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Branching of *Escherichia coli* Cells Arises from Multiple Sites of Inert Peptidoglycan†

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Some strains of *Escherichia coli* defective for *dacA*, the gene coding for penicillin-binding protein 5, exhibit a strong branching phenotype when cell division is blocked. Since such branch formation implies a differentiation of polar caps at ectopic locations in the cell envelope, we analyzed murein segregation and observed a strong correlation between areas of inert murein and these morphological anomalies. In particular, the tips of branches exhibited the same properties as those described for polar caps of wild-type cells, i.e., the synthesis and turnover of murein were inhibited. Also, the mobility of cell envelope proteins was apparently constrained in areas with morphological defects. Polar regions of branching cells and sacculi had aberrant morphologies with a very high frequency. Of special interest was that areas of inert murein at polar caps were often split by areas of active synthesis, a situation unlike that observed in wild-type cells. These observations suggest that in *dacA* mutants, branches and other morphological anomalies may arise from split polar caps or by de novo generation of new poles built around inert peptidoglycan patches in the side walls of the cell.

Escherichia coli is a morphologically simple organism with a cylindrical body of uniform diameter terminated by two semispherical polar caps. Growth of *E. coli* occurs by the periodic succession of elongation and division events (1, 2). Cells divide at their midpoint once the chromosome is replicated and the initial length is doubled (8). Cellular morphology is remarkably constant, and observation of aberrant shapes is extremely rare. However, certain mutant strains show a marked tendency to generate branched cells under specific growth conditions (4, 9, 14, 19). Branching is an extreme phenotype characterized by the presence of cells with Y or X shapes and cells with kinks or buds of irregular diameter.

Observation of branching *E. coli* cells dates back more than 30 years (17), but the mechanism of branching remains unclear. In spite of early proposals (19), formation of branches is apparently unrelated to the division process. Branching points do not correlate with division sites, and neither of the essential division proteins FtsI or FtsZ is required for branch formation (9). As an alternative hypothesis, it has been proposed that branches may develop from small asymmetries in the cell wall (9). The fact that strains with extensive branching are affected in cell wall metabolism supports this idea (9, 14, 15).

Recent work on the role of the low-molecular-weight penicillin-binding proteins led to the construction of mutant strains with a strong branching phenotype (14, 15). Generation of morphological anomalies in these strains requires multiple mutations but is ultimately conditioned by the activity of penicillin-binding protein 5 (PBP 5), coded for by the *dacA* gene. In isogenic couples differing in the activity of PBP 5, Δ*dacA* strains have a strong phenotype, whereas the parental strains have normal morphology (15).

As pointed out before (9), branching as well as other morphological aberrations can be understood as the consequence of local disturbances in the metabolism of the murein sacculus, the major morphogenetic structure of the bacterial cell wall (10, 18). Formation of branches implies the generation of new poles at the tips of the branches and of stable bifurcation points in the cell envelope. Indeed, it seems quite reasonable to assume that the poles are the older regions of the growing branches. Whether or not branch poles and normal poles are equivalent is unknown at present. Nevertheless, how a new polar region can develop at an ectopic location in the cell surface is by no means evident.

The polar regions of *E. coli* apparently constitute highly differentiated topological domains of the cell envelope: in the sacculus, murein at the poles is stable over time and metabolically inert (5); in the outer membrane, the mobility of at least some proteins is severely restricted at the poles (M. A. de Pedro, submitted for publication); in the periplasm, molecular diffusion between the lateral and the polar regions is restricted (6), and in the cytoplasmic membrane, poles are enriched in cardiolipin (12). Furthermore, poles are apparently rather stable structures which segregate in a conservative way at cell division and can be traced for several generations.

Differentiation of the new poles occurs concomitantly with septum formation. At the earlier stages of division, localized murein synthesis is activated at the division site and remains active until septum completion, when zonal synthesis stops and the new poles become as inert and stable as the old ones (5). Therefore, the situation is quite paradoxical. On the one hand, generation of poles is tightly linked to division in normal cells, but generation of branches and consequently of new branch poles does not seem to be so.

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† For a commentary on this article, see page 1125 in this issue.

