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Murein Segregation in *Escherichia coli*†

MIGUEL A. DE PEDRO,[1]* JOSÉ CARLOS QUINTELA,[1] JOACHIM-VOLKER HÖLTJE,[2] AND HEINZ SCHWARZ[2]

Centro de Biología Molecular "Severo Ochoa" Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Facultad de Ciencias, 28049 Madrid, Spain,[1] and Max-Planck-Institut für Entwicklungsbiologie, 72076 Tübingen, Germany[2]

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Peptidoglycan (murein) segregation has been studied by means of a new labeling method. The method relies on the ability of *Escherichia coli* cells to incorporate D-Cys into macromolecular murein. The incorporation depends on a periplasmic amino acid exchange reaction. At low concentrations, D-Cys is innocuous to the cell. The distribution of modified murein in purified sacculi can be traced and visualized by immunodetection of the -SH groups by fluorescence and electron microscopy techniques. Analysis of murein segregation in wild-type and cell division mutant strains revealed that murein in polar caps is metabolically inert and is segregated in a conservative fashion. Elongation of the sacculus apparently occurs by diffuse insertion of precursors over the cylindrical part of the cell surface. At the initiation of cell division, there is a FtsZ-dependent localized activation of murein synthesis at the potential division sites. Penicillin-binding protein 3 and the products of the division genes *ftsA* and *ftsQ* are dispensable for the activation of division sites. As a consequence, under restrictive conditions *ftsA*, *ftsI*, or *ftsQ* mutants generate filamentous sacculi with rings of all-new murein at the positions where septa would otherwise develop.

The peptidoglycan (murein) layer of the bacterial cell envelope forms a giant, bag-shaped macromolecule, the sacculus, surrounding the cell. The sacculus is a covalently closed structure in which long polysaccharide chains are cross-linked by peptide bridges in a netlike fashion. The sacculus is the principal stress-bearing and shape-maintaining element of the cell wall, and it plays an essential role in bacterial morphogenesis. Metabolism of the sacculus is a complex process not only because of the large number of enzymes and metabolites involved but mostly because of regulatory requirements and topological constraints (1, 24, 29, 37, 44).

Growth of *Escherichia coli* occurs by the periodic succession of elongation and division events (1, 11). Cells divide at their midpoint once the chromosome is replicated and the initial mass (length) is doubled (15, 16). Cell elongation demands the concomitant enlargement of the sacculus, and cell division requires the formation of a transverse septum at the center of the sacculus. As the sacculus supports the turgor pressure of the cell, its enlargement and formation of septa must proceed while avoiding the generation of discontinuities to prevent cell lysis (24, 27). In addition, the sacculus is subjected to a dynamic metabolic activity comprising maturation, turnover, and growth phase-dependent structural changes (14, 19–21, 23, 26, 41).

Insertion of new precursors by the concerted action of biosynthetic proteins (penicillin-binding proteins [PBPs]) and murein-hydrolytic enzymes promotes the elongation of the sacculus (24, 37, 46). However, a detailed picture of the insertion process is still missing. Incorporation of new precursors is likely to occur in the form of multistrand, cross-linked groups which are first bound to the sacculus in a relaxed state. Cleavage of specific bonds in the murein fabric would then bring the newly inserted strands into the stress-bearing layer, enlarging the sacculus. Assuming a circumferential orientation for the murein glycan strands, incorporation would result in an increased length of the sacculus (5, 22, 24, 40, 48).

The critical event of cell division is the generation of a transverse cell wall, the septum, which leads to binary fission of the mother cell. The division process is subjected to complex regulatory mechanisms to ensure the proper location and timing of the septation event (1, 11, 43). Disturbance of the cell cycle triggers regulatory loops which modulate or halt division, helping the cell to cope with changing environmental conditions (33). The septation process implies a drastic change in both the sacculus and the murein biosynthetic apparatus. At the initiation of septation, a region of the sacculus differentiates to permit the ingrowth of a circumferential invagination mediated by specific enzymes, which eventually develops into the septum (10, 13, 32).

Understanding the mode of growth of the sacculus and the generation of septa is an important issue in bacterial physiology. Both longitudinal growth and septation imply the introduction of new materials into a preexisting structure. Therefore, substantial information could be obtained from the segregation pattern of murein throughout successive cell cycles. However, this approach has been hampered by the difficulty in differentiating new and old murein in the sacculi. The best results have been obtained by means of electron microscopic autoradiography of radiolabeled sacculi. It has been proposed that incorporation of precursors occurs at random, except at constrictions in dividing cells, where synthetic activity was apparently higher (4, 38, 45, 50, 51). Nevertheless, the autoradiographic approach has a spatial resolution intrinsically limited by the specific activity of the precursors and by the free-flight trajectory of emitted radiation. These limitations have sometimes been put forward to criticize the autoradiographic results.

Here we describe a new approach to the study of murein segregation and its application to the study of cell wall growth in *E. coli*. The method relies on the ability of *E. coli* cells to

* Corresponding author. Mailing address: Centro de Biología Molecular "Severo Ochoa," Facultad de Ciencias U.A.M., Campus de Cantoblanco, 28049 Madrid, Spain. Phone: (34-1) 3978083. Fax: (34-1) 3978087. E-mail: madepedro@mvax.cbm.uam.es.

† Dedicated to Uli Schwarz, who constantly encouraged and supported our research.

incorporate into the sacculus D-amino acids when present in the growth medium (7, 8, 47). Incorporation of D-amino acids takes place by an amino acid exchange reaction in the periplasm. The terminal D-Ala residue in the peptide chain of muropeptides is exchanged by a residue of the foreign D-amino acid. Only at high concentrations are D-amino acids deleterious for cell growth and murein synthesis. However, at low concentrations (<1 mg/ml), cells are rather tolerant to the modification of murein (8, 9). This property can be exploited to label sacculi with an easily detectable molecule not naturally found in murein. Detection of the modifying residue in particular regions after a chase period would define the murein as old. Regions without label would correspond to material inserted during the chase period. The distribution of the label would therefore reflect the biosynthetic activity of different regions in the sacculus. We used D-Cys as the labeling compound. This amino acid is easy to detect with reagents suitable for immunodetection in purified sacculi either by optical or electron microscopy techniques.

The results presented here indicate that the method is straightforward, has good spatial resolution, and is flexible enough to be applied in a large number of experimental setups. Furthermore, the method could be applied to other bacteria (*Salmonella typhimurium*, *Enterobacter cloacae*, and *Pseudomonas aeruginosa*) where incorporation of D-amino acids has been detected (6).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* strains used in this work were TOE1 [F^- *ara argE ftsQ1*(Ts) *galK his lacY leu mtl proA rpsL supE thi thyA tsx xyl*] (2), TOE13 [as TOE1 but *ftsA*(Ts) instead of *ftsQ1*(Ts)] (2), LMC1012 [F^- *ara* Δ(*argF-lac*)*U169 deoC1 dnaX2016*(Ts) *flbB5301 lysA1 ptsF25 rbsR relA1 rpsL150 zba*::*Tn10*] (35, 36), MC6RP1 (K-12, F^- *dra drm leuA lysA proA thi thr*) (42), and its derivatives MC6RP41 (*ftsI*^{655}) and MC6RP61 (*ftsZ*) (17). Cultures were routinely grown in Luria-Bertani medium (30) at 37 (30°C for the thermosensitive strains) in gyratory water baths. Growth was monitored by measuring the optical density of the cultures at 550 nm (OD_{550}).

D-Cysteine labeling of murein. Flasks containing appropriate volumes of prewarmed medium were inoculated ($OD_{550} \approx 0.03$) from overnight cultures of the selected bacterial strain and incubated until the OD_{550} doubled. At that moment, D-Cys was added to the cultures to 100 μg/ml (final concentration), and the cultures were further incubated until the OD_{550} reached 0.7, equivalent to 3.5 mass doublings. Cultures were further processed according to the specific requirements of each experiment. Under the conditions described, D-Cys had no detectable effects on the growth rate, morphology, rate of murein synthesis, or amount of murein per cell (data not shown). High-pressure liquid chromatography analysis of murein (23, 41) at the end of the labeling period indicated that between 5 and 6% of muropeptides with a tetrapeptidic side chain contained D-Cys instead of D-Ala. The murein composition was otherwise normal in all parameters.

Purification of sacculi. Cultures (40 to 80 ml, ca. 10^{10} cells) were harvested by centrifugation (10,000 × *g* for 3 min) at the temperature of growth. The pellets were quickly resuspended into 3 ml of NaCl (0.9 g/liter) and dropped into a flask containing 6 ml of boiling 8% (wt/vol) sodium dodecyl sulfate (SDS). The samples were kept boiling for 15 min. To avoid desiccation, distilled water was added, taking care not to interrupt boiling. Samples were transferred to capped thick-wall test tubes (20-ml nominal capacity) and kept closed on a boiling-water bath for 6 h. After addition of small magnetic stirrers, the tubes were kept overnight at room temperature with gentle stirring. Next morning, the tubes were again incubated in a boiling-water bath for 1 h, and once the temperature fell to about 40°C, sacculi (9 ml of the suspension) were sedimented by centrifugation on a desktop ultracentrifuge (Beckman TL100) (400.000 × *g* for 15 min at 30°C), resuspended in a total volume of 2.5 ml of 4% (wt/vol) SDS in a closed tube, and incubated for 4 h in a boiling-water bath. The sacculi were sedimented as above, and the washing procedure was repeated once again but with 1% SDS. The sacculi were resuspended in 2 ml of 50 mM sodium phosphate (pH 7.3). The samples were treated with α-chymotrypsin (100 μg/ml) and incubated first for 4 h at 37°C and then overnight after addition of a second dose of enzyme (100 μg/ml). Next morning, the sacculus suspensions were made up to 3 ml with 6% (wt/vol) SDS and incubated for 4 h in a boiling-water bath. The sacculi were sedimented by centrifugation as above, resuspended in 1% (wt/vol) SDS, incubated for 2 h in a boiling-water bath, and sedimented as before. The final pellet was resuspended in 100 μl of distilled water and preserved at 4°C for immediate use or mixed 1:1 with ethylene glycol for longer storage at −20°C. Periodic

