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Identification of Cell Wall Subunits in *Bacillus subtilis* and Analysis of Their Segregation During Growth

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Continuous as well as pulse-labeling and chase experiments with *Bacillus subtilis* demonstrated that the cell wall (both peptidoglycan and teichoic acid) is composed of a limited number of blocks which, once completed, segregate during subsequent growth without undergoing any mixing with newly synthesized blocks. This observation suggests that new wall material is inserted in a limited number of zones. Previously reported observations which suggested diffuse intercalation of new wall material are reinterpreted on the basis of our results. Experiments performed on different media showed that the number of segregation units per unit of cell length and thus the density of insertion zones increases with medium richness. This finding suggests analogies between the regulation of cell wall and DNA synthesis.

The nature of bacterial envelope synthesis has been abundantly investigated since Jacob et al. (14) proposed a theoretical model of zonal insertion of new surface components, aiming to provide a mechanism for the regular segregation of chromosomes into daughter cells. In a previous note, we reported an autoradiographic analysis of cell wall segregation in *Bacillus subtilis* (22), which strongly suggested zonal insertion of new wall material and showed a good deal of analogy with the already established mode of cell wall synthesis in gram-positive cocci (7, 12).

In the present study we have extended our observations on media of different richness, each characterized by a fixed average number of replication forks of DNA per chromosome (26). Our aim was to determine the number of insertion zones per cell as a function of medium richness and to investigate the correlation between the number of DNA replication forks and that of cell wall insertion zones. A relationship between these parameters would suggest that DNA and cell wall synthesis have common regulatory mechanisms and could provide some evidence about the role of the cell envelope in DNA segregation.

Experiments reported below show that the number of wall insertion zones per cell is greater than one and increases with the richness of the medium. This has permitted a reassessment of previously reported observations which were interpreted in favor of diffuse intercalation of new cell wall (2, 4, 8, 9, 15). Our data also provide information on the size of segregation blocks of cell wall and underline the necessity to distinguish between the inside and the outside wall surfaces. Both of these factors are pertinent to any model aiming to describe cell wall biosynthesis in gram-positive rod-shaped bacteria.

MATERIALS AND METHODS

Strains. Most of the experiments were performed with *B. subtilis* strain FJ3 *metC3 lyt-1* (10), which has a considerably reduced cell wall turnover in all media used (H. M. Pooley and X. Favre, unpublished data). Continuous labeling and chase experiments in rich medium (see below) were performed on strain Ni15 *trpC2 thyA thyB xin-15 lyt-15*, a mutant of *B. subtilis* with reduced cell wall turnover in rich medium (20). In our experimental conditions both *lyt* strains form long chains (Fig. 1). Strain 168 *trpC2 lyt*$^+$ (25) was used as a reference strain with normal turnover.

Media. Rich medium contained 0.5% (wt/vol) Casamino Acids, 0.5% (wt/vol) glucose, and 0.01 M potassium phosphate; 100 ml of medium was supplemented with 0.1 ml of a salt mixture containing 25 g of $MgSO_4 \cdot 7H_2O$, 100 mg of $FeSO_4 \cdot 7H_2O$, 100 mg of $ZnSO_4 \cdot 7H_2O$, 10 mg of $MnSO_4 \cdot 4H_2O$, 1 mg of $CusO_4 \cdot 5H_2O$, and 0.2 mg of $K_2Cr_2O_7$ per 100 ml of water (derived from 19). Final pH was 7.5. When required, thymine and tryptophan were added at 40 μg/ml and 30 μg/ml, respectively.

Minimal medium contained 0.015 M $(NH_4)_2SO_4$, 0.5% glucose, and 0.01 M potassium phosphate. It was supplemented with the same amount of the salt mixture described above. Final pH was 7.2. When required, 40 μg of thymine or methionine per ml and 30 μg of tryptophan per ml were added.

The medium of intermediate richness was obtained by supplementing the minimal medium with 1% sodium glutamate and 40 μg each of leucine, cysteine, and alanine per ml.

Continuous labeling and chase experiments. Throughout the experiments cultures were grown at 44°C with aeration and maintained in exponential growth phase. By appropriate dilutions into fresh medium, the cell titer was always maintained below 5 × 10^7/ml in rich medium and 10^8/ml in minimal medi-

um. Cell wall was labeled during four to five generations in media containing appropriate concentrations of *N*-acetyl-D-[1-^{3}H]glucosamine ([^{3}H]GlcNAc) (see below and Tables 1 and 2); final concentrations of total (labeled and unlabeled) GlcNAc were 60 μM in rich medium and 250 μM in minimal medium, sufficient to maintain steady-state labeling. At 0 min, cells were harvested by filtration, washed, and diluted appropriately in prewarmed medium containing 100 μM (rich medium) or 250 μM (minimal medium) GlcNAc. After the desired chase periods, samples were filtered, treated for 1 min with cold trichloroacetic acid (5%, wt/vol), washed with distilled water, and processed for autoradiography.

