JOURNAL OF BACTERIOLOGY, Oct. 1985, p. 130–135
0021-9193/85/100130-06$02.00/0

Vol. 164, No. 1

Cell Wall and DNA Cosegregation in *Bacillus subtilis* Studied by Electron Microscope Autoradiography

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Received 9 April 1985/Accepted 18 July 1985

Cells of a *Bacillus subtilis* mutant deficient in both major autolytic enzyme activities were continuously labeled in either cell wall or DNA or both cell wall and DNA. After appropriate periods of chase in minimal as well as in rich medium, thin sections of cells were autoradiographed and examined by electron microscopy. The resolution of the method was adequate to distinguish labeled DNA units from cell wall units. The latter, which could be easily identified, were shown to segregate symmetrically, suggesting a zonal mode of new wall insertion. DNA units could also be clearly recognized despite a limited fragmentation; they segregated asymmetrically with respect to the nearest septum. Analysis of cells simultaneously labeled in cell wall and DNA provided clear visual evidence of their regular but asymmetrical cosegregation, confirming a previous report obtained by light microscope autoradiography (J.-M. Schlaeppi and D. Karamata, J. Bacteriol. 152:1231–1240, 1982). In addition to labeled wall units, electron microscopy of thin sections of aligned cells has revealed fibrillar networks of wall material which are frequently associated with the cell surface. Most likely, these structures correspond to wall sloughed off by the turnover mechanism but not yet degraded to filterable or acid-soluble components.

Identification of cell wall and DNA segregation units (18, 25, 26), i.e., polar sheaths and DNA strands, in exponentially growing cells of *Bacillus subtilis* and the analysis of their segregation pattern have revealed an asymmetrical 100% cosegregation of DNA with cell wall units (25). The latter were in a twofold excess over DNA units, so that of two wall units of the same age, only one seemed to be associated with a DNA strand (25). These experiments extended previous electron microscopic observations of an association between DNA and the cell envelope (14, 15) and are in full agreement with more recent biochemical and genetic studies which revealed specific DNA-envelope complexes both in gram-positive (6, 7, 29) and gram-negative (12) bacteria. However, in the DNA-cell wall cosegregation studies mentioned above (25), both cell wall and DNA segregation units were always tritium labeled and identified by light microscope autoradiography. Thus, no distinction could be made between grains originating from one or the other of the components, and the conclusions stand upon analyses of grain distributions along chains of cells, each derived from a single cell at the beginning of chase.

In the present contribution, we have examined the same problem by taking advantage of high-resolution electron microscope autoradiography. Analysis of grain distributions on thin sections allowed a good visualization and distinction between labeled cell wall and nucleoplasm. Analysis of the segregation of labeled units during prolonged periods of chase provided clear visual evidence of the cosegregation pattern of cell wall and DNA, confirming a previous report (25).

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MATERIALS AND METHODS

Strains, media, and labeling conditions. All experiments were performed with strain FJ3, a *metC3 lyt-1* mutant of *B. subtilis* deficient in autolytic enzyme activities (9) which forms long chains of cells that exhibit a considerably reduced cell wall turnover (26). Cells were grown in two media, a rich Casamino Acids-glucose medium and a minimal salts-glucose medium described previously (25, 26). Cultures were grown at 44°C with aeration and maintained in the exponential growth phase by appropriate dilution into fresh medium (25).

Following previous practice (25), each culture was divided into three equal 2-ml volumes containing, respectively, the following: (i) *N*-acetyl-D-[1-^{3}H]glucosamine ([^{3}H]GlcNAc) to label the cell wall, (ii) [*methyl*-^{3}H]thymidine ([^{3}H]dThd) and 2′-deoxyadenosine (dAdo) to label DNA, and (iii) [^{3}H]GlcNAc, [^{3}H]dThd, and dAdo to label both the cell wall and DNA.