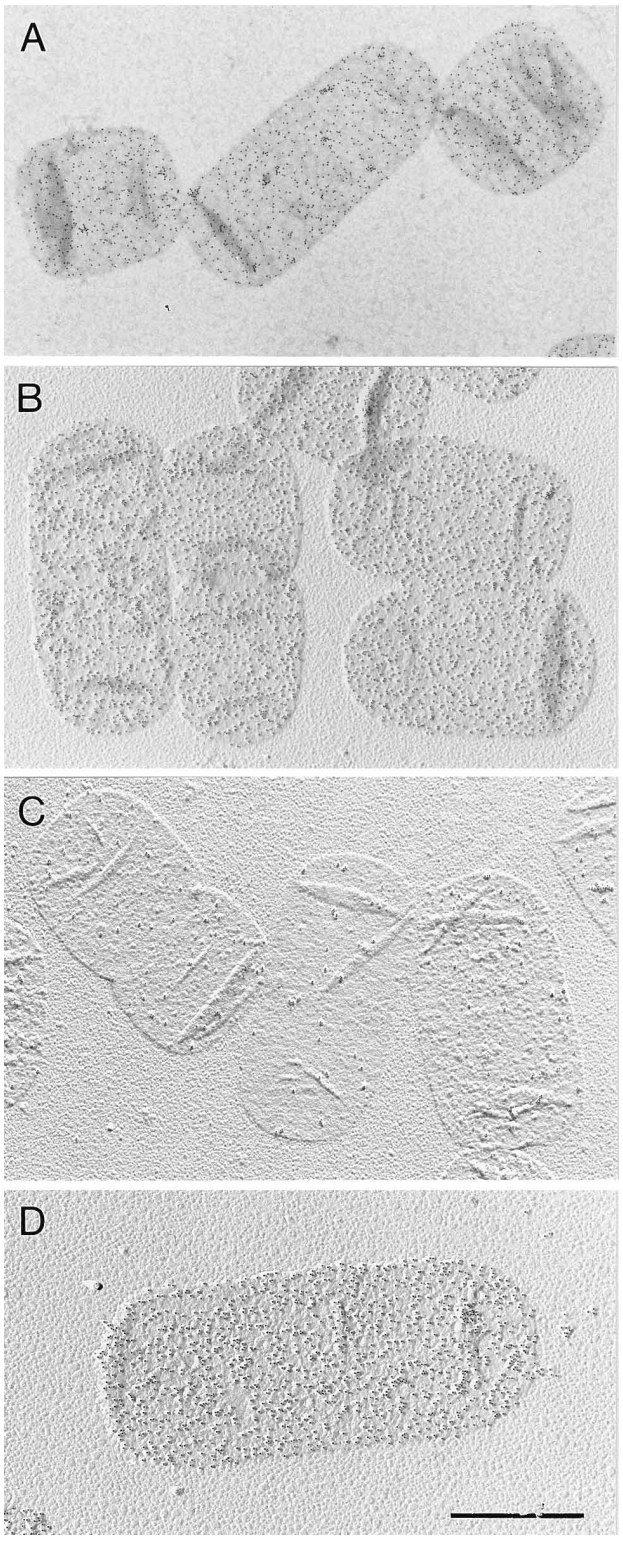

FIG. 1. Detection by immunoelectron microscopy of D-Cys residues in D-Cys-containing sacculi of *E. coli* MC6RP1. Sacculi from cells grown in LB and in LB plus D-Cys for 3.5 mass doublings were purified, subjected to NaH_4B reduction, biotinylated either with biotin-HPDH (SH specific) or with NHS-LC-biotin (NH_2 specific), and subjected to protein A–10-nm gold immunolabeling and electron microscopy as described in Materials and Methods. (A) D-Cys-labeled sacculi, biotin-HPDH, uranyl acetate staining. (B) D-Cys-labeled sacculi, biotin-HPDH, uranyl acetate staining, and C/Pt shadowing. (C) Unlabeled sacculi, biotin-HPDH, uranyl acetate staining, and C/Pt shadowing. (D) Unlabeled sacculi, NHS-LC-biotin, uranyl acetate staining, and C/Pt shadowing. Bar, 1 μm.

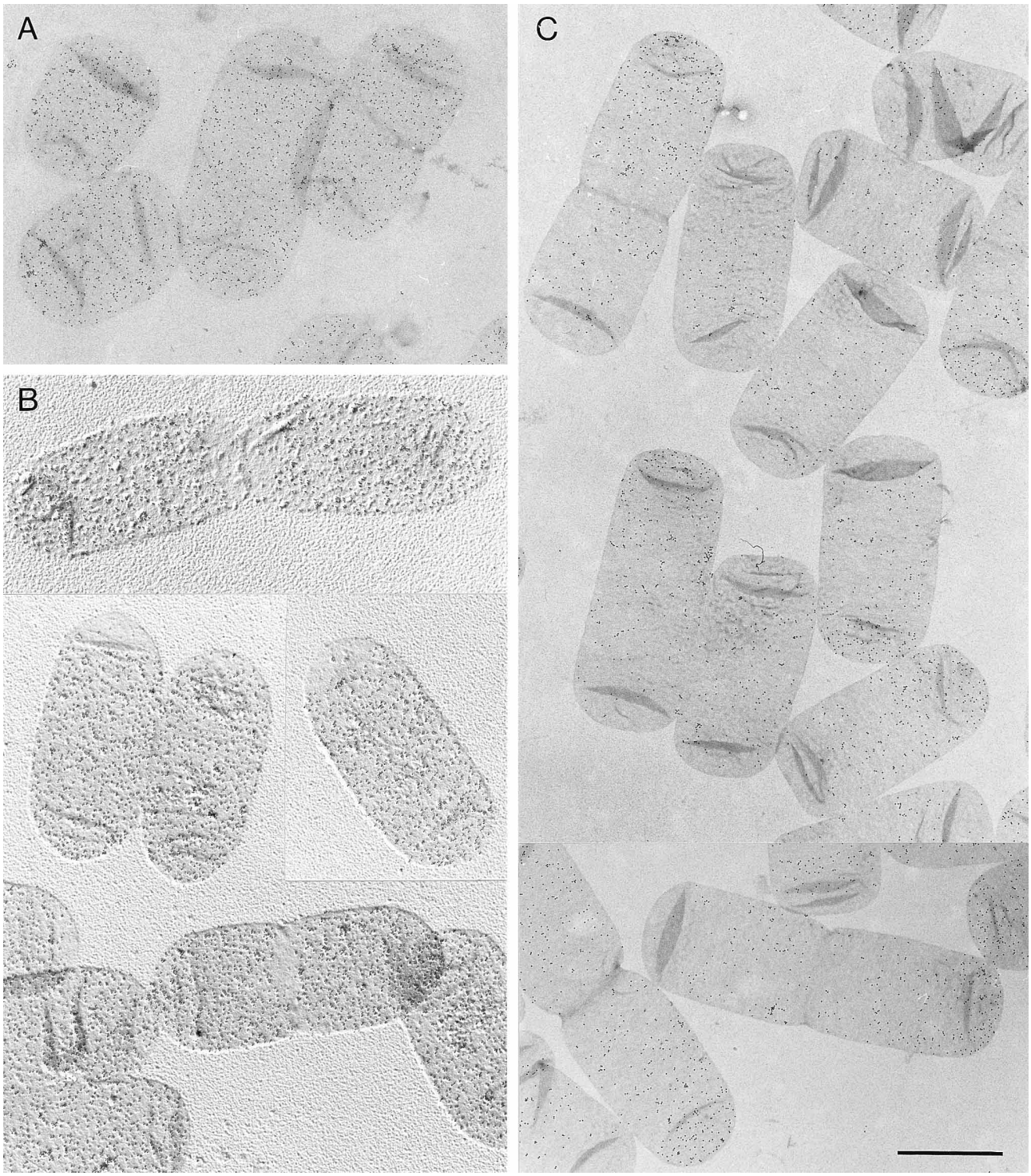

FIG. 2. Immunoelectron microscopy of sacculi from MC6RP1 cells grown in the presence of D-Cys, followed by short chase times. A culture of MC6RP1 was labeled with D-Cys, and upon removal of the D-amino acid, the cells were used to inoculate subcultures of D-Cys-free medium. At the end of the labeling period and after one and two doublings in cell mass, sacculi were purified, reduced with NaH_4B, biotinylated with biotin-HPDH, and subjected to protein A–10-nm gold immunolabeling and electron microscopy as described in Materials and Methods. (A) Sacculi at the end of the incubation in D-Cys, uranyl acetate staining. (B) Sacculi from cells chased for one mass doubling, uranyl acetate staining, and C/Pt shadowing. (C) Sacculi from cells chased for two mass doublings, uranyl acetate staining. Bar, 1 μm.

examination of the ethylene glycol-stored sacculi under the electron microscope indicated appropriate conservation (data not shown).