[^{3}H]GlcNAc specific activities and cell concentrations were chosen so as to obtain at the end of each chase period comparable cell titers of about 2×10^7 to 5×10^7/ml in rich medium and 6×10^7 to 10×10^7/ml in minimal medium and, upon processing for autoradiography, comparable grain densities per cell.

Teichoic acids were labeled with [2(*n*)-^{3}H]glycerol in rich medium supplemented with 50 μg of unlabeled glycerol per ml (24). The experimental design was identical to that described above. Prior to processing for autoradiography, phospholipids, also labeled by this procedure, were extracted by standard chloroform-methanol treatment (18).

Pulse labeling and chase experiments. Cells were maintained in exponential growth phase at 44°C in media containing neither cold glucosamine (GlcN) nor GlcNAc. At the time of addition of 2.5 μM D-[6-^{3}H]GlcN hydrochloride (specific activity, 20 to 40 Ci/mmol), cell titer was 4×10^7/ml in rich medium, 10^8/ml in minimal medium, and 6×10^7/ml in the medium of intermediate richness. After a 2-min labeling period, cells were filtered, washed, and resuspended in appropriate volumes of unlabeled medium supplemented with 100 μM GlcN. Dilutions were calculated so as to obtain at each time of sampling during the period of chase a maximal cell titer of about 4×10^7/ml in rich medium, 10^8/ml in minimal medium, and 6×10^7/ml in the medium of intermediate richness. Samples withdrawn after appropriate chase periods were processed as described above.

Measurements of trichloroacetic acid (5%, wt/vol)-precipitable radioactivity showed that label continued to be incorporated during a short period after resuspension as a result of an intracellular precursor pool. We determined that the actual labeling periods were 3.5 min (in the case of a nominal 2-min pulse in rich medium) and 3 min (2-min pulse in minimal medium).

Preparation of cells for autoradiography. Unless otherwise stated, cells were prepared as follows: trichloroacetic acid-treated samples were suspended in 1 ml of RNase (bovine pancreas, 2 mg/ml), and after 45 min of incubation at 37°C, 1 ml of trypsin (2 mg/ml) was added and the incubation was continued for a further 90 min. This treatment eliminated label incorporated into proteins (a relatively important fraction in minimal medium). Afterwards, samples were filtered, washed, treated with trichloroacetic acid (5%, wt/vol) for 45 s, washed again, spread on slides, and dried at 60°C for 1 h.

Slides were coated with the Ilford-L4 emulsion (17), and after 2 to 6 days of exposure at 20°C, they were developed with the D-19 Kodak developer for 7 min at 20°C, fixed, and washed.

After development, septa were stained as follows: slides were dipped in 0.02% (wt/vol) crystal violet for 75 s, washed with water, dried, dipped in 0.1% (wt/vol) fuchsin for 2 min, washed, and finally dried again. Slides were examined with a Zeiss photomicroscope III, and pictures of an adequate number of chains of cells were taken. After development and enlargement of these pictures, the original photographed chains were relocated on the slide; cross walls as well as grains (counted by adjusting the focus throughout the sample) were traced onto the photographs. Cross walls, which stained red, were clearly distinguishable from black grains.

Occasionally, when the RNase/trypsin treatment was omitted, septa were stained prior to processing for autoradiography in the following way: trichloroacetic acid-treated bacteria were suspended for 5 min in 10% (wt/vol) tannic acid, harvested by centrifugation, washed twice with water, suspended in 0.02% (wt/vol) crystal violet for 90 s, washed again, and finally spread on slides and dried. Since the stain was leached during development of the photographic emulsion, slides were photographed before they were processed for autoradiography. Photographed chains of cells were subsequently relocated for observation and tracing of grains (see 22).

Statistical analysis of grain distributions. In experiments with cells grown in minimal medium, grains per whole cell were counted. In other media, the midpoint between neighboring septa was determined and grains per half cell were counted. Grain distributions per cell (or per half cell) were compared with the Poisson distribution by the χ^2 test. As in previous studies, the variability in grains per cell (or per half cell), due solely to variation in cell length, was reduced by dividing the population into two classes: cells shorter than the mean length (class I) and cells longer than or equal to the mean length (class II) in each medium (see Tables 1, 2, 5, and 7).