In rich medium, the labeling conditions were as follows: (i) 0.24 μmol of [^{3}H]GlcNAc (specific activity, 0.64 Ci/mmol); (ii) 3.4 nmol of [^{3}H]dThd (specific activity, 58 Ci/mmol) with 3 μmol of dAdo; (iii) 0.24 μmol of [^{3}H]GlcNAc (specific activity, 0.64 Ci/mmol) and 3.4 nmol of [^{3}H]dThd (specific activity, 58 Ci/mmol) with 3 μmol of dAdo. In minimal medium, the conditions were as follows: (i) 0.48 μmol of [^{3}H]GlcNAc (specific activity, 0.41 Ci/mmol); (ii) 2.6 nmol of [^{3}H]dThd (specific activity, 58 Ci/mmol) with 3 μmol of dAdo; (iii) 0.48 μmol of [^{3}H]GlcNAc (specific activity, 0.41 Ci/mmol) and 2.6 nmol of [^{3}H]dThd (specific activity, 58 Ci/mmol) with 3 μmol of dAdo. The presence of dAdo in minimal medium had an adverse effect on cell growth; generation times, normally 40 min, increased to 47 min (see below). No such effect was observed in rich medium (25). At the end of the labeling period (four to five generations), cultures were harvested by filtration, washed, and appropriately diluted in prewarmed, unlabeled medium as previously described (25). After 3.8 and 5 generations of chase in

minimal and rich media, respectively, cells were prefixed by the addition of OsO_4 (final concentration, 0.1%) directly to the medium and processed for electron microscope autoradiography.

Electron microscope autoradiography. (i) Fixation, alignment of cells, embedding, and thin sections. Prefixed cells were washed once with 1% tryptone and fixed overnight in 1% OsO_4 (21). To align chains of cells in the plane of thin sections, samples were prepared as described by Whitehouse et al. (28) with the following modifications. First, cells were fixed and washed before the agar steps (4); second, they were suspended in 0.5 ml of 1.8% Noble agar at 55°C before being processed for alignment. After 2 h of soaking in uranyl acetate (0.5%), samples were dehydrated in acetone, washed in propylene oxide, and embedded in Epon 812 (Fluka AG) by a modification of the original method described by Luft (16). The embedding material was as follows: 13.3 g of Epon 812, 3.76 g of dodecenyl succinic anhydride, 8.98 g of methyl nadic anhydride, and 0.35 ml of 2,4,6-tri(dimethylaminomethyl)phenol (J. Fakan, personal communication). After polymerization, blocks were cut with a diamond knife on a Reichert-Jung ultramicrotome. Thin sections with pale gold interference colors were picked up on collodion-coated glass slides (11).

(ii) Autoradiography. With a "dipping machine," glass slides were dipped at constant speed in the photographic emulsion (1 part of Ilford L-4 mixed with 4 parts of degassed distilled water). Slides were exposed in a dry atmosphere at 4°C for 14 to 17 days, developed with the Kodak D-19 developer for 2 min at 20°C, fixed, washed, and dried. Collodion films were partially detached by floating on distilled water so that a collodion-covered grid could be introduced under the thin sections. The latter were subsequently stained with uranyl acetate (4%) and lead citrate (1%) (19).

(iii) Analysis of the autoradiograms. Thin sections were examined with a Philips 201 C electron microscope at 100 kV. Micrographs were enlarged ×5,000 to ×30,000 for the analysis of silver grain distributions. Only sections parallel to the major cell axis were taken into account. As described by Salpeter et al. (23), the midpoint of a developed grain was assigned to the center of the smallest circle which fully circumscribed the grain. However, in some cases of fully labeled complete cell wall and DNA segregation units, in which grains were too close to each other to be visualized separately, their number was assessed by drawing circles whose diameters corresponded to the average diameter determined on clearly distinguishable individual grains.

The emulsion background was found to represent, on the average, one grain for 40 μm^2 or 1 grain for 35 cells in minimal medium and 1 grain for 15 cells in rich medium. Therefore, one grain located inside the cytoplasm of each 35th cell in minimal medium or 15th in rich medium of a randomly numbered series of thin sections was excluded from the analysis.

