As predicted above, the distance in micrometers between labeled poles increased exponentially as cell cultures grew exponentially. Theoretically, the distance between labeled poles should exactly double each generation. The data show a slightly steeper slope than the theoretical doubling, but this is still not significantly different than the doubling time.

Figure 7 shows the results of similar experiments with the autolytic-sufficient strain C33 in which autoradiographs were used to determine the percentage of cell poles that retained labeled GlcNAc after growth in unlabeled medium. Because of the density of the silver grains and the time necessary for cylindrical turnover to occur (about one generation), all cells appeared to have grains in all poles after 1.0 to 1.5 generations of exponential growth. However, after this preliminary period, the fraction of poles retaining label followed the predicted value by decreasing after each generation by a factor of two. In this experiment, between 375 and 675 cell poles were observed for each time point shown. The results are in good agreement with the predicted values (see above).

One additional treatment of the data was performed for the autolysin-sufficient strain C33. The autoradiographs of each sample group of strain C33 were photographed and enlarged (×2,500). A grid that sliced the cell into four theoretical sections (3 mm per section, 1.2 μm of cell length) was placed over the cell images so that a pole was in section number one (Fig. 8). Grains in each section were counted, and the average number of grains per slice was calculated. At 0 and 0.6 generations after the transfer to fresh medium, the grains were evenly distributed throughout all slices (each slice contained 24 to 26% of the total grains counted). By 1.4 and 2.1 generations, the cell pole was found to contain a significantly higher percentage of grains than the cylinder. After 2.8 generations, 94.8% of the grains were found in the first slice, with no more than 1.7% in any other slice. A computer analysis of the rates of loss of grains per generation for the individual slices revealed that only slice one was distinctly different (95% confidence level). Slices two, three, and four exhibited the same rates of loss of grains and were not significantly different, showing that none of these regions were preferentially conserved or turned over during division. The computer was also capable of making the same

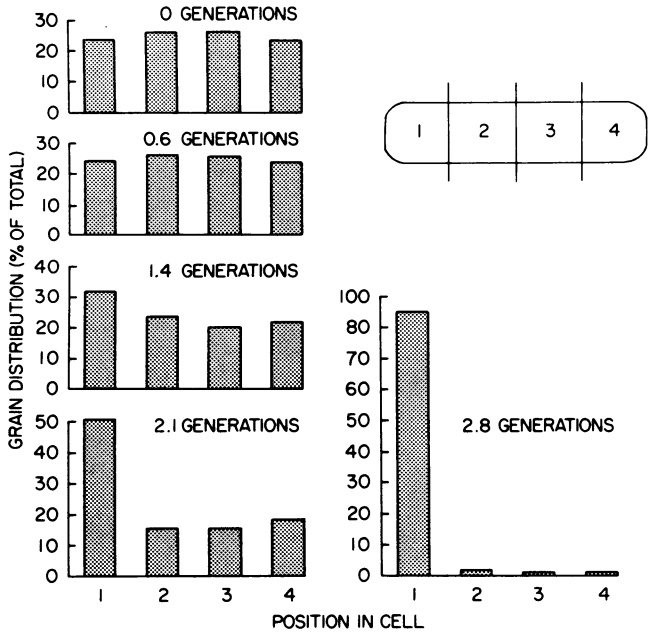

FIG. 8. Distribution of label after incorporation of GlcNAc in cells of strain C33. Exponential-phase cultures of strain C33 were grown in Penassay medium, labeled with GlcNAc for two generations, filtered, washed, and suspended in fresh prewarmed medium. Samples were taken for each sample group. Photographs were taken under the light microscope (×1,000) and enlargements (final magnification, ×2,500) were made (see also Fig. 6). A grid was placed over the selected and photographed cell which divided the cell image into four sections of 3.0 mm each. The cell pole was arbitrarily placed in section 1. Silver grains within each section were recorded. The average number of silver grains per section was calculated. The data are expressed as the percentage of the total number of grains per cell found in each section. Each section represents 1.2 μm of cell length. The number of generations of exponential growth in fresh medium is shown for each group. For each sample group, at least 100 separate cells were used for calculations. When cells (or chains of cells) of length sufficient to give 7 to 11 slices were subjected to similar analyses, profiles identical to those shown in the figure were obtained, showing that cell length did not bias the results.

calculations when data from zero generations (or 0 plus 0.6 generations) were deleted. Results from these calculations confirmed that only slice one was different. These latter calculations were made to ensure that the washing and resuspension procedures did not give rise to perturbations which could modify grain distributions.

Finally, the data were subjected to a *t* test for grain distributions as described by Mauck et al. (44) for *Bacillus megaterium*. Statistically significant differences were found only when slice one was compared with the other slices.