Definition of a cell wall segregation unit. A segregation unit was defined as a cluster of grains or a single grain, observed along chains of labeled cells after approximately five generations of chase, which was separated from neighboring grain clusters (or single grains) by at least 1 μm. Clusters centered on a septum or spreading over both sides of a septum were considered two separate segregation units.

Determination of the number of cell wall segregation units per cell. To eliminate grains due to emulsion background and to that fraction of the label incorporated into protein, the following corrections were performed. (i) For every development the emulsion background was counted. On average, it corresponded to one grain per 17 cells in minimal medium and per 8 cells in rich medium. Therefore, starting at one end of each chain of cells, a grain was removed every 17 or 8 cells. Should the relevant cell be devoid of grains, the nearest grain to this cell was removed. (ii) RNase/trypsin digestion did not remove all the label incorporated into protein. We found that, after protein digestion in 2-min pulse and chase experiments, about 1% (rich medium) and 2% (minimal medium) of the grains were due to label in residual protein. In continuous labeling and chase experiments, the proportion of grains due to protein which remained after trypsin treatment was 3.5% and 10% in rich and minimal medium, respectively. The calculated number of

grains due to protein was divided by the number of cells in the population examined to obtain the ratio $1/n$. Since at the end of the chase period proteins were fairly uniformly distributed along the chains, a grain was removed from every nth cell.

Segregation units so defined correspond to cell wall blocks visualized by grains. The observed number was an underestimate since it did not include the class of segregation units with zero grains (0 grain class). However, from the distribution of grains into clusters (not cells), which was a Poisson distribution in the case of pulse and chase experiments, the 0 grain class of segregation units was calculated in each such experiment and added to the total number of counted units.

The mean number of segregation units per cell was calculated from the average number of units per cell at the time of sampling, corrected for the exponential increase in cell numbers during the corresponding chase period. For example, in the case of a five-generation chase period, each cell on the average has given rise to 32 cells. To obtain the mean number of segregation units per cell at the beginning of the chase, the determined average number of segregation units per cell at the time of sampling was multiplied by 32.

Since in pulse experiments a significant fraction of the label was incorporated after the beginning of the chase as a result of an intracellular precursor pool (see above), the chase period was calculated from the moment when the labeled precursors had been chased out of the pool (see above).

RESULTS

Segregation during a chase period of cell wall material incorporated in continuously labeled cultures. (i) Nature of segregation of cell wall material. Exponentially growing cultures of *B. subtilis* mutants Ni15 and FJ3 were labeled with [^{3}H]GlcNAc of appropriate specific activity for four to five generations. During the subsequent chase period in unlabeled medium, samples were withdrawn at intervals and the distribution of labeled cell wall was determined by light microscope autoradiography (see Materials and Methods). For these experiments we took advantage of Lyt$^-$ strains Ni15 and FJ3, which exhibit a considerably reduced cell wall turnover and thus retain sufficient numbers of grains to allow statistical analysis after periods of chase of over five generations. Results of typical continuous labeling and chase experiments obtained with strain Ni15 in rich medium (doubling time of 20 min at 44°C) and strain FJ3 in minimal medium (doubling time of 40 min at 44°C) are shown in Tables 1 and 2, respectively.

In rich medium statistical analysis shows that after five generations of chase grain distributions were clearly non-Poisson. In minimal medium grain distributions had already become clearly non-Poisson after three generations of chase.

This result, which confirms our previous report (22), is incompatible with models advocating diffuse intercalation of new wall material and strongly suggests that in both rich and minimal media new wall is inserted in a limited number of growth zones. We would like to stress that cell wall segregation appeared only after a rather long delay. Indeed, statistical analysis (Tables 1 and 2) shows clearly that after up to three (60 min) and two (80 min) generations of chase in rich and minimal media, repectively, grain distributions were not significantly different from the Poisson distribution. However, visual inspection of autoradiographs revealed segregation of new and old wall material in both rich and minimal media about 40 min before it could be demonstrated by statistical methods.