Recently, high-resolution murein and outer membrane labeling methods suitable for pulse and chase experiments have been developed and applied to the study of segregation of both cell envelope components (5). Here these methods have been applied to study branching in *E. coli* PBP 5 mutant strains as a further attempt to elucidate the mechanisms of bacterial morphogenesis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used were *E. coli* CS109 (W1485 *rpoS rph-1*) and its derivative CS801-4 (CS109 *yrfE yrfF mrcA dacA dacB dacC dacD pbpG ampC ampH*) (4, 15). Cultures were grown at 37°C in Luria-Bertani (LB) medium (13) in a gyratory water bath. Growth was monitored by measuring the optical density at 550 nm (OD_{550}) at regular intervals.

D-Cysteine labeling of murein. Labeling was performed as before (5). Flasks containing appropriate volumes of LB 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-cysteine (100 μg/ml) was added to the cultures, which were further incubated until they reached an OD_{550} of 0.8. To remove D-cysteine, cultures were centrifuged for 5 min at 20,000 × *g* at the same temperature as used for growth, resuspended into an equal volume of D-cysteine-free medium warmed to the growth temperature, centrifuged again as before, and finally resuspended in medium as appropriate for each experiment. When required, cell division was inhibited by addition of 1 μg of aztreonam per ml to the medium (7).

Purification and immunolabeling of murein. Culture samples containing about 3×10^9 cells were withdrawn after appropriate chase times and processed for murein purification and biotinylation of incorporated D-cysteine as described before (5). Distribution of biotin residues over the sacculus surface was visualized by immunolabeling of biotin residues and either confocal or electron microscopy.

For confocal microscopy, sacculi were first uniformly labeled with the NH_2-specific reagent Oregon Green 488 carboxylic acid succinimidyl ester (Molecular Probes Europe BV, Leiden, The Netherlands) to permit visualization of whole sacculi. To do so, sacculi were mixed 1:1 with 0.2 M $NaHCO_3$ and labeled with Oregon Green 488 carboxylic acid succinimidyl ester (50 μg/ml final concentration) for 10 min. Sacculi were washed by repeated cycles of centrifugation and resuspension in water. To label and visualize the areas rich in biotinylated muropeptides, drops (20 μl) of adequately diluted sacculus suspension were deposited onto glass coverslips and further processed for immunolabeling with rabbit antibiotin antiserum and indocarbocyanine-conjugated goat anti-rabbit immunoglobulin antibody as described before (5). Confocal microscopy was performed with a Leica TCS-NT confocal microscope fitted with a 100×, N.A. 1.4 objective lens.

For electron microscopy, sacculi were also processed as before (5) except that they were spread on carbon-pioloform-covered nickel grids (200 mesh), and immunolabeling with rabbit antibiotin antiserum and protein A-6-nm gold complexes was followed by silver enhancement (3, 16) to improve detection of gold grains at low magnification. Samples were observed in a Philips CM10 electron microscope at 60 kV acceleration voltage.

In vivo labeling of cells with Texas Red-X succinimidyl ester. Culture samples were centrifuged (8,000 × *g*, 4 min, 30°C), and cell pellets were resuspended at a cell density of ca. 10^{10} cells/ml in 0.1 M $NaHCO_3$ and mixed 1:50 (dye to cell) with a 2.5-mg/ml solution of Texas Red-X succinimidyl ester (Molecular Probes Europe BV, Leiden, The Netherlands) in dimethyl sulfoxide. The reaction mixture was kept in the dark at room temperature for 5 min, and the reaction was stopped by adding 50 μl of 50-mg/ml lysine in water per ml of labeling mixture. Samples were centrifuged as above, and cell pellets were resuspended in equal volumes of LB medium warmed to 37°C and centrifuged once again. The pellets were resuspended as before and diluted as required for each experiment.

Manipulation of Texas Red-labeled cells was done under dim light, and flasks with labeled cultures were wrapped in thick aluminum foil for further incubation. When required, cell division was blocked by addition of 1 μg of aztreonam per ml to the medium. Samples (1 ml) were removed at periodic intervals, mixed 1:1 with 0.5% formaldehyde in phosphate-buffered saline (PBS), and stored at 4°C in the dark. Small drops (2 to 5 μl) of bacterial suspension were spread on poly-lysine-covered glass slides and allowed to air dry. Slides were washed extensively with water, air dried, and mounted with Sigma mounting medium (Sigma Diagnostics, St. Louis, Mo.).