As previously described (26), a significant proportion of the [^{3}H]GlcNAc can be solubilized by trypsin treatment of trichloroacetic acid-permeabilized cells. It was estimated that 10 to 15% and 20 to 25% of the label was incorporated into the protein fraction of cells grown in rich and minimal media, respectively. However, the trypsin treatment performed at the end of the chase period for light microscope autoradiography (25, 26) could not be used for electron microscope autoradiography, since it grossly affected cell morphology. Therefore, when specified, grains due to labeled protein were substracted, following previous practice (26). In addition, we would like to point out that earlier analysis focused on fully labeled cell wall units which were not fragmented and dispersed among cells synthesized during the chase period (26). Since during that period, labeled proteins were fairly uniformly distributed among the cells, we calculated from previous data (26) that, in cells containing fully labeled cell wall polar sheaths, the background due to labeled protein represents only about 7 and 4% of the grains in minimal and rich media, respectively.

TABLE 1. Grain distribution in nucleoplasm, cell wall, and outside areas of thin sections of cells continuously labeled with [^{3}H]GlcNAc or [^{3}H]dThd and chased in rich or minimal medium[a]

Label	Medium	No. of grains (%) in the following area		
		Nucleoplasm	Cell wall	Outside
[^{3}H]GlcNAc	Minimal	61 (16.6)	220 (59.9)	86 (23.4)
	Rich	68 (16.7)	228 (55.9)	112 (27.5)
[^{3}H]dThd	Minimal	135 (51.9)	99 (38.0)	26 (10.0)
	Rich	146 (52.0)	110 (39.1)	25 (8.9)

[a] Thin sections of cells were divided into nucleoplasm, cell wall, and outside areas. The median line of the cell wall (longitudinal axis of symmetry) was referred to as the zero axis in all measurements. The nucleoplasm area corresponds to the nucleoplasm, as revealed by the Ryter-Kellenberger staining technique (21), and the cytoplasm with the exclusion of a 150-nm wide band adjacent to the zero axis. The cell wall area includes the cell wall and its inside and outside surroundings within a distance of 150 nm from the zero axis. The outside area corresponds to the outside region situated between 150 and 800 nm from the zero axis. Grains due to the emulsion background were substracted as described in Materials and Methods.

RESULTS

Resolution and quantitative aspects of the method. Following previous practice (5, 23), the resolution of electron microscope autoradiography was determined from cell walls continuously labeled with [^{3}H]GlcNAc. Since our analysis was performed on sections of whole cells, the latter were labeled in rich medium to minimize incorporation of the label into protein, which was about 10 to 15% in previously described growth conditions (26). The distribution of grains on both sides of the cylindrical part of the wall (poles and their immediate neighborhood were not taken into account) was determined in an area surrounding the cell and extending up to 800 nm. The area was divided in 50-nm steps. Of 456 grains examined, 50% were located within 142 nm of the median line of the cell wall. Accordingly, in our experiments, we considered that all grains located within 150 nm of a labeled structure originated from the latter, i.e., that the resolution was about 150 nm. Although in our experiments labeled wall was not a true "line source" as defined by Salpeter and McHenry (24), our value of 142 nm was close to their reported value of 145 nm (23, 24).

Since the main purpose of the experiments described below was to distinguish grains due to [^{3}H]GlcNAc-labeled cell walls from those due to [^{3}H]dThd-labeled DNA, we have defined three areas—"nucleoplasm area," "cell wall area," and "outside area" (grains falling outside the cell)—and determined the grain distribution among them in cells labeled in either cell wall or DNA (Table 1). It appeared that only about 17% of the grains originating from the cell wall were located in the nucleoplasm area, whereas about 48% of the grains originating from labeled DNA were located within the cell wall area or even outside the cell. The latter result is in good agreement with a previous report (22). Although these figures allow a distinction between grains due to DNA or wall, visual inspection of autoradiographs reveals an appreciably more favorable situation. As it happens, after four to