(ii) Estimation of the number of cell wall segregation units. Microscopic observation of autoradiographs of cells continuously labeled with [^{3}H]GlcNAc and chased for about five generations revealed mostly well separated clusters of grains or isolated grains along chains of cells (Fig. 1C). Assuming that each cluster (or individual grain) separated from neighboring ones by 1 μm or more (resolving power of the autoradiographic technique used in our experiments) corresponds to one segregation unit of cell wall, we have calculated the average number of segregation units per cell at the beginning of chase. Data presented in Table 3 show that a cell wall in rich and minimal media consists of about 30 and 8 cell wall segregation units, respectively. Since no allowance for cell wall units not revealed by this technique (0 grain class) was made, the actual figures could even be slightly higher, although we believe that they do represent upper limits because of the very fine criteria of resolution used to define a segregation unit. To eliminate the possibility that these unexpectedly high figures were due to some kind of nonspecific fragmentation which might be expected to increase with time, we have checked and confirmed that the above-determined numbers of segregation units per cell remained unchanged even after eight generations of chase (data not presented).

(iii) Nature and size of segregation units. Inspection of autoradiographs of FJ3 cells continuously labeled and chased for about five generations in rich as well as in minimal medium revealed unusually dense grain clusters located exclusively at one or both ends of long chains of cells (Fig. 1C). In both media the number of these clusters equaled twice the number of cells at the beginning of the chase so that at first it could be assumed that they correspond to poles present at that time (22). However, theoretical estimates of polar areas (both cell poles) in corresponding media, calculated by the method of Burdett and Higgins (5), provide figures of 12% and 26% of the cell surface, whereas Table 4 shows that grains contained in these large

TABLE 1. Statistical analysis of grain distributions of continuously labeled and chased cultures of strain Ni15 in rich medium

Time of chasing in generations (min)	Class I (cell length <4.2 μm)		Class II (cell length ≥4.2 μm)		Mean no. of grains/half cell for entire population	Sp act[b] of [^{3}H]GlcNAc (Ci/mmol)	No. of cells examined
	Mean no. of grains/half cell	Probability[a] that distribution is Poisson (%)	Mean no. of grains/half cell	Probability[a] that distribution is Poisson (%)			
0 (0)	5.5	92	8.5	84	6.9	0.065	214
0 (0)	1.5	86	2.6	76	2.0	0.020	188
1.5 (30)	5.0	66	7.1	88	5.9	0.170	201
3 (60)	4.8	33	7.0	69	5.6	0.450	362
5 (100)	2.1	<0.05	3.2	<0.05	2.8[c]	1.375	515

[a] Obtained by χ^2 test.
[b] Specific activities were chosen so as to obtain comparable grain densities at each time of sampling except in one experiment (0 generations of chase, line 2) in which specific activity was low and grains were mostly separate.
[c] Total loss of radioactivity due to turnover is about 20% in 5 generations (20).

clusters represent at least 20% and 48% of the total number of grains incorporated into cell wall in rich and minimal media, respectively. (Our previously published figure of 13% with strain Ni15 in rich medium [22] resulted from the fact that grains due to background and to label incorporated into protein were not subtracted.) Therefore, it appears that these large segregation units represent some sort of "polar sheaths" (poles plus part of the cylindrical surface) which, in the case of mutants with reduced wall turnover, once synthesized, segregate without undergoing any subsequent fragmentation or mixing with new wall material. Such "polar sheaths," designated as "C's," have already been observed on fluorescent-antibody-labeled *B. cereus* (6). Allowing for different specific activities in rich and minimal media, we have calculated that, irrespective of medium richness, "polar sheaths" consist of fairly comparable amounts of cell wall material (Table 4). We believe that the smaller segregation units represent such structures not completed at the beginning of chase. Nevertheless, we would like to point out that in minimal medium over two-thirds of the total number of grains are found in large clusters located at ends as well as in the middle of chains (see Fig. 1C).

(iv) Segregation of teichoic acids labeled with [^{3}H]glycerol. Insertion of new cell wall material was also examined by continuous labeling and chase experiments, with [2-^{3}H]glycerol as radioactive marker for teichoic acids (24). Strain Ni15 growing exponentially at 44°C in rich medium, supplemented with 50 μg of glycerol per ml, was labeled for five generations with [^{3}H]glycerol of appropriate specific activity. After resuspension in medium containing unlabeled glycerol, samples were withdrawn at 0 and 100 min (5 generations) and processed for autoradiography. Analysis of grain distributions (Table 5) shows that in analogous growth conditions segregation patterns of [^{3}H]glycerol (representative of the polyglycerol phosphate teichoic acid) and [^{3}H]GlcNAc (mainly representative of peptidoglycan) were identical (Table 1). This is in perfect agreement with the well-established covalent linkage between these two wall polymers (23).