Biotinylation of thiol groups in purified sacculi. To regenerate the thiol groups oxidized during cell growth and purification of sacculi, a reduction step was performed before biotinylation. Aliquots (50 μl) of sacculus suspensions were mixed with 450 μl of 50 mM sodium carbonate–bicarbonate buffer (pH 8.5) and incubated for 30 min at room temperature after addition of 1 mg of NaH_4B. Excess NaH_4B was eliminated by addition of enough (≈50 μl) 0.6 M H_3PO_4 to lower the pH to 3. After 5 min, the pH was increased to 8.5 by addition of 0.5 M $NaHCO_3$ (≈120 μl), and 0.3 ml of a 2.2-mg/ml solution of *N*-(6-[biotinamido]hexyl)-3′-(2′-pyridyldithio)propionamide (biotin-HPDH; Pierce, Rockford, Ill.) in dimethyl sulfoxide was added to each sample. After 45 min, the

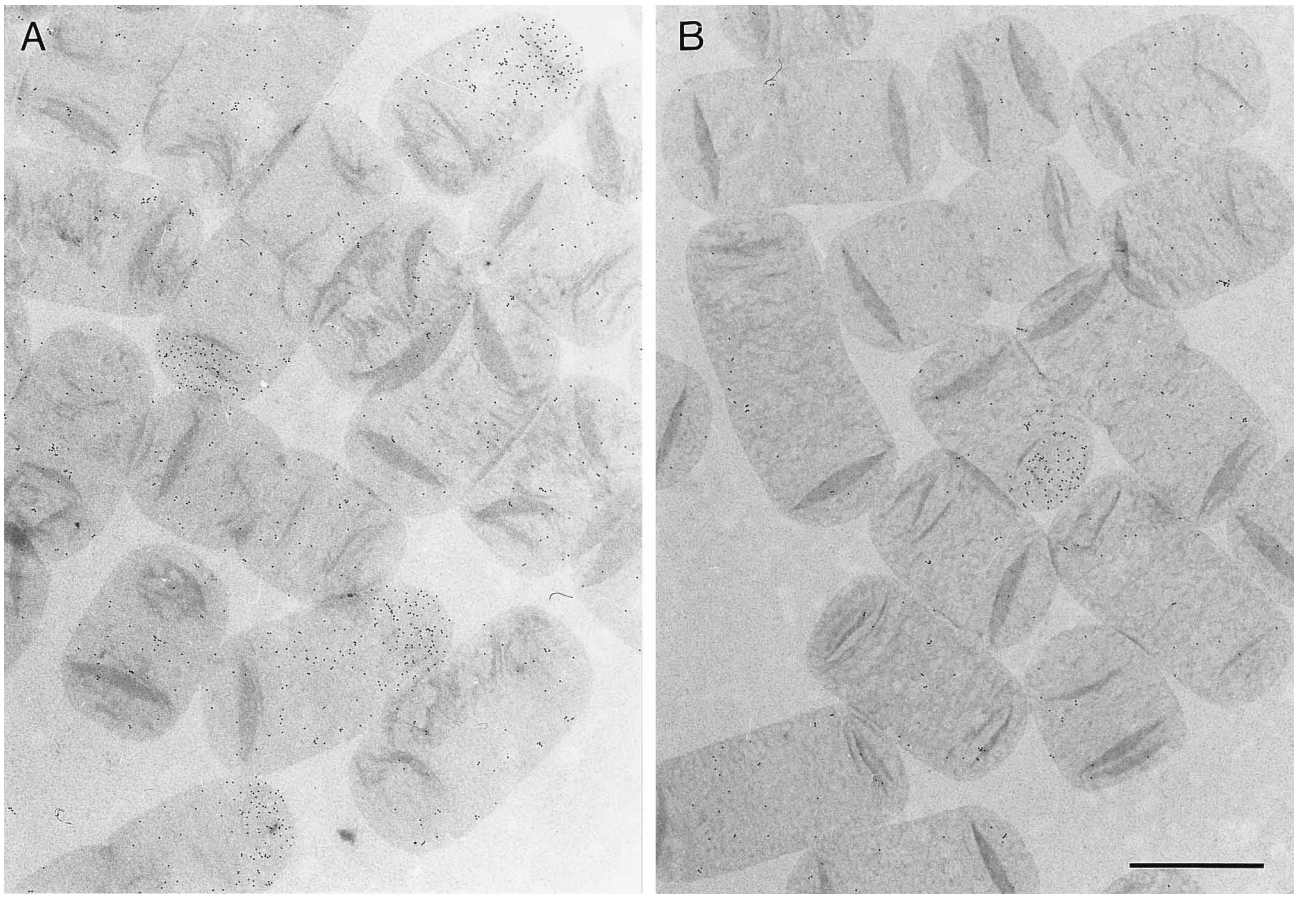

FIG. 3. Immunoelectron microscopy of sacculi from MC6RP1 cells grown in the presence of D-Cys, followed by long chase times. Additional subcultures from the same D-Cys-labeled culture described in Fig. 2 were incubated for four and five mass doublings in D-Cys-free medium. Sacculi were purified and processed as in Fig. 2 for the detection of D-Cys residues by protein A–10-nm gold immunolabeling and electron microscopy. (A) Sacculi from cells chased for four mass doublings, uranyl acetate staining. (B) Sacculi from cells chased for five mass doublings, uranyl acetate staining. Bar, 1 μm.

samples were briefly centrifuged (10 to 15 s in an Eppendorf centrifuge) to remove the white precipitate formed during the reaction. The supernatants were made up to 2.5 ml with distilled water and, after addition of 100 μl of 6% SDS, sedimented by centrifugation (Beckman TL100 centrifuge; 400,000 × *g* for 15 min at 30°C). The sacculi were resuspended in 50 μl of distilled water and immediately used for immunodetection or stored as above.

Biotinylation of free amino groups in purified sacculi. Aliquots of 50 μl of sacculus suspension were mixed with 450 μl of 50 mM sodium carbonate–bicarbonate buffer (pH 8.5). After addition of 0.8 mg of succinimidyl-6-(biotin-amido)hexanoate (NHS-LC-biotin; Pierce), the samples were incubated for 2 h at room temperature. They were diluted with water to a final volume of 2.5 ml and mixed with 0.1 ml of 6% (wt/vol) SDS. The sacculi were recovered by centrifugation (Beckman TL100 centrifuge; 400.000 × *g* for 15 min at 30°C), resuspended in 50 μl of water, and used or stored as above.

Immunofluorescence of biotinylated sacculi. Drops (15 μl) of sacculus suspensions diluted 1:5 in water were placed onto glow-discharged (10 min) circular glass coverslips and let for 15 min. The liquid was removed by suction, and the coverslips were allowed to air dry for 5 min. The sacculus deposition area was covered with drops (25 μl) of PBG (0.5% [wt/vol] bovine serum albumin and 0.2% [wt/vol] gelatin in phosphate-buffered saline [PBS; 8g of NaCl per liter, 0.2 g of KCl per liter, 1.44 g of Na_2HPO_4 per liter, 0.24 g of KH_2PO_4 per liter; pH 7.4]) for 15 min and then with drops (25 μl) of rabbit anti-biotin antiserum diluted 1:300 in PBG. After an incubation period of 60 min, the primary antiserum was removed, and the areas of interest were washed with PBG (five times with 25 μl each) and then covered for 45 min in darkness with 25 μl of fluorescently labeled (fluorescein or Cy3 [Jackson Immuno Research Labs, West Grove, Pa.) goat anti-rabbit antiserum diluted 1:500 in PBG. The samples were rinsed five times with double-distilled water, dried, and mounted with Moviol plus 1,4-diazabicyclo [2.2.2] octane (50 mg/ml). Observations were performed in a Zeiss Axioplan fluorescence microscope with a 100×/1.3 Neofluar objective. Pictures were taken on Kodak T-Max 100 film.

Staining of bacterial nucleoids. Aliquots (0.5 ml) of bacterial culture were fixed by being mixed (1:1) with 0.4% (vol/vol) formaldehyde in 0.9% (wt/vol) NaCl at 4°C and were kept in the cold until used. Samples (200 μl) of fixed cells were mixed with 500 μl of PBS and sedimented by centrifugation (1 min in an Eppendorf desktop centrifuge). The pellet was resuspended in 75 μl of PBS, mixed 1:1 with 50 μg of 4′,6-diamidino-2-phenylindole (DAPI) per ml in PBS, and incubated for 15 min in the dark. PBS (850 μl) was added to each sample, and the cells were centrifuged as above. The cells were resuspended in an equal volume of PBS and centrifuged as above, and the pellets were resuspended in 100 μl of PBS. Drops (5 to 10 μl) were placed onto agarose (1% wt/vol)-covered glass slides, and excess liquid was carefully removed with filter paper after 1 min. The samples were covered with coverglasses and observed under the fluorescence microscope.

Protein A-gold immunolabeling and electron microscopy of sacculi. Carbon-pioloform-coated copper grids (200-mesh) were glow discharged (10 min) and floated for 15 min onto drops of sacculus suspensions appropriately diluted (1:3 to 1:10) in double-distilled water. The grids were removed, excess liquid was soaked onto filter paper, and the grids were allowed to air dry for 10 min. They were washed three times by flotation on drops of distilled water, floated for 15 min on PBG and for 60 min on drops of rabbit anti-biotin antiserum diluted to 1:300 in PBG. Then the grids were washed five times by flotation on PBG and floated on a solution of 10-nm gold-protein A in PBG. The grids were washed five times in PBG and three times in water, stained by floating for 1 min on 1% (wt/vol) uranyl acetate in water, briefly washed in water, and air dried. For the observation of unlabeled sacculi, grids containing sample were washed in water (three times) and directly stained as above. To better visualize the flatness of the sacculi, the grids were in some instances subjected to carbon-platinum (C/Pt) shadowing at an angle of 15°. Microscopic observations were performed on a Philips CM10 transmission electron microscope at an acceleration voltage of 40 or 60 kV. For statistical evaluation of the label distribution in *dnaX* mutant cells, micrographs of sacculi labeled with protein A–6-nm gold were analyzed with a software program (SIS Soft-Imaging-Software, Muenster, Germany). Gold particles along the longitudinal axis of the sacculi were counted with a counting frame of 200 by 400 nm, starting from the center of the cell.

Muramidase digestion of murein sacculi. Purified sacculi were deposited on the surface of carbon-Formvar-coated electron microscope copper grids as above. The grids were floated for the desired time on drops (50 μl) of a solution of cellosyl muramidase (Hoechst, Frankfurt am Main, Germany) (14) at 5 or 10 μg/ml in 20 mM sodium phosphate buffer (pH 4.9) at room temperature. The buffer for enzyme solutions was kept at room temperature, and solutions were prepared immediately before use. At the end of the incubation, the grids were picked up, excess enzyme solution was removed on filter paper, and the grids were rapidly washed three times in drops of precooled (4 to 6°C) double-distilled water, stained for 30 s by flotation onto drops of 1% (wt/vol) uranyl acetate in water, dried, and observed under the electron microscope.