Observation of the distribution of fluorescence over the cell surface was performed either by epifluorescence/phase-contrast microscopy or by confocal microscopy. In the former case, we used an Olympus BX50 microscope fitted with a 60× N.A. 1.25 Olympus UPlan Fl objective and AD photographic system. Pictures were taken on Fuji Provia 1600 film and digitized on a Nikon Coolscan III slide scanner (Nikon Co., Tokyo, Japan). Confocal microscopy was performed as indicated above for labeled sacculi.

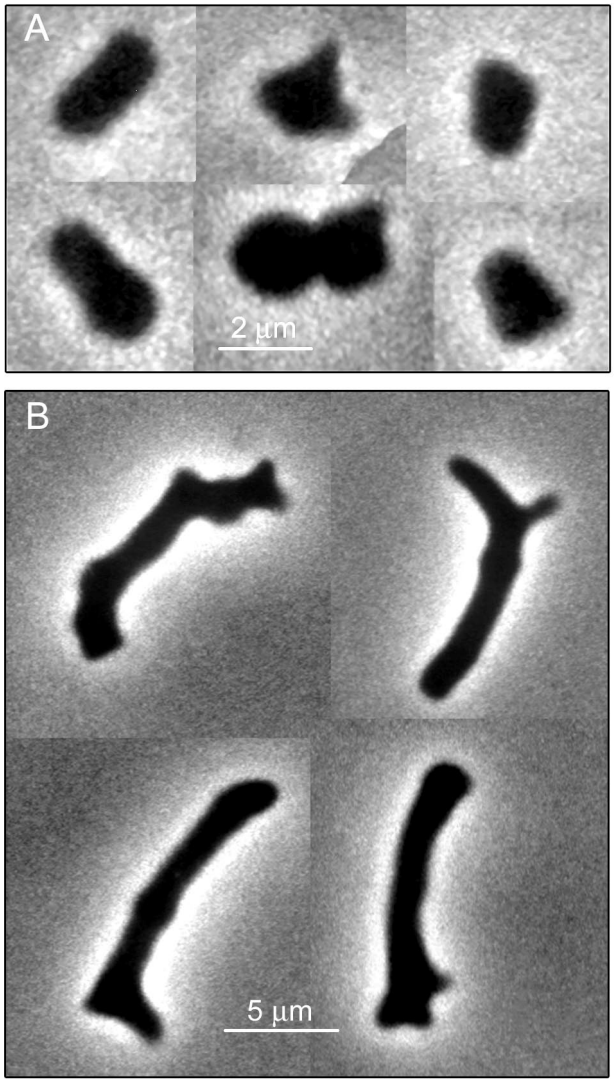

FIG. 1. Morphology of *E. coli* CS801-4 cells in the presence and absence of the cell division inhibitor aztreonam. *E. coli* CS801-4 cells were grown in LB medium and in LB plus 1 μg of aztreonam per ml for two and a half mass doubling times, and samples were observed and photographed under the phase-contrast microscope. The negatives were digitized, and mosaics with the images of selected cells were constructed with Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, Calif.) software. (A) Cells from the untreated culture. (B) Cells from the aztreonam-treated culture.

RESULTS

Morphology of *E. coli* CS801-4. Growth of *E. coli* CS801-4 in the presence of the cell division inhibitor aztreonam resulted in extensive morphological alterations, including a high proportion of branching cells, in the whole of the bacterial population, as observed previously (Fig. 1) (4, 14, 15). In the absence