Segregation of pulse-labeled cell wall material. To determine the average number of wall segre-

TABLE 2. Statistical analysis of grain distributions of continuously labeled and chased cultures of strain FJ3 in minimal medium

Time of chasing in generations (min)	Class I (cell length <1.9 μm)		Class II (cell length ≥1.9 μm)		Mean no. of grains/cell for entire population	Sp act[b] of [^{3}H]GlcNAc (Ci/mmol)	No. of cells examined
	Mean no. of grains/cell	Probability[a] that distribution is Poisson (%)	Mean no. of grains/cell	Probability[a] that distribution is Poisson (%)			
0 (0)	2.9	84	4.6	89	4.0	0.026	270
2 (80)	4.4	20	5.2	25	4.8	0.100	249
3 (120)	3.5	<0.05	4.7	<0.05	4.2	0.204	335
5 (200)	0.8	<0.05	1.7	<0.05	1.3[c]	0.350	447

[a] Obtained by χ^2 test.
[b] Specific activities were chosen so as to obtain comparable grain densities at each time of sampling.
[c] Total loss of radioactivity due to turnover is about 20% after 5 generations.

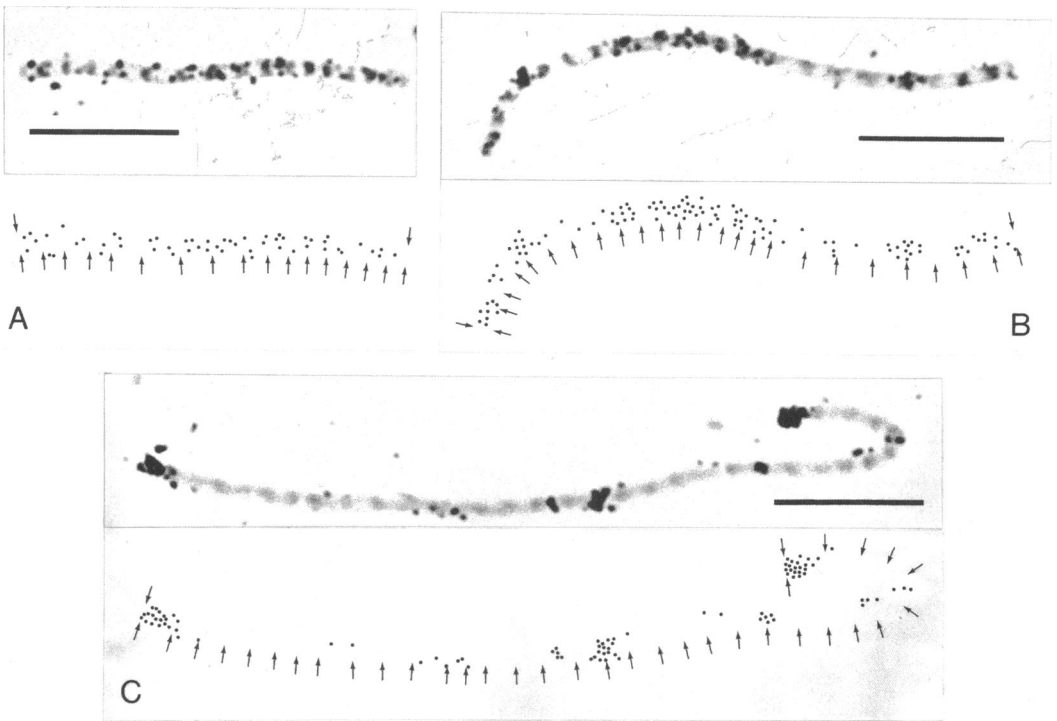

FIG. 1. Cells of *B. subtilis* FJ3 were grown at 44°C in minimal medium and labeled with [^{3}H]GlcNAc for 4 generations. After 0, 2, and 4.5 generations of chase in unlabeled medium, cells were harvested, treated with trypsin, and prepared for autoradiography. After development, septa (arrows) were stained with crystal violet and fuchsin. A, B, and C correspond to chase periods of 0, 2, and 4.5 generations, respectively. Bar = 10 μm.