Removal of radioactivity from cells labeled with GlcNAc. The incorporation of radioactive GlcNAc into cell wall has been well established for the genus *Bacillus* (15, 21, 43, 44). These studies were concerned primarily with the use of *N*-acetyl-D-[^{14}C]glucosamine or GlcNAc as a short-term label. Some studies have suggested that a small amount of GlcNAc can be converted into protein in *B. subtilis* (21, 44, 47). This small amount of protein, although negligible for 1 to 3 generations, may cause serious errors if not properly removed before autoradiography. Schlaeppi et al. (57) found that some radioactivity could be removed from cells by trypsin when the cells were labeled with GlcNAc. We pulse-labeled cultures of *B. subtilis* 168 and FJ6 with GlcNAc and then divided the cultures into two parts. One part was harvested immediately, whereas the other part was chased for four generations with unlabeled *N*-acetyl-D-glucosamine. The cells were washed and subjected to breakage in the French press or treated directly with proteases (Table 6). The results show that, indeed, the chased sample contained more protease-soluble radioactivity than did the pulsed cells. For example, in *B. subtilis* 168, the amount of trypsin plus subtilisin-soluble radioactivity in the pulsed cells was ca. 6.9%, whereas the radioactivity in the pulse-chased cells was ca. 19.1% of the total. Strain FJ6 seemed to contain fewer counts in a protease-susceptible form. In addition, when the cells were first ruptured in the pressure cell, the amount of protease-soluble radioactivity increased substantially in both the pulsed and the pulsed-chased cells. Importantly, it can be seen that subtilisin is capable of removing trypsin-refractory material from the cells, suggesting that trypsin alone is not totally effective in cleaving all wall-associated protein. Subtilisin has previously been shown to remove some, but not all, of the radioactivity from detergent-extracted, ^{35}S-labeled cell walls (48). Furthermore, as expected, the proportion of radioactivity solubilized by lysozyme is much lower in the pulse-labeled and chased cells than in the pulse-labeled cells. The results show that pulsed and chased cells contain a significantly greater amount of protease-susceptible label than do pulsed cells.

TABLE 6. Removal of radioactivity from intact cells of *B. subtilis* 168 and FJ6 labeled with GlcNAc[a]

Enzyme treatment for whole and broken cells[b]	% original cell-associated radioactivity after the following treatments of the indicated strains[c]:			
	Pulse-labeled		Pulse-labeled and chased	
	168	FJ6	168	FJ6
Trypsin soluble				
Whole	1.4	0.1	9.8	6.1
Broken[d]	6.3	3.8	11.4	9.4
Subtilisin soluble				
Whole	5.5	0.2	9.3	5.9
Broken	6.5	6.3	16.3	8.3
Lysozyme soluble				
Whole	87.3	95.9	73.3	90.0
Broken	84.5	90.3	57.8	86.3
Lysozyme insoluble				
Whole	3.0	6.8	10.1	4.1
Broken	4.8	3.6	9.9	3.9

[a] Exponential-phase cells, growing in Penassay broth, were labeled for 5 min with 1.0 μCi/ml (final activity) of GlcNAc (2.94 Ci/mmol) and harvested by centrifugation or chased for four generations with 10 mM unlabeled *N*-acetyl-D-glucosamine. The K_m for GlcNAc uptake in strain 168 is 3.7 μM (47). Harvested cells were washed twice with cold water and then incubated at 100°C for 30 min in water to inactivate autolysins. Washed and heat-inactivated cells were freeze-dried.

[b] Cell suspensions, 1.0 mg/ml in 50 mM potassium phosphate adjusted to pH 7.2, were sequentially treated with 50 μg/ml (final concentrations) of proteases or lysozyme at 37°C. Digestion with trypsin was for 5 h, whereas subtilisin treatment was for 18 h, and the lysozyme digestion time was for 3 h.

[c] Values indicate the percentage of the original cell-associated radioactivity. The initial radioactivity in the pulsed cells of strain 168 was 287,377 cpm/mg. Radioactivity in the pulsed and chased cells was 14,300 cpm/mg. For strain FJ6, the initial radioactivity was 1,060,300 cpm/mg, whereas the pulse-chased cells contained 61,270 cpm/mg. After each treatment, both soluble and insoluble fractions were counted in triplicate. Quench corrections were made by the addition of an amount of GlcNAc to each vial which approximately corresponded to the activity in the unknown sample.

[d] Cells suspended in phosphate buffer were subjected to breakage in the French pressure cell operated at 15,000 lb/in^2.