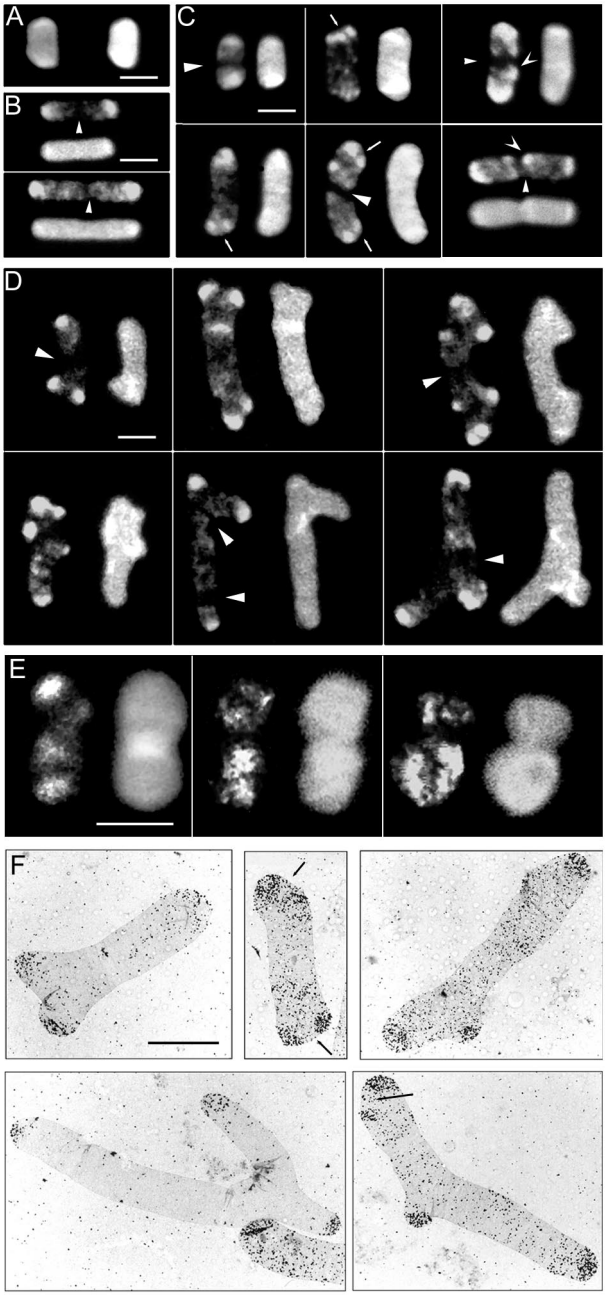

FIG. 2. Segregation of murein in D-cysteine-labeled sacculi from *E. coli* CS801-4. Cells labeled with D-cysteine were transferred to D-cysteine-free LB medium with and without 1 μg of aztreonam per ml. Samples were removed when the OD_{550} increased by three- and fivefold, and murein was purified and processed for immunodetection of D-cysteine-containing and total murein as described in the text. As a control, a culture of the parental strain CS109 was D-cysteine labeled and chased in parallel. Samples were removed at the initiation of the chase period and when the OD increased by threefold. Sacculi were observed either by confocal (A to E) or by electron (F) microscopy. Fluorescence pictures were captured in two channels; one would image the distribution of D-cysteine-containing murein (left or upper image in each frame), and the second would image total murein (right or bottom image in each frame). Mosaics depicting selected sacculi were constructed with Adobe Photoshop software. (A) CS801-4 sacculi at the initiation of the chase period. (B) Sacculi from CS109 chased in the presence of aztreonam for a threefold increase in OD. (C) Sacculi from CS801-4 chased in the presence of aztreonam for a threefold increase in OD. (D) Sacculi from CS801-4 chased in the presence of aztreonam for a fivefold increase in OD. (E) Sacculi from CS801-4 chased in the absence of aztreonam for a threefold increase in OD. (F) Sacculi from CS801-4 chased in the presence of aztreonam for a fivefold increase in OD. Silver grains reveal areas of D-cysteine-containing murein. The electron microscopic negatives were digitized and further processed for the mosaic picture with Adobe Photoshop software. Bars, 2.5 μm. Triangular arrowheads indicate potential division sites made up of "all new" murein, V-shaped arrowheads indicate regions of conserved murein outside the poles, and small arrows indicate apparently split poles.

of the inhibitor, cells had a rather normal appearance at first sight. However, a closer look revealed anomalous morphologies with a high frequency. About 20% of the cells had detectable irregularities like those shown in Fig. 1. Inhibition of penicillin-binding protein 3 was not an absolute requirement to elicit branch formation in CS801-4. Inhibition of cell division with nalidixic acid or norfloxacin also led to cells of aberrant morphology, although growth was affected and cells seldom reached the size of aztreonam-treated ones (data not shown).