RESULTS

Immunodetection of D-cysteine modified muropeptides in purified sacculi. Most experiments described below are based on a common experimental setup, essentially a label-and-chase strategy. Cells were first incubated in the presence of D-Cys. As a consequence, murein sacculi were uniformly labeled with D-Cys residues. The cells were next transferred into D-Cys-free medium and subjected to the specific experimental treatment, and cell growth was rapidly stopped. Murein incorporated into the sacculus during the chase period would therefore be essentially free of D-Cys. In the following steps, sacculi were purified, deproteinized, reduced with NaH_4B, and biotinylated with an -SH-specific reagent. At the end of the treatment, the regions with old murein would be biotinylated whereas the regions with new murein would not. The distribution of biotin molecules in the sacculi could be visualized by immunofluorescence or immunoelectron microscopy. The experimental details for each step are described in Materials and Methods.

The micrographs in Fig. 1 illustrate critical aspects of the labeling procedure. Sacculi purified as described were spread flat on grids, without signs of structural damage. Biotinylation of sacculi with biotin-HPDP and immunodetection with anti-biotin antibody and protein A-gold yielded samples with a high density of homogeneously distributed gold particles and a remarkably low background (Fig. 1A and B). Omission of the borohydride treatment resulted in a significant (≈50%) reduction of labeling density. The homogeneous distribution of gold particles confirmed the uniformity of the incorporation of D-Cys over the surface of the sacculi, a crucial aspect for the validation of the method. Biotinylation of D-Cys residues and immunodetection of biotin with anti-biotin antibody and protein A-gold were both highly specific. The number of gold grains in sacculi from cells without D-Cys subjected to an identical treatment was close to background (Fig. 1C). However, biotinylation of the same sample with an NH_2-specific reagent yielded heavily labeled sacculi, indicating that the immunolabeling conditions were appropriate (Fig. 1D).

Segregation of murein in sacculi from exponentially growing cells of *E. coli* MC6RP1. A culture of *E. coli* MC6RP1 was labeled with D-Cys as described above, and subcultures were chased in Cys-free medium for different periods. Samples were immunolabeled and observed under the electron microscope as described above. The results are shown in Fig. 2. Sacculi prepared from cells at the end of the incubation with D-Cys were uniformly labeled with gold particles, irrespective of their size and degree of constriction (Fig. 2A). Examination of sacculi chased for one mass doubling revealed some interesting peculiarities (Fig. 2B). In many cells, the presence of well-defined areas deprived of gold grains was evident. The gold-free areas evolved either as gaps at the central position in constricting sacculi or at one pole in the smaller cells. Labeling patterns were more complex in the sample chased for two mass doublings (Fig. 2C). Nevertheless, all the sacculi fell into one of the following categories: one unlabeled polar cap, one unlabeled cap and a central gap, or two unlabeled polar caps.

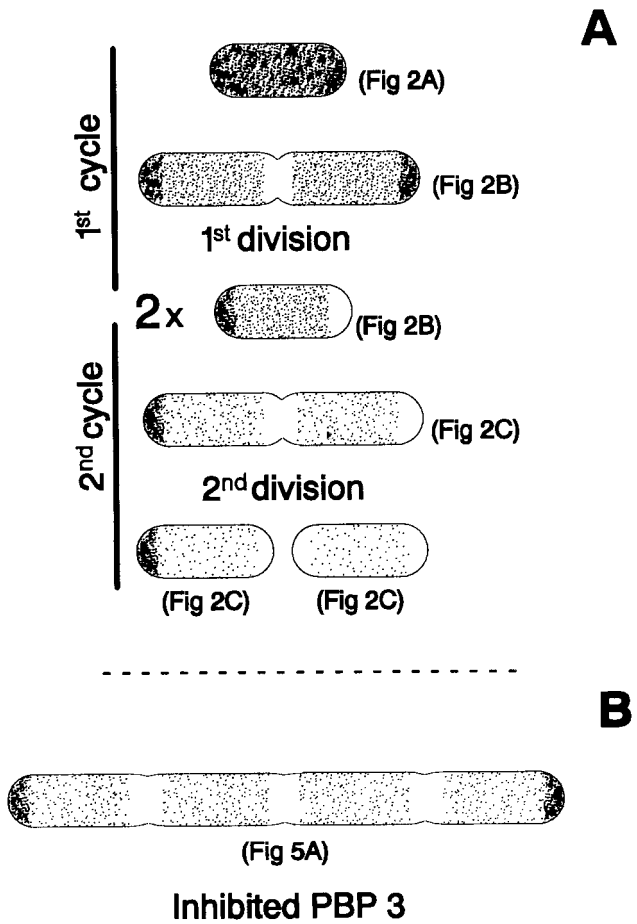

FIG. 4. Schematic interpretation of murein segregation in *E. coli* cells subjected to a D-Cys label-and-chase experiment. (A) Theoretical distribution pattern over time of gold grains in a D-Cys-labeled cell chased for two mass doublings, according to the assumptions presented in the text. (B) Formation of filaments with multiple, regularly arranged gaps caused by inhibition of PBP 3. The correspondence among particular steps in the scheme and the actual pictures of sacculi is indicated in parentheses.

Very interestingly, the distribution of gold in the labeled areas was uniform but was denser in the poles than in the cylindrical wall (Fig. 2C). After longer chase periods, four and five mass doublings, labeling was too poor to give defined patterns, with the notable exception of the polar caps (Fig. 3A and B). In both cases, most polar caps were unlabeled, but in some cells one of them remained heavily labeled. In the sample chased for five mass doubling times, 4.5% of the sacculi (16 from 365) had one labeled polar cap. This is close to the expected value (6.2%), assuming that the polar cap murein was conserved throughout the chase period. Furthermore, the numbers of gold grains in the labeled poles of extensively chased and unchased sacculi were very similar: 49.5 ± 10.4 and 45.6 ± 9.9 grains/cap for unchased cells (28 poles) and cells chased for four mass doublings (22 poles), respectively. The boundary between labeled polar caps and unlabeled cylindrical murein was remarkably sharp, in particular in the sacculi chased for the longest period (Fig. 3B), suggesting an abrupt transition between new and old murein.

The evolution of the segregation pattern observed could be understood on the basis of the following assumptions: (i) murein in the polar caps is stable; (ii) at a particular moment in the cell cycle, a region of localized synthesis differentiates at