DISCUSSION

The purpose of this study was to determine how cell walls are assembled in gram-positive rods, as typified by *B. subtilis*. The data were gathered by use of both autolysin-sufficient and -deficient strains. In addition, two completely independent probes, one for cell wall teichoic acid and one for peptidoglycan, were utilized in the experimental design. The autolysin-deficient bacterium forms long chains of cells facilitating the study of cell surface patterns and also has much lower rates of cylinder cell wall turnover. In contrast to other studies (2, 3, 11, 12, 16, 22, 25, 33), limitation of nutrients, addition of antibiotics, or other manipulations that disturb balanced growth were not necessary to bring about the desired cell surface changes. The general conclusions are the same for the two strains, regardless of the probe employed. (i) Cell poles tend to resist turnover. (ii) All regions of the cell side walls turn over at sites randomly distributed over all the available external surface area. (iii) The boundary between cell wall that is relatively resistant to turnover and cell wall that is not resistant to turnover appears to be near the pole-cylinder junction. (iv) The data support the view, as originally proposed by Fan et al. (26) and supported experimentally by Pooley (50–52), Archibald and Coapes (3), Jolliffe et al. (31), Doyle et al. (21), and De Boer et al. (15), that much wall synthesis occurs in a radial mode consistent with an inside-to-outside growth model for the elongation of gram-positive cylindrical walls of bacilli (34, 37, 38, 55).

When the temperature-sensitive phosphoglucomutase cells were shifted from permissive to nonpermissive conditions, a loss in cell wall hexose occurred (Table 2). The loss in hexose is accompanied by loss of agglutinability by ConA (Table 5) and by a loss in the stainability of the organisms by the fluorescein-conjugated lectin (Fig. 3 and 4). In strains C33 and BUL96, there appeared to be a gradual loss of

stainability by Fl-ConA over the cylindrical regions but not at poles and septa. These results provide evidence for the conservation of poles in *B. subtilis* and support a view that the cell wall is differentiated in this bacterium. Archibald and colleagues (2, 3, 59) have previously demonstrated the resistance of cell poles to turnover in autolysin-sufficient strains of *B. subtilis*. Earlier, Fan et al. (24, 25) found that the in vitro autolysis of cell walls of *B. subtilis* tended to enrich for cell poles as hydrolysis proceeded. Burdett (7, 8) recently showed, however, that end walls may be just as susceptible as side walls to autolysins.

Doyle et al. (23) found that cell wall fragments of *B. subtilis* contained tightly bound transforming DNA and that the DNA was enriched for markers near the origin and terminus of replication. Sandler and Keynan (53) have also suggested that there is a coupling of DNA initiation with cell wall synthesis in *B. subtilis*. Koch et al. (39) and Doyle et al. (19) have hypothesized that the polar ends of bacilli should interact (at least indirectly, probably mediated by the membrane) with the chromosome. When wall fragments autolyze enough to yield significant proportions of cell poles, the DNA remains firmly bound to the insoluble residues (19). Schlaeppi and Karamata (56) have also recently suggested that there is a close relationship between the chromosome and the cell wall of *B. subtilis*. There may therefore be a biological need for the conservation of cell poles by gram-positive rods. The fact that it was possible to predict and experimentally verify (Fig. 7) the percentage of conserved poles in an exponentially increasing cell population argues for the view that poles remain relatively resistant to autolysins in vivo. Results from autoradiography studies were also in accord with the premise that the cylinders of autolysin-sufficient cells contain little wall which is more than several generations old, whereas the poles may contain wall which is many generations old. When the autolysin-sufficient strain C33 was labeled with GlcNAc and the position of the label monitored by autoradiography, it was observed that the distribution of the silver grains reflected a conservation of radioactivity at the poles and a random loss of radioactivity over the cylinder (Fig. 8) of the separated cells. Moreover, the quantitative determination of the numbers of poles possessing silver grains after a pulse of GlcNAc gave a value corresponding closely with predictions of conservation of pole (Fig. 7).

The chain-forming strain BUL96 was also studied by autoradiography after growth of GlcNAc-labeled cells in unlabeled medium. The results suggested that material in the cylinder was "diluted" by length extension or turned over during growth, whereas polar material was conserved (Fig. 4 and 5). Because BUL96 forms chains of cells that do not readily separate, it was possible to measure the distance between conserved regions of the wall as a function of growth. If septa or poles were resistant to turnover, the distances between septa should double each generation. Within experimental error, the foregoing prediction was verified with photographic combinations of autoradiography and phase-contrast microscopy (to locate silver grain clusters and septa) (Fig. 6). The patterns of fluorescence of ConA also revealed the topographic distribution of cell surface α-D-glucosylated teichoic acid. Both methods yielded results, which suggest that cell wall assembly in the cylinders of *B. subtilis* occurs by uniform insertion on the inner wall face. It is interesting to note that Schlaeppi et al. (57) have found that segregation patterns of wall teichoic acid in cultures of *B. subtilis* uniformly labeled with [^{3}H]glycerol were identical to those of GlcNAc-labeled cells.