Murein segregation in branching *E. coli* CS801-4. The pattern of murein segregation in branching cells of CS801-4 was studied with the D-cysteine labeling technique (4). Cells labeled with D-cysteine were diluted to an OD_{550} of 0.15 and incubated in the presence of aztreonam in D-cysteine-free medium. Samples were removed immediately after dilution and at an OD_{550} of 0.45 and 0.9. As a control, murein was also purified from a culture of the parental strain CS109 treated in parallel. Murein was purified, immunolabeled, and subjected to confocal and electron microscopy as described in Materials and Methods. Aberrant cell morphologies were accurately reflected in the shapes of isolated sacculi (Fig. 2). At short times of antibiotic treatment, most sacculi increased in diameter and exhibited small buds close to the poles. Bent sacculi and sacculi of irregular diameter (width) were also frequent (Fig. 2C). Branches as well as sacculi with multiple buds became evident only at longer times (Fig. 2D and F). Sacculi from the parental strain CS109 developed normal, straight filaments of uniform diameter in the presence of the division inhibitor (Fig. 2B).

When the fate of D-cysteine-labeled murein was analyzed in sacculi from antibiotic-treated cells, we found that morphological abnormalities correlated with strongly labeled areas in both confocal and electron microscopy photographs (Fig. 2). Long-term conservation of label denotes no or severely reduced incorporation of precursors in the corresponding area throughout the chase for D-cysteine. Interestingly, in samples chased for a short time, the polar regions of a large proportion of sacculi had a split appearance, (Fig. 2C and 2F, small arrows), and patches of strongly labeled murein were observed at atypical locations, that is, outside of the polar regions (Fig. 2C, V-shaped arrowheads). Quite often these areas were close to putative division sites, as deduced by their proximity to the middle of the cell.

Murein segregation in dividing cells was also abnormal in the mutant. Although resolution was not quite as good as for division-inhibited cells, it could be seen that, in many cells, patches of conserved murein occupied atypical locations outside of the poles and that conserved regions at the poles themselves were often split or asymmetrical (Fig. 2E). In contrast,

murein segregation in the parental strain was as described previously; that is, murein at polar regions was conserved, areas devoid of label developed at division sites, and label was gradually lost from the lateral wall during the chase (Fig. 2B) (5). Sacculi from cells chased for a long time frequently exhibited multiple buds at what should correspond to the early polar areas (Fig. 2D), and branches were often detected closer to one cell end than to the middle. Areas of label-devoid murein could be seen in many of the antibiotic-treated sacculi at positions apparently corresponding to division sites. However, these were frequently irregular and/or skewed instead of perpendicular to the axis of the sacculus (Fig. 2C and 2D, arrowheads). At the resolution attainable, no peculiarities were detected in the bifurcation points.

Labeling and segregation of surface proteins in *E. coli* CS801-4. Recent evidence indicated that the mobility of outer membrane proteins is severely restricted at the cell poles, probably through interactions with the subjacent inert murein (de Pedro, submitted). To determine whether or not the regions of stable murein associated with morphological abnormalities shared this property, cells of *E. coli* CS801-4 and the parental strain CS109 were labeled with the fluorescent dye Texas Red X-succinimidyl ester and then allowed to grow in LB plus 1 μg of aztreonam per ml. Samples were removed at appropriate times, and the distribution of fluorescence over the cell surface was observed by epifluorescence and confocal microscopy (Fig. 3).

Cells of both the parental and mutant strains showed an intense and uniform fluorescence before the chase (Fig. 3A and 3C). After a chase period, allowing for a fivefold increase in OD, it became evident that in CS109 the fluorescent signal was much stronger at the poles than in the cylindrical body of the cells, which grew as long and uniform filaments (Fig. 3B). Cells of CS801-4 chased for the same length of time developed the usual malformations, and most interestingly, fluorescence intensity was highest at the tips of the branches and at the regions where morphological alterations developed (Fig. 3D and 3E). These results indicated that diffusion of Texas Red-labeled molecules was indeed restrained at those locations.