gation units per cell undergoing synthesis at any moment of time, [^{3}H]GlcN of high specific activity (20 to 40 Ci/mmol) was incorporated during a 2-min pulse into exponentially growing *B. subtilis* FJ3 in rich, minimal, and intermediate media. After chase periods of up to five generations, samples were prepared for autoradiography and grain distributions were examined. First, grains due to background and due to label incorporated into protein were eliminated, and then segregation units were counted and their average number per cell at the time of exhaustion of the pool was calculated (see Materials and Methods). Since the distribution of grains in clusters (not cells or half cells) appeared to be a Poisson distribution, the 0 grain class (segregation units not visualized by grains) was calculated and added to each corresponding total number of units visualized by grains. By this method, we found that an average cell growing in rich and minimal media synthesized about 24 and 8 cell wall segregation units, respectively (Table 6). Comparison of these figures and those obtained with continuously labeled cells (Table 3), which were not corrected for the 0 grain class, shows that in rich medium pulse and chase experiments

TABLE 3. Number of wall segregation units per cell of *B. subtilis* FJ3 obtained from continuous labeling and chase experiments in rich and in minimal media[a]

Medium	Generation time (min)	Avg cell length (μm)	Avg no. of grains/ segregation unit	Mean no. of segregation units/cell[b]	No. of cells examined
Rich	20	4.0	3.0	29.6	931
Minimal	40	1.9	3.2	8.4	1718

[a] Determined after 5 and 4.5 generations of chase in rich and minimal media, respectively.
[b] After correction for grains due to background and labeled protein. Cells grown in both rich and minimal media were subjected to trypsin treatment.

TABLE 4. Analysis of large grain clusters in strain FJ3 after chasing continuously labeled cells in rich and minimal media[a]

Medium	No. of cells examined	Corresponding no. of cells at beginning of chase	No. of dense grain clusters observed	Theoretical % of cell surface corresponding to polar area (both poles)[b]	% of total no. of grains contained in end clusters	Avg no. of grains/cluster	Sp act of [^{3}H]GlcNAc (Ci/mmol)	Time of development of autoradiographs (h)	Normalized no. of grains/cluster[c]
Rich	1,107[d]	34	70	12	20	13.8	0.653	48	13.8
						17.5	0.653	70	12.0
Minimal	1,244[d]	57	121	26	48	12.8	0.387	100	10.4
						6.3	0.235	70	12.0

[a] Determined after 5 and 4.5 generations of chase in rich and minimal media, respectively.
[b] Determined as described in reference 5.
[c] For a specific activity of 0.653 Ci/mmol and a development time of 48 h.
[d] Data pooled from 2 separate experiments.

yielded consistently lower figures: 24 units as compared to 30 obtained with continuously labeled cells. This observation suggests that not all segregation units were labeled during a pulse and is compatible with our observation that "polar sheaths" (see above) once synthesized do not undergo any mixing with new cell wall material. The fact that the above considerations do not seem to apply to data obtained in minimal medium, where both types of experiments provided an almost identical figure (8 segregation units per cell), will be discussed later (see Discussion).

Kinetic studies of segregation as a function of time (data not presented), identical to those performed with continuously labeled cells (Tables 1 and 2), showed that grain distributions of pulse-incorporated material in rich and in minimal media had already become non-Poisson 3 and 1.5 to 2.5 generations, respectively, after the beginning of the chase. This relatively short segregation delay could be due to the following: firstly, continuously labeled cells segregate large labeled blocks which can easily give rise to grains in neighboring cells devoid of any label, and secondly, the number of cell wall segregation units labeled during a pulse in rich medium is smaller than that obtained by continuous labeling (see Discussion).

Segregation of pulse-labeled cell wall in *B. subtilis* 168 *lyt*$^+$ (normal cell wall turnover). The observation that segregation of pulse-incorporated label could be established after a relatively short delay (see above) prompted us to investigate the mode of cell wall synthesis in strains with wild-type turnover. In such strains turnover of pulse-incorporated material starts about a generation after the beginning of chase and then proceeds at a rate of about 25% of pulse-incorporated label per generation (21). A pulse and chase experiment using [^{3}H]GlcN was performed on strain 168 (*lyt*$^+$) growing in the medium of intermediate richness. Data presented in Table 7 show that after 45 min (1.7 generations) of chase the distribution of grains incorporated during a 2-min pulse was non-Poisson, demonstrating that the wall of cells with wild-type turnover, like that of *lyt* mutants, consists of a limited number of segregation units. Likewise, pulse and chase experiments performed with strain Ni15 in minimal medium, in which it exhibits normal turnover (J.-M. Schlaeppi and H. M. Pooley, unpublished data), gave, after 1.8 generations of chase, non-Poisson grain distributions compatible with zonal insertion.