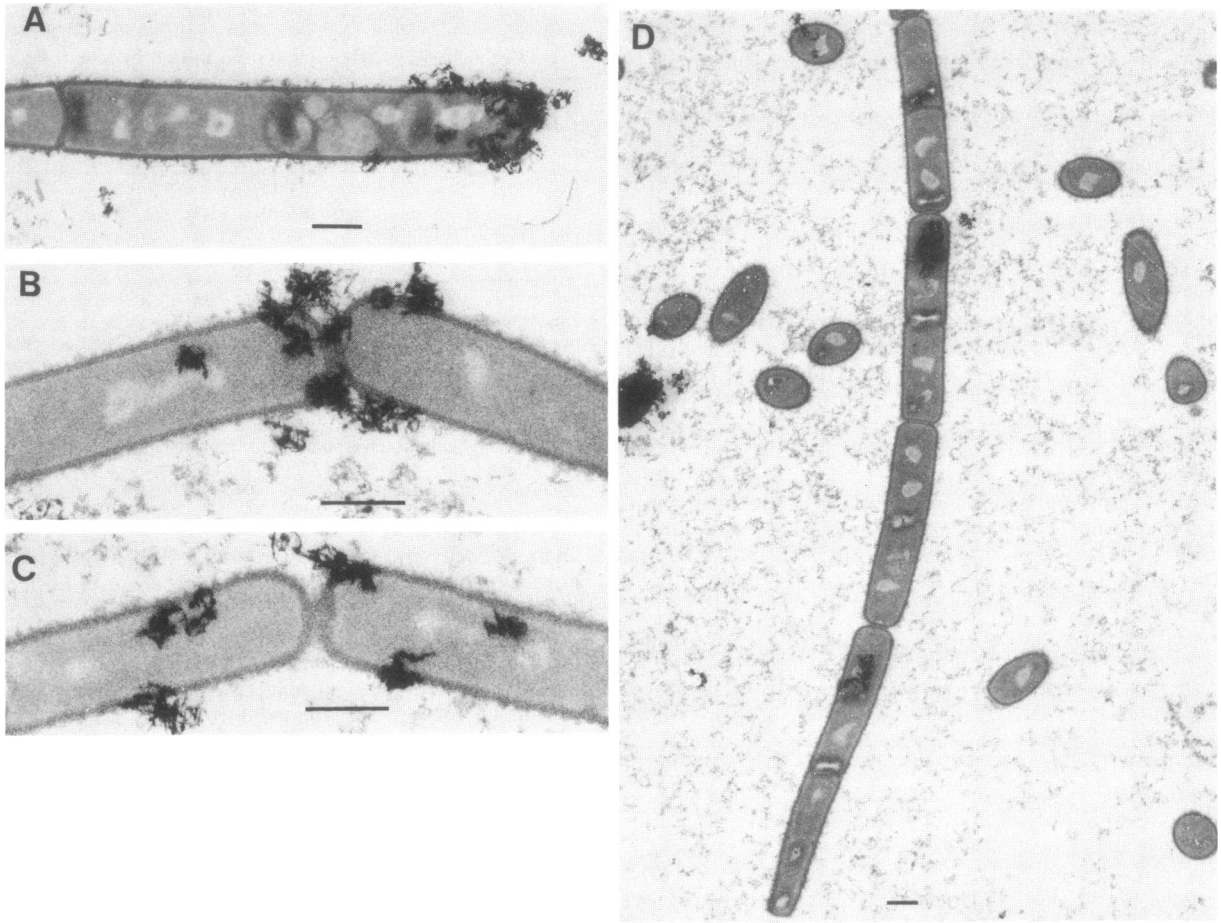

FIG. 1. Cells of *B. subtilis* FJ3 *lyt-1 metC3* growing exponentially at 44°C were labeled during four or five generations with either [^{3}H]GlcNAc (A to C) or [^{3}H]dThd (D) of appropriate specific activity. After a period of chase of 5 generations in rich medium (A) or 3.8 generations in minimal medium (B to D), cells were fixed with OsO_4, aligned, embedded, and processed for thin section autoradiography. Bar = 0.5 μm.

five generations of chase, labeled cell wall units are often longitudinally separated from the corresponding nucleus (Fig. 1D), and an appreciable excess of grains originating from the cell wall are situated outside the cell (see below).

Segregation of cell wall units. Inspection of thin sections of *B. subtilis* FJ3 labeled during 4 to 5 generations with [^{3}H]GlcNAc and chased during 3.8 and 5 generations in minimal and rich media, respectively, revealed mainly large clusters of grains located at ends of aligned chains of cells as well as inside such chains. Grains in clusters located at chain ends were confined to cell poles and to a portion of cylindrical wall in the immediate vicinity of the pole (Fig. 1A). Grain clusters inside chains (Fig. 1B) were distributed symmetrically with respect to a labeled septum (data not shown). These observations, obtained in both rich and minimal media, suggest that such large clusters correspond to cell wall segregation units fully synthesized at the beginning of chase (26). During the chase period, chain separation takes place generally at the oldest poles to yield chains with clusters at their ends. However, at the end of the chase period, a proportion of labeled units remains unseparated and gives rise to symmetrical interchain clusters. Thus, segregation of cell wall subunits proceeded according to a symmetrical pattern in full agreement with figures obtained by light microscope autoradiography (25, 26).