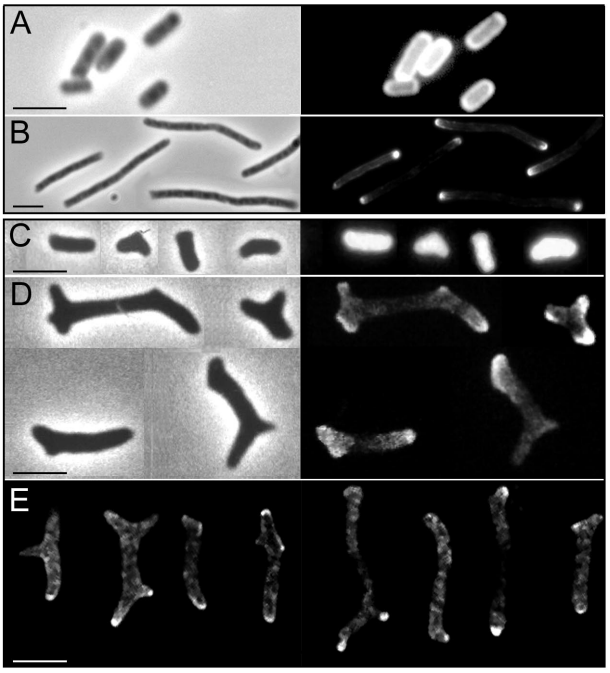

FIG. 3. Segregation of Texas Red succinimidyl ester-labeled surface components. Cells of CS801-4 and its parental strain CS109 were labeled with Texas Red X-succinimidyl ester, transferred to LB medium plus 1 μg of aztreonam per ml, and incubated at 37°C. Samples were removed immediately after dilution and when the OD had increased by fivefold. Cells were fixed and further processed for phase-contrast/epifluorescence or confocal microscopy as described in the text. (A) Unchased cells of CS109; (B) chased cells of CS109; (C) unchased cells of CS801-4; (D) chased cells of CS801-4. Right panels show the fluorescence image for Texas Red in the cells visualized by phase contrast in the left panels. (E) Selected cells of CS801-4 as observed by confocal microscopy. The optical planes showed are those apparently corresponding to the central sections of the cells. It is relevant that CS801-4 cells do not extend flat on the glass slides because they are bent in more than one plane. Nominal thickness of the optical sections was 0.53 μm. Bars, 5 μm. The figure was assembled from digitized photographic slides (phase contrast and epifluorescence) or from digital images (confocal) with Adobe Photoshop software.

DISCUSSION

The object of the present study was to find out whether generation of morphological abnormalities in *E. coli* cells with a branching phenotype was linked to topologically singular regions of the cell envelope. The morphogenetic relevance of the sacculus suggested a causal role for this particular component, and therefore, we studied murein and cell surface protein segregation in branching cells to gain information on the origin of branches.

Branching is characterized by the presence of cells with a Y or X shape. The key question was whether or not all polar caps in branching cells were equivalent. Differentiation of poles in normal cells is the final stage of cell division, but branch formation seems to be independent of cell division (9). Therefore, some other mechanism should operate for the formation of branch polar caps, which could be differentiated on the basis of murein stability. Polar murein is extremely stable in normal cells (5), and it has been argued that its inertness might be crucial for the determination of the rod shape (11).

The strain CS801-4 was chosen for our experiments because virtually all cells displayed the expected phenotypes upon cell division inhibition and more than 25% of them exhibited fully developed branches after incubation for two to three doublings in cell mass (4). It is relevant that abnormal morphologies were not found exclusively in division-inhibited cells and could be easily detected in a considerable proportion of dividing cells. Therefore, inhibition of cell division might accentuate the phenotype but is not a strict requirement. Sacculi isolated from both dividing and nondividing cells closely reflected the cellular morphologies, as expected. It is interesting that when long branches developed, the diameter of the branch was often quite uniform (e.g., Fig. 2F) and the bifurcation points were neatly defined in the sacculi. However, the polar regions showed significant changes in many sacculi, irrespective of the presence of aztreonam and of the duration of incubation with the division inhibitor.

Analysis of murein segregation in CS801-4 revealed a significantly altered pattern, in particular in sacculi from cells chased

in the presence of aztreonam. The most remarkable fact was the close association of morphological defects and areas of strongly labeled murein even in cells chased for long periods. Indeed, patches of either strong fluorescence or accumulation of protein A-gold were found in any single malformation irrespective of the kind (branch, bud, kink, etc.) or location. Furthermore, in well-developed branches, only the tips retained a strong signal. A strong signal identifies areas of the sacculus where insertion of new (unlabeled) precursors has been very low or null throughout the chase period. Therefore, our results indicated that morphological abnormalities develop from regions of the sacculus where murein metabolism is severely reduced.