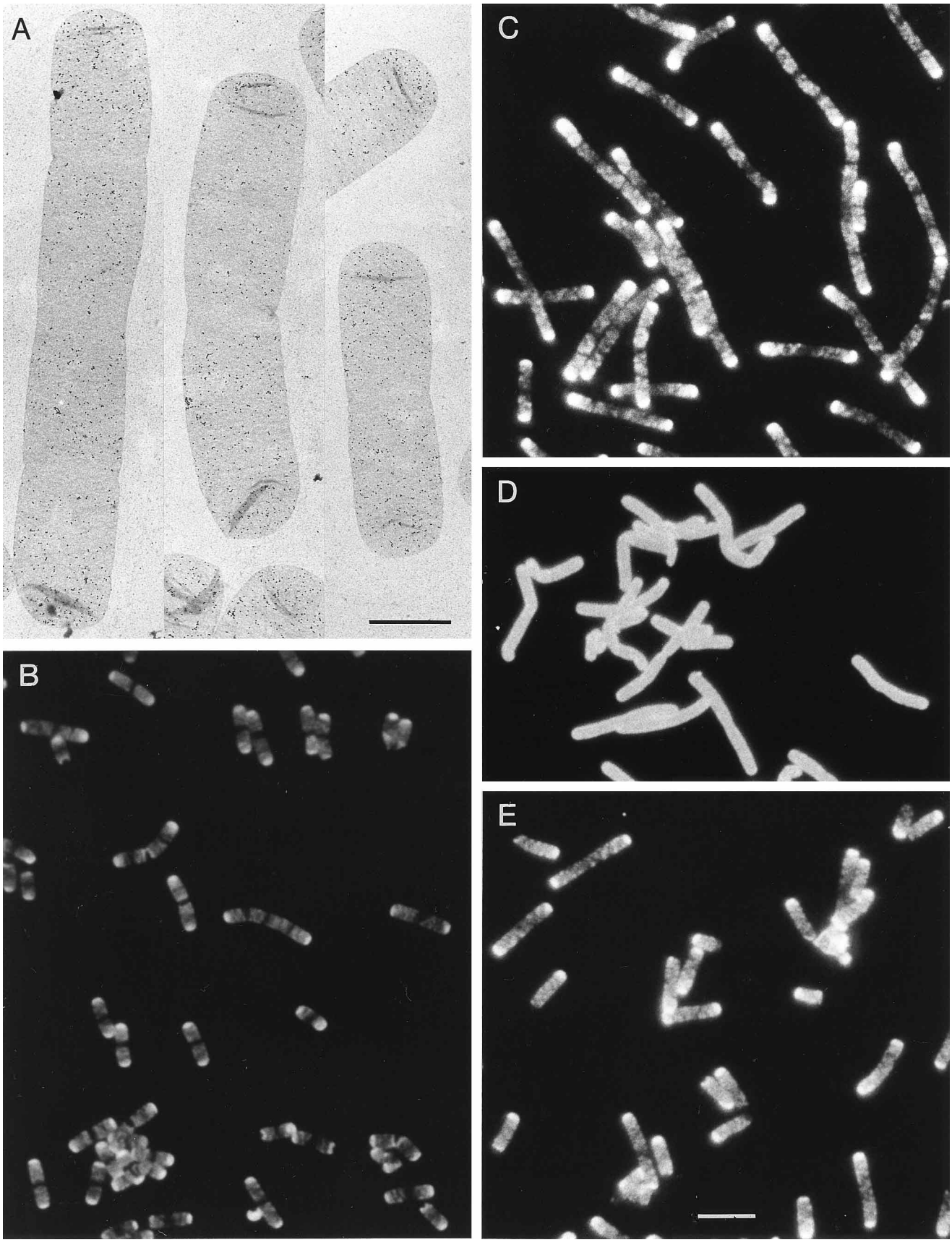
A
B
C
D
E

FIG. 5. Immunodetection of D-Cys residues in sacculi from MC6RP1 cells labeled with D-Cys and chased in the presence of cell division inhibitors. A culture of MC6RP1 was labeled with D-Cys, and upon removal of the D-amino acid, the cells were used to inoculate subcultures of D-Cys-free and D-Cys-containing medium which in addition contained either 1 μg of aztreonam per ml or 40 μg of nalidixic acid per ml. After one and two doublings in cell mass, sacculi were purified, reduced with NaH_4B, and biotinylated with biotin-HPDH, and biotinylated D-Cys residues were visualized either by immunofluorescence after staining with Cy3-labeled goat anti-rabbit antibody or by protein A–10-nm gold immunolabeling and electron microscopy. (A) Immunoelectron microscopy of sacculi from cells chased for one mass doubling in the presence of aztreonam, uranyl acetate staining. (B and C) Immunofluorescence of sacculi from cells chased for one (B) or two (C) mass doublings in the presence of aztreonam. (D) Immunofluorescence of sacculi from cells grown for two mass doublings in the presence of aztreonam plus D-Cys. (E) Immunofluorescence of sacculi from cells chased for one mass doubling in the presence of nalidixic acid. Bars, 1 μm (electron micrographs) and 5 μm (fluorescence micrographs).

the potential division site and generates a septum made of new murein; and (iii) longitudinal expansion of the cylindrical part of the cell wall occurs by diffuse insertion. The scheme in Fig. 4A illustrates the theoretical evolution of sacculi according to our postulates and shows how it correlates with the patterns of murein segregation actually observed (Fig. 2).

Segregation of murein in sacculi from *E. coli* MC6RP1 treated with cell division inhibitors. To obtain complementary information, experiments were performed with cells treated with division inhibitors during the chase period. Two inhibitors with different mechanisms of action were selected, the β-lactam aztreonam and nalidixic acid. Aztreonam inhibits penicillin-binding protein 3 (PBP 3), the septum-making enzyme (18). The primary effect of nalidixic acid is the inhibition of DNA replication and consequently of the early steps of cell division via FtsZ (25, 31, 49). Electron microscopy analysis of filaments has an intrinsic difficulty. At the low magnifications appropriate to visualize filaments, the gold grains become difficult to detect. Use of larger gold conjugates did not solve the problem, as it led to low labeling densities. Fortunately, immunofluorescence proved to be an adequate alternative technique.

The results obtained with aztreonam (1 μg/ml)-treated cells are shown in Fig. 5. After a chase period of one mass doubling, most sacculi showed a neat central gap deprived of antigenic material, with the poles more intensely labeled than the cylindrical wall (Fig. 5A and B). Particularly interesting was the presence in the larger sacculi of two additional unlabeled gaps that developed at one-quarter and three-quarters of the sacculus length. These sacculi correspond to cells close to a division event at the beginning of the chase, which were approaching a second round of division at harvest time. After longer chase times, virtually all the sacculi had multiple, regularly spaced, unlabeled gaps (Fig. 5C). In addition, the contrast between the polar caps and cylindrical wall was higher, as would be expected if polar murein were indeed stable. In contrast, the label distribution was homogeneous in filaments grown in the presence of D-Cys (Fig. 5D), supporting a uniform incorporation of label all over the cell surface. The labeling features of aztreonam-induced filaments are schematically shown in Fig. 4B. A rather different picture emerged from the analysis of nalidixic acid (40 μg/ml)-treated samples. The most striking difference was the absence of gaps in sacculi from treated cells (Fig. 5E). Nevertheless, differential labeling of the polar caps and lateral wall was clearly observed (Fig. 5E). Sacculi from control cells kept in D-Cys plus nalidixic acid showed a uniform surface labeling like those from aztreonam treated cells (data not shown). The differential effect of aztreonam and nalidixic acid suggested that gap formation might be dependent on an early event in the septation process.

Gaps have a defined size. In aztreonam-induced filaments, the gaps at the center (oldest) and at the "fourths" (younger) of the sacculi apparently reached similar sizes (Fig. 5B and C). This would indicate that the gap-generating mechanism was active for a limited period. Otherwise, one would expect the oldest gap (central) to be significantly larger than younger ones in all instances, because the gap size should increase in proportion to the cell size for as long as the cell grows. Figure 6 shows a plot of gap width against cell length for D-Cys-labeled sacculi chased for one mass doubling in the presence of aztreonam (see above). Measurements were made on electron micrographs of protein A-gold-labeled sacculi. Proportionality between both parameters holds only for a reduced range of cell lengths (from 2.5 to 4.5 μm). Below this range, no gaps were found, and above this range, the gap width remained constant at ca. 0.5 μm.

Segregation of murein in sacculi from cell division mutants. Murein segregation in thermosensitive mutants with mutations in the division genes *ftsA*, *ftsI*, *ftsQ*, and *ftsZ* was also studied (1, 2, 17). The experimental layout was as above, but cultures were grown in D-Cys medium at 30°C and transferred to 42°C at the initiation of the chase period. The murein segregation patterns in *ftsI* and azthreonam-induced filaments were expected to be similar, because PBP3 would be impaired in both cases. In fact, sacculi from the *ftsI* strain MC6RP41 showed segregation patterns (Fig. 7A) identical to those of aztreonam treated MC6RP1 cells (Fig. 5B and C). The most distinctive features were the presence of regularly arrayed gaps and the persistence of heavily labeled polar caps (Fig. 7A). Sacculi from *ftsA* and *ftsQ* mutants exhibited segregation patterns similar to that of the *ftsI* mutant (Fig. 7B and C). Nevertheless, in the *ftsQ* mutant, filaments with an asymmetrical distribution of gaps were frequent (Fig. 7C). Analysis of sacculi from the *ftsZ* mutant (Fig. 7D) revealed a segregation pattern reminiscent of nalidixic acid-induced filaments (Fig. 5E). The cell poles of *ftsZ* sacculi remained intensely labeled even in the longer filaments, whereas the cylindrical wall showed a uniform labeling. Periodically arranged gaps were absent in *ftsZ* filaments. However, a small number of sacculi (≈15%) showed a thin

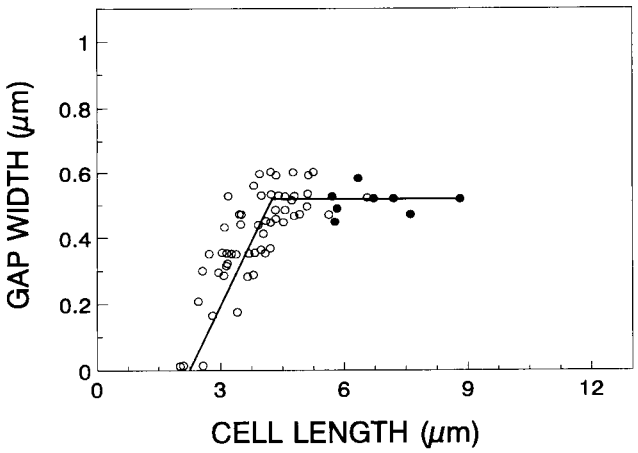

FIG. 6. Gap versus sacculus size. The central gap of sacculi from MC6RP1 cells labeled with D-Cys and chased for one mass doubling in the presence of aztreonam was measured in electron micrographs upon protein A–10-nm gold immunolabeling and electron microscopy. Open circles, measurements in cells with only one (central) gap; solid circles, central gap of cells with three gaps.