In addition to large clusters, autoradiographs revealed isolated grains or small clusters which were located mainly on the cylindrical portion of the wall and not on the septum. Despite their low numbers, such grains presented most often an apparently symmetrical distribution with respect to the neighboring septum (Fig. 1C). Therefore, as previously suggested (25), they might be related to the cell wall units partially synthesized at the beginning of chase.

Inspection of thin sections, described above, revealed a considerable amount of electron-dense material scattered in large areas surrounding the cells (Fig. 1 and 2). Since this material is frequently associated with autoradiographic grains in [^{3}H]GlcNAc-labeled cells (grain density is about six times higher than background) but not in those labeled with [^{3}H]dThd, we believe that it represents cell wall debris in the process of being sloughed off. It could be released in large lumps during growth or possibly during processing for electron microscopy.

Segregation of DNA units. Exponentially growing cells of *B. subtilis* FJ3 were labeled with [^{3}H]dThd during five generations and, after an appropriate chase period, processed for autoradiography. To assess the symmetry of the segregation of labeled DNA with respect to the nearest neighboring septum, we selected only clusters of grains situated inside chains of aligned cells. After 3.8 generations

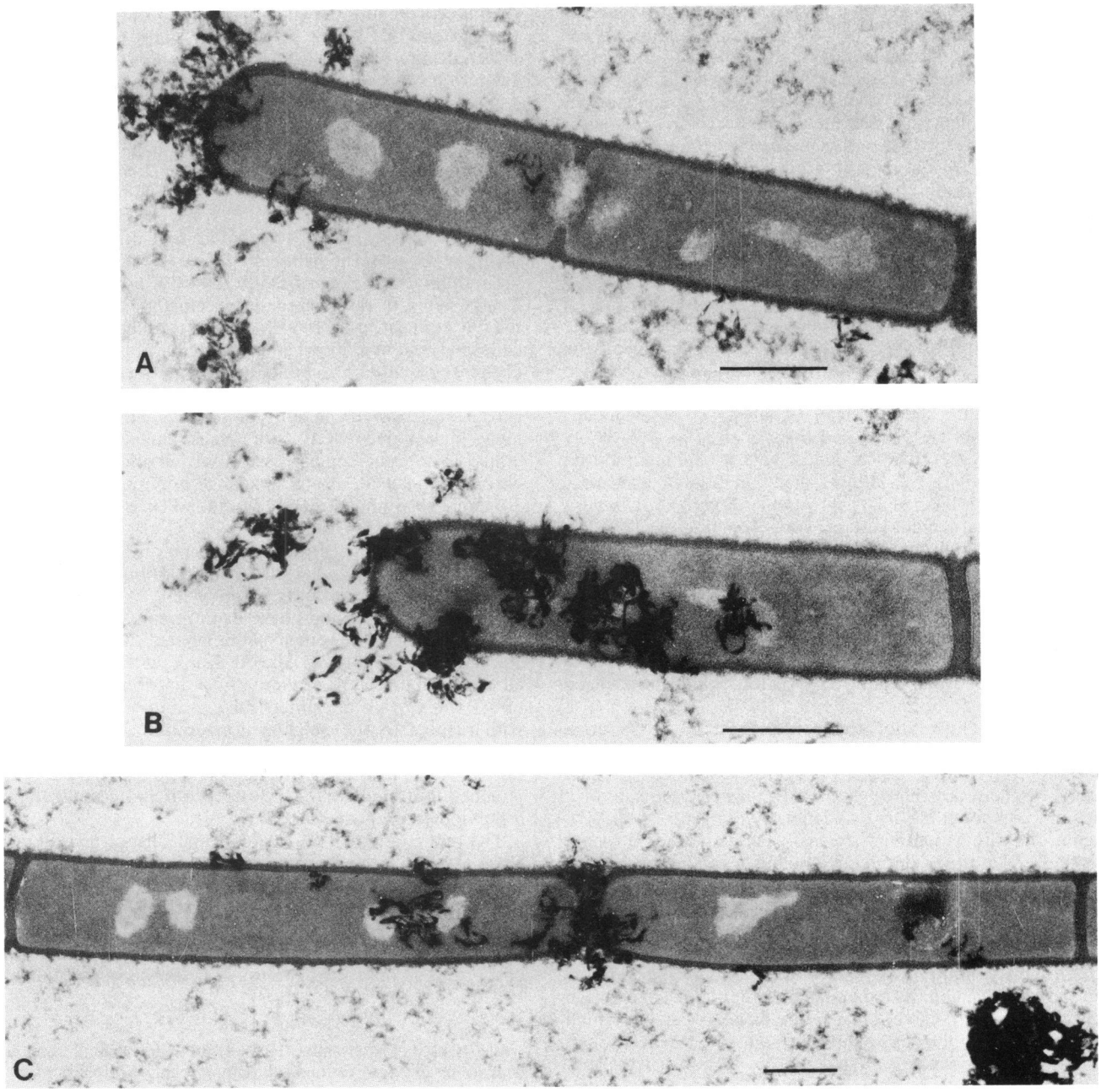

FIG. 2. Cells of *B. subtilis* FJ3 were labeled simultaneously with [^{3}H]GlcNAc and [^{3}H]dThd during 4 to 5 generations and chased during 3.8 generations in minimal medium (A and B) or 5 generations in rich medium (C). Bar = 0.5 μm.

of chase in minimal medium, 184 of 193 figures examined were completely asymmetrical, i.e., grains were located in the nucleoplasm area on only one side of the septum (Fig. 1D). In the remaining nine cases, grains were found in nucleoplasm areas of contiguous cells, but in unequal amounts (ratios of nearly 1:10). Such figures are most likely due to DNA sister strand exchange (8), since in our experimental conditions DNA undergoes fragmentation of about 5% per generation in minimal medium (26). A comparable situation was observed in rich medium. After five generations of chase, 169 of 189 analyzed figures were completely assymmetrical, whereas in the remaining ones, grain distributions with respect to the neighboring septum were similar to those recorded for the minimal medium (see above).