In wild-type cells the polar caps are the only regions of conserved and inert murein and, as stated before, are formed at the time of septation. Patches of inert murein in the sacculus of the *dacA* mutant could distort the geometry of the nearby surface, thereby creating radial forces from the patch as new precursors are inserted in the surrounding area. These distorted areas could end up as the buds and kinks observed in the mutants. Development of branches as such could be understood as a particular case in which a bud could grow further by continuous and regular insertion of precursors around the tip and into the newly developing "stalk." Therefore, our results support the idea that malformations develop from local distortions of murein metabolism, as proposed by Gullbrand et al. (9). However, the question remains as to why regions of inert murein are found outside the poles.

Poles are the products of cell division. The physiological balance between DD- and LD-carboxypeptidases seems to be important for the location of potential division sites (10). Alteration of the balance between these activities could affect the ability of the biosynthetic complexes to identify and discriminate areas where synthesis must or must not proceed. Therefore, active synthesis might be wrongly triggered inside the polar areas, and vice versa, regions of lateral wall might be erroneously identified as polar, and consequently insertion of precursors would be prevented at those locations. A number of observations back this proposal. We repeatedly observed morphological alterations at the polar regions of cells and sacculi, and the regions of inert murein at the cell poles were very often split. Indeed, in cells chased for long periods, it was common to see structures resembling twofold-split poles. Furthermore, areas of strongly labeled murein were frequently observed in the central regions of the sacculi in the mutant but never in strains with normal morphology (Fig. 2D) (5).

In cells with impaired penicillin-binding protein 3 activity, activation of zonal murein synthesis at the division site results in the formation of rings of all new murein at the potential division sites, which in murein segregation experiments are identified as label-devoid areas (5). Sacculi of *E. coli* CS801-4 labeled with D-cysteine and chased in the presence of aztreonam showed label-devoid areas which could correspond to the rings described in cells with normal morphology (arrowheads in Fig. 2B, 2C, and 2D). However, bands were often slanted and irregular. Nevertheless, the fact that CS801-4 cells divide efficiently indicates that zonal murein synthesis sites, although possibly distorted, are competent for septation.

Evidence supporting the idea that diffusion of (some) outer membrane proteins is restricted in the polar areas of the *E. coli* cell envelope has recently been gathered (de Pedro, submitted). Reaction of growing cells with the fluorescent NH_2-specific reagent Texas Red X-succinimidyl ester resulted in the labeling of cell surface proteins without compromising cell viability. When the fate of the labeled molecules was followed over time, it was found that fluorescence was retained in the polar regions more efficiently than in the rest of the cell, as illustrated in Fig. 3B for CS109. The high residence time of labeled molecules at the polar regions suggests that they are efficiently trapped, because otherwise the signal intensity would decrease as in the rest of the cell surface. When we applied the same technique to cultures of CS801-4 chased in the presence of aztreonam, we observed a strong correlation between morphological anomalies and areas of high fluorescence intensity. Therefore, it seems that the mobility of labeled molecules is restricted in areas corresponding to patches of inert murein, as it is in the poles of normal cells. Furthermore, as cells are actively growing during the chase period, the very slow dilution rate of the signal means that no new material is inserted into the highly fluorescent areas; in other words, those areas are not expanding or are doing so at a much reduced pace.

A similar compartmentalization of the polar regions was previously observed in the periplasmic space of *E. coli* by means of fluorescence recovery after photobleaching experiments. According to these experiments, some kind of barrier (periseptal annuli) constrains molecular diffusion between the polar and central regions of the periplasmic space (6). The parallelism observed at the levels of outer membrane, murein sacculus, and periplasmic space argues strongly in favor of a causal link and supports the idea that poles are highly differentiated topological domains of the cell envelope.

Based on the observations presented, we conclude that circumstances leading to a branching phenotype alter the ability of the cell to properly discriminate those regions where insertion of murein precursors must be blocked from those where it should proceed. Morphological anomalies may arise from the cylindrical side walls because synthesis around randomly placed inert patches of murein would extend the side wall in an irregular fashion and from the poles, where insertion of new precursors splits and deforms these otherwise inert regions.

The cause for the erroneous identification of inert murein remains unknown. From the results with CS801-4, we suspect that the phenomenon arises from either a structural alteration of the murein or a specific requirement for PBP 5 itself. The mutations in CS801-4 cause the structure of murein to be deeply modified. However, the fact that a CS801-4 isogenic strain with a functional PBP 5 has a similarly altered murein composition but retains normal morphology (data not shown) favors the idea of a specific requirement for PBP 5 as a key element endowing biosynthetic complexes with the ability to recognize the areas where murein insertion must be prevented.

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