DISCUSSION

Experiments reported above demonstrate that the cell wall of *B. subtilis* consists of a limited number of fairly large blocks which, once com-

TABLE 5. Statistical analysis of grain distributions of [2-^{3}H]glycerol continuously labeled and chased cultures of strain Ni15 in rich medium

Time of chasing in generations (min)	Class I (cell length <4.2 μm)		Class II (cell length ≥4.2 μm)		Mean no. of grains/half cell for entire population	Sp act of [2-^{3}H]glycerol (Ci/mmol)	No. of cells examined
	Mean no. of grains/half cell	Probability[a] that distribution is Poisson (%)	Mean no. of grains/half cell	Probability[a] that distribution is Poisson (%)			
0 (0)	3.3	95	5.7	88	4.5	0.015	106
5 (100)	1.0	<0.05	1.6	<0.05	1.3	0.178	200

[a] Obtained by χ^2 test.

pleted, segregate during subsequent growth without undergoing any mixing with newly synthesized blocks; these results are incompatible with models according to which the synthesis of the cylindrical part of the cell wall of rod-shaped gram-positive bacteria takes place by diffuse intercalation (2, 4, 8, 9, 15). It appears that the number of wall segregation units of a cell in an exponential growth phase depends upon medium richness; continuous labeling and chase experiments (Table 3) provide a figure of 30 segregation units per cell in rich medium (doubling time of 20 min at 44°C) and 8 segregation units per cell in minimal medium (doubling time of 40 min). This surprisingly high number of observed segregation units per cell and the limited resolution of light microscope autoradiography largely account for the relatively long delays (Tables 1 and 2) required for statistical (as well as visual) identification of wall segregation units and thus the recognition of the discrete nature of cell wall synthesis. Indeed, if we consider that a segregation unit has an average extent of 1 μm and that two units are counted as separate only when distant from each other by 1 μm (assumed to be the resolution limit of the autoradiographic method), we calculate that segregation of about 30 units, present in every cell at the beginning of a chase, would require over four generations, which is in excellent agreement with our data (Table 1). Likewise, in minimal medium, we deduce that three generations of chase are required to observe segregation of 8 units. These relatively long delays could easily explain the failure to obtain evidence of the discrete nature of cell wall insertion by authors who used relatively short chase periods (9, 15).

Although our data strongly imply the existence of discrete insertion zones of new cell wall material, they provide no direct information regarding the number of wall segregation units generated by each zone or the localization and the size of such zones. The latter question can be approached by electron microscope autoradiography (8). Such experiments performed on *B. megaterium* showed that about 40% of labeled precursors were incorporated in a narrow central zone of the cell. The remainder were shown by statistical analysis to be uniformly distributed over the cell surface. We believe that a direct comparison of these experiments with present results is difficult because the cell populations used by De Chastellier et al. (8) may have been no longer in exponential growth phase: reported cell densities would have corresponded to early stationary phase (A. Demierre, personal communication) and cells were short in comparison with another report (15). Nevertheless, if we assume, following the authors (8), that cells were in log phase at the time of pulse, attribution of that heavily labeled central zone to cross wall synthesis exclusively (8) would imply that the latter (equivalent to two nascent poles) accounts for 40% of the total cell wall. This appears incompatible with cell geometry; average cell dimensions of *B. megaterium* and Table 4 provide much lower figures. Therefore, we believe that grains in the central area cannot all be

TABLE 6. Number of wall segregation units of *B. subtilis* FJ3 labeled by a 2-min pulse of [^{3}H]GlcN in rich, intermediate, and minimal media[a]

Medium	Generation time (min)	Avg cell length (μm)	Avg no. of grains/segregation unit	Mean no. of segregation units/cell		No. of cells examined
				After correcting for grains due to background and labeled protein	Corrected for 0 grain class	
Rich	20	4.0	2.0	19.2	24.7	943
Intermediate	30	3.1	2.4	11.3	12.7	251
Minimal	40	1.9	1.9	6.2	8.2	358

[a] Determined after five generations of chase.