Incidentally, in minimal medium, 7 of 200 chains presented large clusters of apparently equal size on both sides of the septum. We believe that these figures do not reveal a randomness in the DNA-cell wall association but correspond to cells which have died during the experiment due to the toxicity of dAdo (see Materials and Methods). Moreover, such figures were never found in experiments performed in rich medium, where dAdo does not affect the growth rate (25). In conclusion, high-resolution autoradiography confirms the asymmetrical segregation of DNA units (25).

Analysis of doubly labeled cell wall and DNA cosegregation. From our preliminary considerations (see above and Table 1), it follows that the resolution of electron microscope autoradiography is sufficient to recognize whether or not

labeled cell wall segregation units are associated with labeled DNA segregation units. Thus, to examine the nature of cell wall and DNA cosegregation, we grew strain FJ3 during four to five generations in both minimal and rich media containing [^{3}H]GlcNAc as well as [^{3}H]dTdh. After 3.8 and 5 generations of chase in minimal and rich media, respectively, cells were processed for autoradiography. Again, as with cells labeled with [^{3}H]GlcNAc only, large grain clusters located at the ends or inside long chains of cells were easily recognized (Fig. 2A to C). Quantitative distinction between cell poles "filled" with labeled DNA or devoid of it was made as follows. When, under identical conditions, cell wall only was labeled, we determined that large segregation units in minimal and rich media consisted, on the average, of 10.8 and 9.4 grains, respectively. Of these grains, 17% were located in the nucleoplasm area (Table 1). Therefore, depending on the size of the cluster (<10 or >10), we assigned one or two grains, respectively, located in the nucleoplasm area to label incorporated into the cell wall. Analysis of labeled cells situated at chain ends revealed that 43 of 84 and 65 of 127 wall units were devoid of labeled DNA in minimal and rich media, respectively. It follows that the ratio of wall units associated with labeled DNA to those devoid of it is very close to 1, in agreement with previous observations (25). We would like to point out that the vast majority of figures were clear-cut, i.e., that the nucleoplasm area contained either 0 to 1 grain or more than 4 grains. The small but repeatedly observed excess of empty poles labeled only in the cell wall is likely to be due to septa which were synthesized at the beginning of the labeling period, i.e., after completion of the replication of corresponding DNA units which took place before isotope addition.