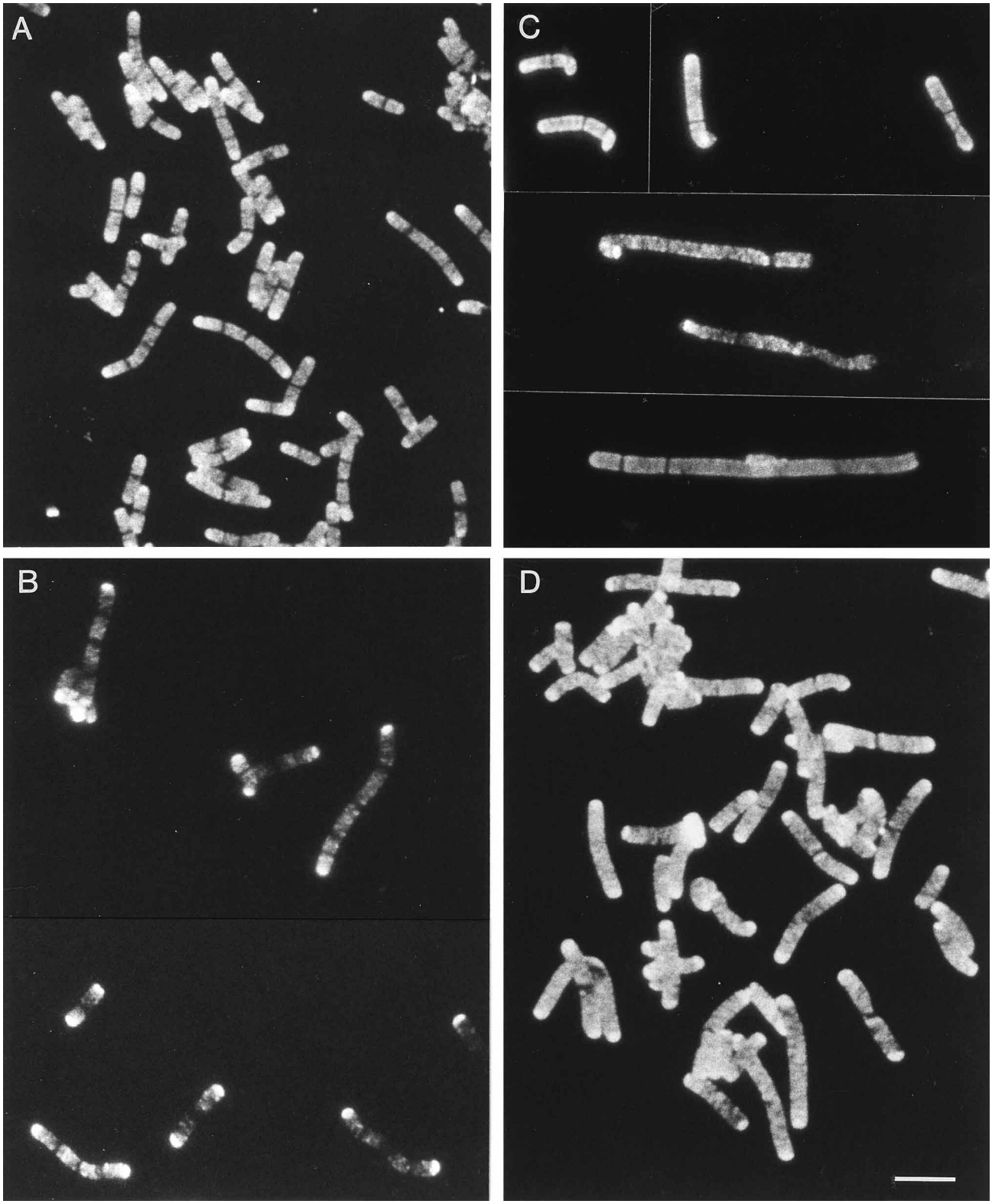

FIG. 7. Immunodetection of D-Cys residues in sacculi from cell division mutant cells labeled with D-Cys and chased at the restrictive temperature. Cultures of strains MC6RP41 (*ftsI*) (A), TOE13 (*ftsA*) (B), TOE1 (*ftsQ*) (C), and MC6RP61 (*ftsZ*) (D) were labeled with D-Cys at 30°C. Upon removal of D-Cys, aliquots were diluted into D-Cys-free medium prewarmed at 42°C and further incubated at 42°C for 1.5 mass doublings. Sacculi were purified and biotinylated with biotin-HPDH, and biotinylated D-Cys residues were visualized by immunofluorescence after staining with either fluorescein- (B and C) or Cy3 (A and D)-labeled goat anti-rabbit antibodies. Bar, 5 μm.

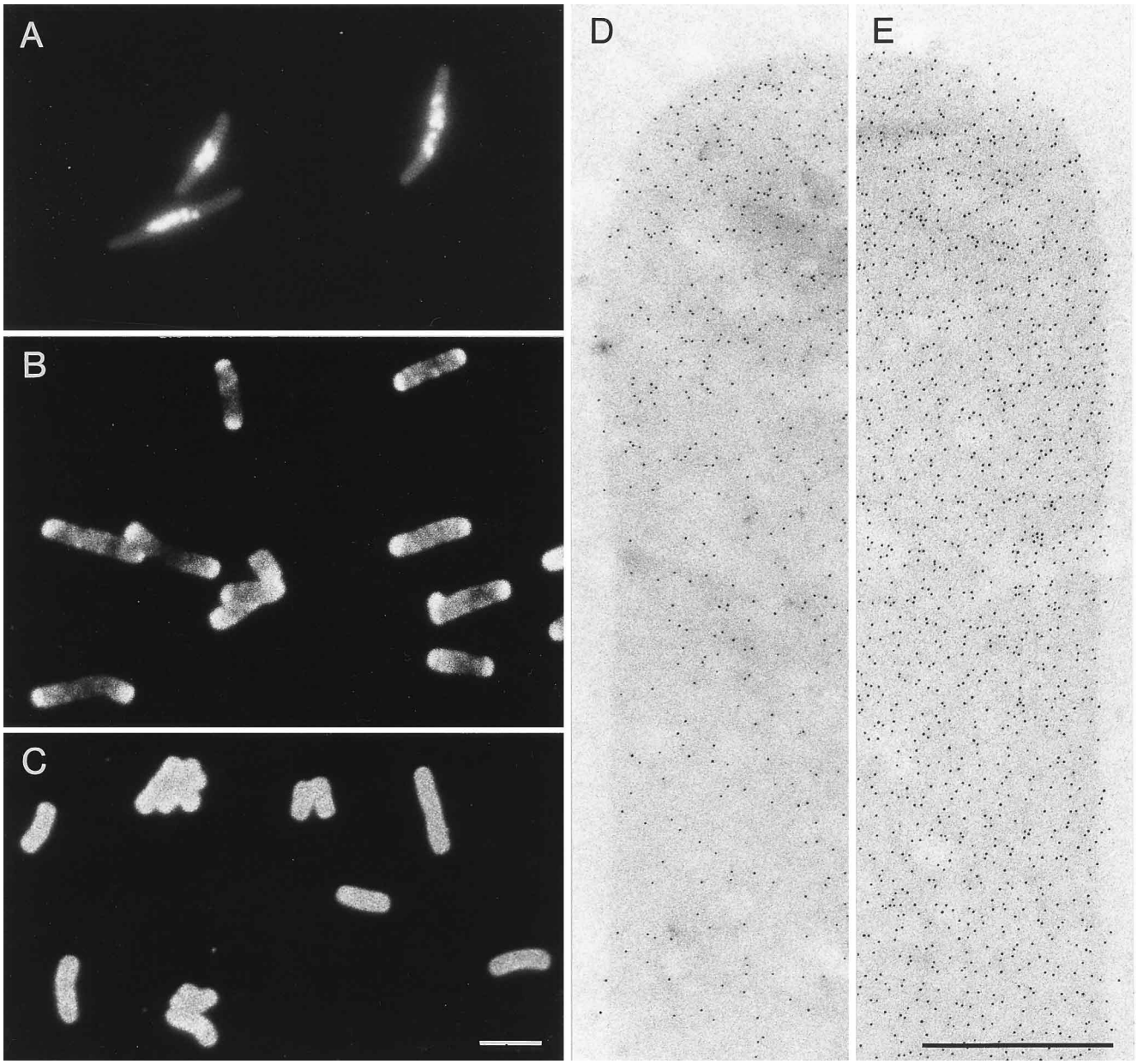

FIG. 8. Immunodetection of D-Cys residues in sacculi from *dnaX* mutant cells labeled with D-Cys and chased at the restrictive temperature. A culture from strain LMC1012 was labeled with D-Cys at 30°C. Upon removal of D-Cys, aliquots were diluted into D-Cys-free or D-Cys-containing medium prewarmed at 42°C and further incubated at 42°C for 1.5 mass doublings. One sample was removed from the culture grown without D-Cys and processed for the detection of nucleoids by DAPI staining and fluorescence microscopy. (A) Sacculi were purified from both subcultures and treated with biotin-HPDH, and biotinylated D-Cys residues were visualized by immunofluorescence after staining with Cy3-labeled goat anti-rabbit antibody or immunoelectron microscopy with protein A–6-nm gold. (B and D) Sacculi chased in the absence of D-Cys. (C and E) Sacculi from cells grown in D-Cys at 42°C. Bars, 5 μm (fluorescence micrographs) and 0.5 μm (electron micrographs).

central gap, most probably corresponding to cells initiating septation at the time of temperature shift up. Sacculi from cells of the strains used which were treated with D-Cys during the incubation at 42°C showed a dense, uniform labeling of the surface in all instances, as illustrated for aztreonam-induced filaments in Fig. 5D (data not shown).

Segregation of murein in sacculi from a DNA replication mutant. Sacculi from the immediate-stop *dnaX* replication mutant LMC1012 were analyzed as for the cell division mutants described above. Upon transfer to the restrictive conditions, the *dnaX* mutant strain formed filaments with a centrally located nucleoid (Fig. 8A). The murein segregation pattern observed was similar to that in the *ftsZ* mutant. Polar caps remained more densely labeled than the cylindrical walls, and gaps were absent (Fig. 8B). Control sacculi from cells grown in the presence of D-Cys at 42°C exhibited a uniformly labeled surface (Fig. 8C). A local inhibitory effect of the nucleoid on murein biosynthesis has been proposed (35). According to this proposal, in mononuclear filaments old murein should be diluted more slowly in the central region than in areas between the nucleoid and the poles. Such an effect was not evident in the pictures of sacculi from *dnaX* filaments (Fig. 8B, D, and E). However, as the effect could be too small to be detected visually, the distribution of old murein along chased (one mass

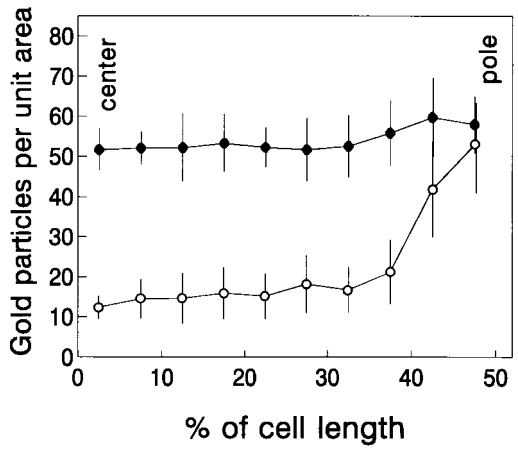

FIG. 9. Distribution of D-Cys-labeled murein in chased and control filaments of the *dnaX* mutant LMC1012. Sacculi from the control (plus D-Cys) and chased (one mass doubling) cultures of LMC1012 described in the legend to Fig. 8 were processed for the detection of D-Cys residues by protein A–6-nm gold immunolabeling and electron microscopy as described in Materials and Methods. High-magnification pictures were taken of straight filaments 4 to 6 μm in length, and the number of gold grains in a measuring window of 200 by 400 nm (length by width) was counted at every 5% of the cell length, following the longitudinal axis of the sacculi. Measuring windows which were only partially occupied by murein (sacculus tips) were not included in the analysis. To avoid orientation biases, each cell was considered to be two independent halves for statistical calculations and plotting. The pictures in Fig. 8D and E are representatives of the ones used for measuring. The number of measured sacculi was 12 and 27 for control and chased cells, respectively. Bars represent σ_n. Symbols: ●, control sacculi; ○, chased sacculi.

doubling) and control filaments was measured on protein A-gold-labeled sacculi such as those shown in Fig. 8D and C. Straight sacculi were selected, and the number of gold grains per unit of area at defined relative positions (each 5% of the total length) along the sacculi were counted. The results, shown in Fig. 9, did not reveal a higher content of old murein in the central region of filaments. On the contrary, the distribution was statistically homogeneous from a distance of 15% of the cell length from the pole. Control filaments also showed a homogeneous distribution, but in this case the density of grains was identical in the poles and any other location of the sacculi. Interestingly, the number of gold grains in the poles was the same in control and chased filaments, as already found for wild-type cells.