TABLE 7. Statistical analysis of grain distribution in 2-min pulse-labeled cultures of *B. subtilis* 168 (*lyt*$^+$) in the medium of intermediate richness

Time of chasing in generations (min)	Class I (cell length <3.4 μm)		Class II (cell length ≥3.4 μm)		Mean no. of grains/half cell for entire population	No. of cells examined
	Mean no. of grains/half cell	Probability[a] that distribution is Poisson (%)	Mean no. of grains/half cell	Probability[a] that distribution is Poisson (%)		
0 (0)	8.3	78	13.8	32	10.9	105
1.7 (45)	2.4	<0.05	4.1	<0.05	3.2	276

[a] Obtained by χ^2 test.

accounted for by cross wall synthesis but would argue in favor of a localized synthesis of the cylindrical part of the cell wall. The proportion of grains outside the central zone, which remains relatively high (about 60%), could be explained partly by nascent secondary zones and partly by some kind of "repair type" of wall synthesis destined to replace wall removed by turnover, which is particularly high in this strain of *B. megaterium* (11). Indeed, in cells with wild-type or high turnover, any segregation block of cell wall (even a complete one) undergoes turnover and must continuously be repaired by incorporation of new material, possibly by synthesis all over the surface (16), since electron micrographs of thin sections reveal a uniform thickness of the cylindrical part of the cell walls of bacilli.

If, according to classical models, we assume that each functional insertion zone is involved in the synthesis of 2 segregation units, we calculate from pulse and chase experiments (Table 6) that the number of insertion zones per cell is equal to 12 and 4 in rich and minimal media, respectively. This provides a ratio of 3 (12/4). We would like to point out that in minimal medium the number of segregation units obtained from pulse and chase experiments was equal to that obtained from continuous labeling and chase experiments (Tables 3 and 6). Since in the latter case all segregation units were labeled (not only those synthesized at the time of the pulse), we believe that even a limited repair-type synthesis in pulse and chase experiments might have led to labeling of "polar sheaths" already completed and thus to an overestimate of the number of insertion zones. Indeed, comparison of the numbers of insertion zones per cell in rich and minimal media obtained by continuous labeling and chase experiments provides a ratio approaching 4 (Table 4). Since the average cell length in rich medium is 4.0 μm and in minimal medium is 1.9 μm, it follows from the latter figure that the number of zones per unit of cell length in rich medium is about two times that in minimal medium. The growth rates in rich and minimal medium being in a 2:1 ratio (doubling times of 20 and 40 min), the rate of cell wall "elongation" could well be regulated by the density of insertion zones, all functioning at the same speed for the growth rates used in our experiments. Results obtained for cells growing in a medium of intermediate richness (Table 6) were in good agreement with this interpretation. This mode of regulation of cell wall synthesis resembles strongly that of DNA replication (26). This analogy does not seem to be a mere coincidence, as evidenced by experiments on cosegregation of DNA and cell wall (J.-M. Schlaeppi and D. Karamata, in preparation), and suggests that a close association between cell wall and DNA could be responsible for regular segregation of the latter.

Using [^{3}H]glycerol-labeled teichoic acids as cell wall marker, we have established the existence of segregation blocks identical to those observed with [^{3}H]GlcNAc-labeled wall. In previously reported experiments (2, 4) which were interpreted in favor of diffuse intercalation of new wall material, teichoic acid synthesis was monitored in a chemostat by altering the phosphate concentration of the medium, and teichoic acid-containing wall was visualized by its capacity to bind bacteriophage. However, both continuously labeled and pulse-labeled cultures, the latter lasting 0.3 generation (1), were analyzed after about 2 generations of chase which, according to our data, should not be sufficient for segregation to have taken place. Nevertheless, several published micrographs reveal phage distributions which could only be due to localized insertion. In particular, "polar sheaths," sometimes up to one-half cell long, are clearly visible and are in perfect agreement with the existence of large segregation blocks demonstrated in our experiments. It is likely that segregation delays would be shorter because the electron microscopic observation of phage-coated wall offers a resolution superior to that of light microscope autoradiography.

The existence of large cell wall segregation blocks in gram-positive rod-shaped bacteria demonstrated in the present study has been shown previously by labeling the cell envelope

with fluorescent antibodies and examining their distribution after appropriate chase periods in media devoid of antibody. Although their experiments were not quantitative, Hughes and Stokes (13), using *B. licheniformis lyt* cells, observed clearly several "wall insertion zones" per cell, and Chung et al. (6), using *B. megaterium* and *B. cereus* in late log phase, observed few zones in which new unlabeled wall appeared at the outer cell surface. It should be stressed that in these experiments, where only the outer layer of the cell envelope was labeled by antibody, segregation of old wall blocks appeared after a relatively short delay. Comparison of these results with the present study, in which all segregation units were labeled and segregation delays were long, makes imperative the distinction between the "outside" and the "inside" of bacterial cell walls, as already suggested by Pooley (21) and Archibald (3). Thus, the possible existence of distinct cell wall layers and their reorganization through movement of one relative to another, with the outer surface as final destination, must be considered in models aiming to describe cell wall synthesis.

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