Analysis of clusters located inside chains of cells offers a confirmation and a striking illustration of the DNA segregation pattern. Indeed, almost all figures examined revealed grains originating from labeled DNA on only one side of a cluster corresponding to two labeled wall units (Fig. 2C). Again, the rare exceptions are easily accounted for by septa synthesized at the beginning of labeling or by the presence in minimal medium of dead cells (see above).

DISCUSSION

High-resolution autoradiography of thin sections of *B. subtilis* cells continuously labeled in their cell walls and then chased has revealed the existence of rather large segregation units which, we believe, correspond to polar caps to which part of the neighboring cylindrical wall is attached. Such structures were recently visualized on sections of thermosensitive *dna* mutants of *B. subtilis* (27). In addition, quantitative analysis (data not shown) showed that grains located along the cyclindrical part cannot be attributed uniquely to labeled adjacent poles as defined by Burdett and Higgins (3) (Fig. 1a and b). Due to low grain numbers, it was not possible to examine cell wall units uncompleted at the beginning of chase (26). However, a completely symmetrical grain distribution with respect to the neighboring septum of both large and small clusters argues strongly in favor of a symmetrical zonal mode of wall insertion as previously suggested (25).

Inspection of thin sections of cells labeled with [^{3}H]GlcNAc and chased for long periods (five generations) revealed that more than two-thirds of the grains were located on the outside of the cell wall and frequently associated with an electron-dense material. These observations are in agreement with those suggesting that wall material incorporated near the cytoplasmic membrane migrates subsequently to the outer wall surface which it occupies one to two generations later (1, 17, 26). In addition, they reveal that wall material may be released in large "lumps." These results seem to be characteristic of lytic, deficient strains (2, 20) as well as those affected in DNA synthesis (27) or cell division (4); they have never been observed on *B. subtilis* cells with wild-type levels of autolysin (2). Nevertheless, this phenomenon may occur in apparently wild-type *B. megaterium* (10). Therefore, we believe that the observed lumps might correspond to wall turnover products which, due to deficient autolysin activity, are not degraded in acid-soluble material and to a large extent remain attached to the cell.

Continuous labeling of DNA followed by a chase period also resulted in rather large grain clusters covering mainly the nucleoplasm area. In 95 to 89% of thin sections of aligned cells analyzed in minimal and rich media, respectively, these clusters presented a completely asymmetrical figure, i.e., all of the grains were located on one side of the septum only (Fig. 1D). However, in addition to large clusters of surprisingly high homogeneity, we occasionally found isolated grains in the nucleoplasm area. We attribute the latter to chromosome sister strand exchange previously reported (8, 30) and already quantified (25) in conditions used in experiments described above.

High-resolution electron microscope autoradiography allowed a satisfactory analysis of the distribution of distinct, complete segregation units of both cell wall and DNA. In continuous labeling and chase experiments, in which either or both of these components were labeled, we observed (i) a 100% cosegregation of labeled DNA with corresponding labeled wall units, (ii) a symmetrical distribution of cell wall units, and (iii) an asymmetrical distribution of DNA units with respect to the neighboring septum. The regular but asymmetrical segregation of DNA molecules, revealing that of two wall units of the same age only one has a DNA strand attached to it, constituted a direct proof and visualization of a previously proposed model (25).

The replicon model postulates zonal surface insertion and DNA attachment to the latter to ensure orderly chromosome segregation (13). Conversely, our direct evidence of DNA and cell wall cosegregation suggests a zonal type of envelope material insertion and strongly infers a multipoint association between DNA and the cell envelope which could be part of a primitive mitotic apparatus in certain procaryotic cells.

ACKNOWLEDGMENTS

We thank J. Fakan and J.-F. Viret for many helpful comments concerning the thin sections and autoradiography preparation.

This work was supported by Fonds National Suisse de la Recherche Scientifique (grants 3.417-0.74 and 3.342-0.78).

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