"On-grid" muramidase digestion of sacculi. The differential stability of murein in polar caps and in the lateral wall detected in the segregation experiments could reflect a differential susceptibility to hydrolytic enzymes. To check this possibility, sacculi from MC6RP1 cells were spread onto electron microscope grids, digested in situ with a muramidase (Cellosyl), and observed under the electron microscope. The results indicated homogeneous susceptibility of murein to muramidase. Irrespective of the extent of digestion, the polar caps were as pockmark with holes as were the cylindrical part of the cells (Fig. 10). Even the apparent size and orientation of the holes were similar.

DISCUSSION

Here we present the application of a new murein-labeling method for the analysis of murein segregation in *E. coli* by immunomicroscopic techniques. The key to the method was the possibility to uniformly label murein with D-Cys. As D-Cys is not a natural component of murein, it makes an effective chemical label. The large number of D-Cys residues introduced per sacculus (roughly 10^5) ensures a high labeling density. The incorporation of label was routinely checked in the experiments reported (data not shown) and was consistently homogeneous. Biotinylation of D-Cys residues, and immunodetection were highly specific. Omission of D-Cys during growth abolished biotinylation with the SH-specific reagent but not with an NH_2-specific one. The very low reactivity for HPDP-biotin of sacculi without D-Cys is indicative of an appropriate deproteinization. Otherwise, labeling of protein Cys residues should be observed.

Analysis of murein segregation in *E. coli* MC6RP1 supports a mechanism of growth in which both diffuse and localized insertion of precursors alternate and/or overlap in the cell cycle (Fig. 4). Precursors would be randomly incorporated over the cylindrical part of the sacculus for most of the cell cycle. At the initiation of septation, a change in the mode of murein synthesis would be triggered, leading to the localized insertion of precursors into the preseptal area. A similar scheme has been proposed on the basis of autoradiographic studies of cells la-

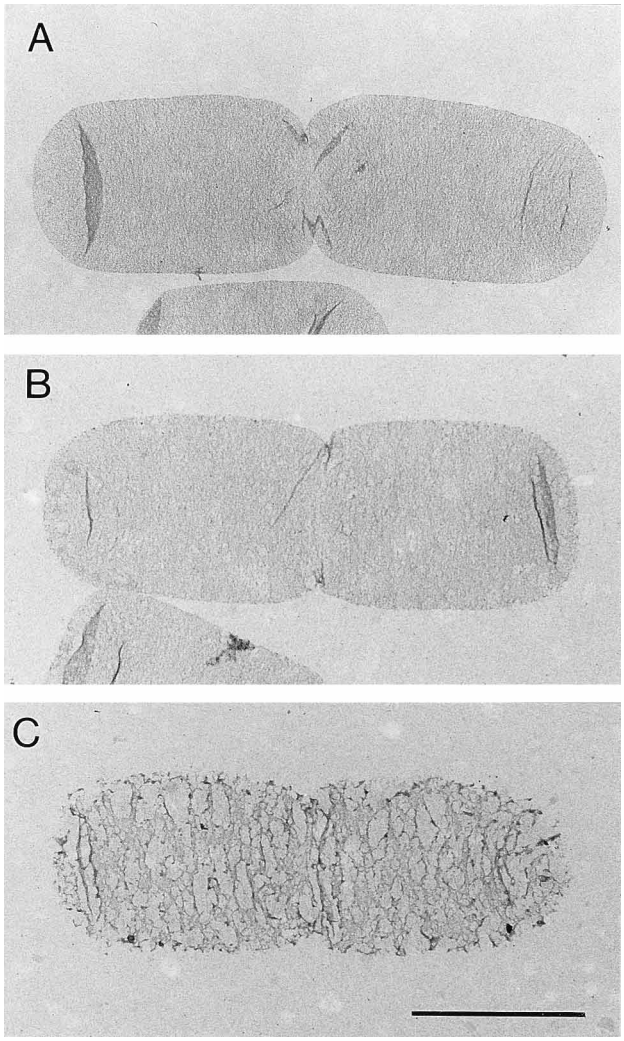

FIG. 10. Muramidase digestion of sacculi. Sacculi purified from an exponentially growing culture of *E. coli* MC6RP1 were laid onto electron microscope grids, digested with muramidase (Cellosyl), stained with uranyl acetate, and observed under the microscope. (A) Undigested control sacculi; (B) sacculi digested for 30 s with 5 μg of muramidase per ml; (C) sacculi digested for 60 s with 10 μg of muramidase per ml. Bar, 1 μm.

beled with a murein radioactive precursor (37, 50, 51). The agreement between the two independent approaches reinforces the hypothesis.

The results of the murein segregation experiments constitute direct experimental evidence in support of a stable nature of the polar cap murein of *E. coli* as previously proposed (28). Persistence of polar murein implies that the rates of synthesis and turnover must be much lower at the poles than in the cylindrical wall. Polar caps are in essence terminated septa. Therefore, it seems reasonable to propose that concomitantly with septum construction, septal murein becomes metabolically inert. Inactivation of murein metabolism in the septal area could occur either by a chemical modification of the murein itself or by the functional isolation (compartmentalization) of polar murein from key biosynthetic enzymes and/or substrates. Available information is still insufficient but would favor the latter alternative. Assuming that minicell and septal mureins are the same thing, the chemical setups of septal and lateral wall mureins have been shown to be very similar (39). Furthermore, the sensitivity of murein to externally added hydrolytic enzymes was homogeneous, not only in the experiments reported here but also in experiments performed with murein hydrolases purified from *E. coli* itself (48). The alternative implies the generation of compartments in the cell envelope where murein metabolism is close to zero. The compartments generated by periseptal annuli would be appropriate for such a function. The timing of generation of periseptal annuli as well as their topology would neatly fulfill the basic requirements (10, 13, 43). Interestingly, conservative segregation of septal murein has been also reported in the gram-positive rod *Bacillus subtilis* (34). Therefore, persistence of polar cap murein is likely to be a widespread property of rod-shaped bacteria.

Analysis of sacculi from filamentous cells further supported our interpretation of murein segregation patterns. Conservation of polar cap murein was even more evident in filament cells, irrespective of the filament-inducing treatment. The ability of aztreonam-treated cells and *ftsI* mutants to generate sacculi with multiple gaps at potential septation sites has important implications. Assuming that gaps represent an early stage of septation, our data confirm previous observations indicating that PBP3 is not required for the initiation of septa (37). In particular, activation of synthesis at preseptal positions would be independent of PBP3. The similarity of murein segregation patterns in *ftsI*, *ftsA*, and *ftsQ* mutants indicates that the products of the later genes are also dispensable for the initiation of septation. In contrast, the segregation patterns observed in nalidixic acid-induced filaments as well as in *ftsZ* and *dnaX* mutants clearly indicated that gap differentiation has a strict requirement for active FtsZ protein. The high frequency of asymmetrical preseptal gaps detected in *ftsQ* filaments at 42°C was an unexpected feature that was not easy to understand. Although a specific investigation is needed to clarify this observation, a polar effect of the *ftsQ* mutation on the expression of the downstream genes *ftsA* and *ftsZ* could lead to such a phenotype (1). A decreased amount of FtsZ could lead to underproduction of gaps. In fact, the levels of FtsZ regulate the frequency of septation events, and a proper balance between FtsA and FtsZ is required for correct division (3, 12).

Gaps formed at the potential division sites in filaments apparently had a maximum defined size. Therefore, murein synthesis in the gap area must have a limited life generating a ring of metabolically inert murein. Otherwise, the gap should grow indefinitely in proportion to the cell length. Inactivation of gap murein could be mediated by the same mechanism inactivating polar murein during normal division events.

Comparison of murein segregation patterns observed in plurinucleoid (*ftsZ*) and mononucleoid (*dnaX*) filament cells provided information about the influence of the nucleoid on murein biosynthetic activity (35). No drastic differences were detected between *ftsZ* and *dnaX* filaments. It has been proposed that the nucleoid could exert a negative effect on murein biosynthetic activity (35). If this were so, and assuming a central distribution of nucleoids in *dnaX* filaments, the central region of filamentous sacculi should be more heavily labeled than the distal regions, because dilution of old material with new one would be smaller. Analysis of the distribution of old murein along chased and control sacculi did not follow the prediction. In contrast, the data supported a homogeneous distribution of old murein except in the immediate vicinity of the cell poles (Fig. 9). Nevertheless, it is important to point out a relevant limitation of the method. The chase time ought to be considerably longer than the labeling pulses used by Mulder and Woldringh (35). Therefore, the possibility exists that the differences generated by subtle changes in the rate of synthesis were obscured by subsequent murein metabolic processes like continued growth and murein recycling.