Previous autoradiographic experiments from the laboratory of Pooley and colleagues (52, 57) suggest that the old wall does not gradually become diluted with new wall, but rather it segregates into a larger number of units after several generations than could be accounted for by the poles present at the time of chase. This is taken as evidence of zonal growth. Our results do not disprove the conclusions of Pooley et al. (52) and Schlaeppi et al. (57), as neither we nor they directly measured sites of incorporation. The data (Fig. 8) show that when the fate of cell wall label is followed for about three generations, there is no distinct area on the cell, except for the pole, that conserves the label. It may not be valid to study grain distributions in cells that have been chased for longer than 3 to 3.5 generations because of the observation that the label may not be incorporated quantitatively into peptidoglycan (Table 6).

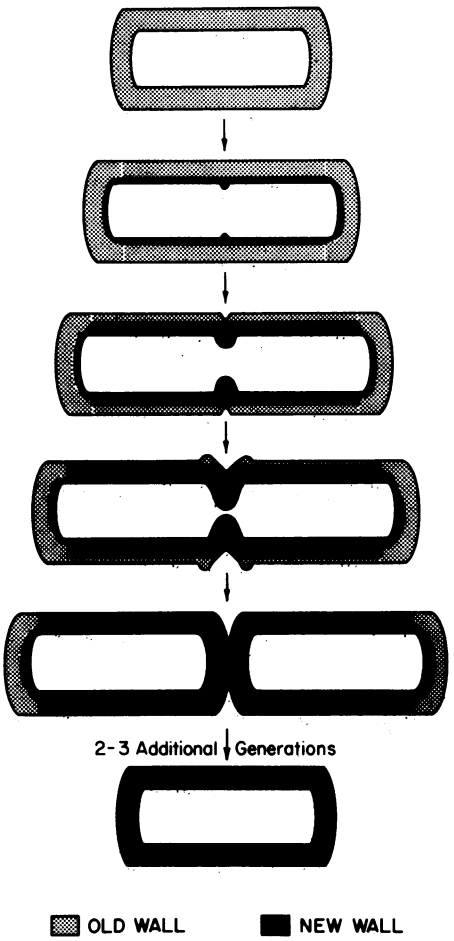

FIG. 9. Model for cell wall growth in *B. subtilis*. A unit cell elongates by random addition of nascent cell wall polymers along the inner face of the cell cylinder. Old outside cylindrical wall is lost as growth proceeds. Septum formation begins with localized (zonal) insertion of newly synthesized wall at a central site inside the confines of the cylindrical wall. As cross-wall formation continues, external cleavage of the cell wall begins. The junctions between new poles and cylindrical wall are now delineated by the formation of wall bands represented by the raised bumps. Finally, the septum is completed, "new" cell wall reaches the cell surface, the wall bands are lost by autolytic digestion, and cell separation occurs. New wall material in old poles appears on the surface only after 2 to 3 generations.

The data and the results from other laboratories support a hybrid model for cell wall replication (Fig. 9). The model states that wall synthesis occurs on the inner face of the cell cylinder, on nascent septa, and very slowly on cell poles. The observed turnover is due to inside-to-outside growth and is much more rapid on the cylinder than on the completed poles. Nascent septa are constituted largely of new wall material and may be assembled in a process analogous to that in streptococci (4, 14). Cylindrical elongation is not by zonal growth, but it is assumed to be a result of uniform insertion of wall precursors to form a completely new innermost layer, with the premise that the new cell wall layer is unstressed, and hence resistant to turnover form, before its migration to the outer face where the autolysins normally function. The data are clearly not compatible with totally zonal (55) wall growth or with totally diffuse (random insertion) growth (14, 29, 34, 55). Moreover, models which may involve asymmetry of surface extension (14, 55) do not appear to be plausible. For example, the unit cell hypothesis (17), which assumes that all elongation occurs from one half of a cylinder, is ruled out for *B. subtilis* by the present data. All zonal models must not be rejected, as some could possibly account for the data reported here. For example, if there were only a few zones of growth and the zones were somehow made to spread, then diffuse or random insertion would be observed. Chatterjee et al. (10) have speculated that the *N*-acetylmuramyl amidase (amidase) could "spread" new wall material by transfer to old wall material. This possibility is presently viewed as unlikely, however, because there was no detectable tendency for label on poles and septa to spread and it is clearly thermodynamically impossible for insertion of wall moieties into regions such that they must bear stress at the time of insertion.

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