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REFERENCES

1. **Ayala, J. A., T. Garrido, M. A. de Pedro, and M. Vicente.** 1994. Molecular biology of bacterial septation, p. 73–102. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science Publishing, Amsterdam, The Netherlands.
2. **Begg, K. J., and W. D. Donachie.** 1985. Cell shape and division in *Escherichia coli*: experiments with shape and division mutants. J. Bacteriol. **163:**615–622.
3. **Bi, E., and J. Lutkenhaus.** 1990. FtsZ regulates frequency of cell division in *Escherichia coli*. J. Bacteriol. **172:**2765–2768.
4. **Burman, L. G., J. Raichler, and J. T. Park.** 1983. Evidence for diffuse growth of the cylindrical portion of the *Escherichia coli* murein sacculus. J. Bacteriol. **155:**983–988.
5. **Burman, L. G., and J. T. Park.** 1984. Molecular model for elongation of the murein sacculus of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **81:**1844–1848.
6. **Caparrós, M.** 1992. Doctoral thesis. Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain.
7. **Caparrós, M., V. Aran, and M. A. de Pedro.** 1992. Incorporation of S-[^{3}H]-methyl-D-cysteine into the peptidoglycan of ether-treated cells of *Escherichia coli*. FEMS Microbiol. Lett. **93:**139–146.
8. **Caparrós, M., A. G. Pisabarro, and M. A. de Pedro.** 1992. Effect of D-amino acids on structure and synthesis of peptidoglycan in *Escherichia coli*. J. Bacteriol. **174:**5549–5559.
9. **Caparrós, M., J. L. M. Torrecuadrada, and M. A. de Pedro.** 1991. Effect of D-amino acids on *Escherichia coli* strains with impaired penicillin-binding proteins. Res. Microbiol. **142:**345–350.
10. **Cook, W. R., and L. I. Rothfield.** 1994. Early stages in development of the *Escherichia coli* cell-division site. Mol. Microbiol. **14:**485–495.
11. **Cooper, S.** 1991. Bacteriol growth and division. Academic Press, Inc., San Diego, Calif.
12. **Dai, K., and J. Lutkenhaus.** 1992. The proper ratio of FtsZ to FtsA is required for cell division to occur in *Escherichia coli*. J. Bacteriol. **174:**6145–6151.
13. **De Boer, P. A., W. R. Cook, and L. I. Rothfield.** 1990. Bacterial cell division. Annu. Rev. Genet. **24:**249–274.
14. **De Pedro, M. A., and U. Schwarz.** 1981. Heterogeneity of newly inserted and preexisting murein in the sacculus of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **78:**5856–5860.
15. **Donachie, W. D.** 1968. Relationship between cell size and time of initiation of DNA replication. Nature **219:**1077–1079.
16. **Donachie, W. D., K. J. Begg, and M. Vicente.** 1976. Cell length, cell growth and cell division. Nature **264:**328–333.

17. **García del Portillo, F., and M. A. de Pedro.** 1990. Differential effect of mutational impairment of penicillin-binding proteins 1A and 1B on *Escherichia coli* strains harboring thermosensitive mutations in the cell division genes *ftsA*, *ftsQ*, *ftsZ*, and *pbpB*. J. Bacteriol. **172:**5863–5870.
18. **Georgopapadakou, N. H., S. A. Smith, and R. B. Sykes.** 1982. Mode of action of azthreonam. Antimicrob. Agents Chemother. **21:**950–956.
19. **Glauner, B., and J.-V. Höltje.** 1990. Growth pattern of the murein sacculus of *Escherichia coli*. J. Biol. Chem. **265:**18988–18996.
20. **Goodell, E. W.** 1985. Recycling of murein by *Escherichia coli*. J. Bacteriol. **163:**305–310.
21. **Goodell, E. W., and U. Schwarz.** 1983. Cleavage and resynthesis of peptide cross-bridges in *Escherichia coli*. J. Bacteriol. **156:**136–140.
22. **Höltje, J.-V.** 1993. "Three for one"—a simple growth mechanism that guarantees a precise copy of the thin, rod shaped murein sacculus of *Escherichia coli*. FEMS Symp. **65:**419–426.
23. **Höltje, J.-V., and B. Glauner.** 1990. Structure and metabolism of the murein sacculus. Res. Microbiol. **141:**75–89.
24. **Höltje, J.-V., and U. Schwarz.** 1985. Biosynthesis and growth of the murein sacculus, p. 77–119. *In* N. Nanninga (ed.), Molecular cytology of *Escherichia coli*. Academic Press, Inc., New York, N.Y.
25. **Hooper, H. C.** 1995. Quinolone mode of action. Drugs **49**(Suppl. 2)**:**10–15.
26. **Jacobs, C., L. J. Huang, E. Bartowsky, S. Normark, and J. T. Park.** 1994. Bacterial cell wall recycling provides cytosolic muropeptides as effectors for beta-lactamase induction. EMBO J. **13:**4684–4694.
27. **Koch, A.** 1988. Biophysics of bacterial wall viewed as stress-bearing fabric. Microbiol. Rev. **52:**337–353.
28. **Koch, A. L., and L. Woldringh.** 1994. The metabolic inertness of the pole wall of a Gram negative rod. J. Theor. Biol. **171:**415–425.
29. **Labischinski, H., and H. Maidhof.** 1994. Bacterial peptidoglycan: overview and evolving concepts, p. 23–39. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science Publishing, Amsterdam, The Netherlands.
30. **Lennox, E. S.** 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1:**190–206.
31. **Lutkenhaus, J.** 1983. Coupling of DNA replication and cell division: *sulB* is an allele of *ftsZ*. J. Bacteriol. **154:**1339–1346.
32. **MacAlister, T. J., W. R. Cook, R. Weigand, and L. I. Rothfield.** 1987. Membrane-murein attachment at the leading edge of the division septum: a second membrane-murein structure associated with morphogenesis of the gram-negative bacterial division septum. J. Bacteriol. **169:**3945–3951.
33. **Maguin, E., J. Lutkenhaus, and R. D'Ari.** 1986. Reversibility of SOS-associated division inhibition in *Escherichia coli*. J. Bacteriol. **166:**733–738.
34. **Mobley, H. L., A. L. Koch, R. J. Doyle, and U. N. Streips.** 1984. Insertion and fate of the cell wall in *Bacillus subtilis*. J. Bacteriol. **158:**169–179.
35. **Mulder, E., and C. L. Woldringh.** 1991. Autoradiographic analysis of diaminopimelic acid incorporation in filamentous cells of *Escherichia coli*: repression of peptidoglycan synthesis around the nucleoid. J. Bacteriol. **173:**4751–4756.
36. **Mullin, D. A., C. L. Woldringh, J. M. Henson, and J. R. Walker.** 1983. Cloning of the *Escherichia coli dna*ZX region and identification of its products. Mol. Gen. Genet. **192:**73–79.
37. **Nanninga, N.** 1991. Cell division and peptidoglycan assembly in *Escherichia coli*. Mol. Microbiol. **5:**791–795.
38. **Nanninga, N., and C. L. Woldringh.** 1985. Cell growth, genome duplication and cell division, p. 259–318. *In* N. Nanninga (ed.), Molecular cytology of *Escherichia coli*. Academic Press, Inc., New York, N.Y.
39. **Obermann, W., and J.-V. Höltje.** 1994. Alterations of murein structure and of penicillin-binding proteins in minicells from *Escherichia coli*. Microbiology **140:**79–87.
40. **Park, J. T., and L. G. Burman.** 1983. Elongation of the murein sacculus of *Escherichia coli*. Ann. Inst. Pasteur Microbiol. **136A:**51–58.
41. **Pisabarro, A. G., M. A. de Pedro, and D. Vázquez.** 1985. Structural modifications in the peptidoglycan of *Escherichia coli* associated with changes in the state of growth of the culture. J. Bacteriol. **161:**238–242.
42. **Prats, R., and M. A. de Pedro.** 1989. Normal growth and division of *Escherichia coli* with a reduced amount of murein. J. Bacteriol. **171:**3740–3745.
43. **Rothfield, L. I., P. De Boer, and W. R. Cook.** 1990. Localization of septation sites. Res. Microbiol. **141:**57–63.
44. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407–477.
45. **Schwarz, U., A. Ryter, A. Rambech, R. Hellio, and Y. Hirota.** 1975. Process of cellular division in *Escherichia coli*: differentiation of growth zones in the sacculus. J. Mol. Biol. **98:**749–759.
46. **Shockman, G. D., and J.-V. Höltje.** 1994. Microbial peptidoglycan (murein) hydrolases, p. 131–166. *In* J.-M. Ghuysen and R. Hakenbeck (ed.), Bacterial cell wall. Elsevier Science Publishing, Amsterdam, The Netherlands.
47. **Tsuruoka, T., A. Tamura, A. Miyata, T. Takei, K. Iwamatsu, S. Inouye, and M. Matsuhashi.** 1984. Penicillin-insensitive incorporation of D-amino acids into cell wall peptidoglycan influences the amount of bound lipoprotein in *Escherichia coli*. J. Bacteriol. **160:**889–894.
48. **Verwer, R. W., N. Nanninga, W. Keck, and U. Schwarz.** 1978. Arrangement of glycan chains in the sacculus of *Escherichia coli*. J. Bacteriol. **136:**723–729.
49. **Walker, G. H.** 1996. The SOS response of *Escherichia coli*, p. 1400–1426. *In* F. D. Neidhardt, R. Curtiss III, J. L. Ingraham, E.C.C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
50. **Wientjes, F. B., and N. Nanninga.** 1989. Rate and topography of peptidoglycan synthesis during cell division in *Escherichia coli*: concept of a leading edge. J. Bacteriol. **171:**3412–3419.
51. **Woldringh, C. L., P. Huls, E. Pas, G. H. Brakenhoff, and N. Nanninga.** 1987. Topography of peptidoglycan synthesis during elongation and polar cap formation in a cell division mutant of *Escherichia coli* MC43100. J. Gen. Microbiol. **133:**575–586.