Plasmolysis During the Division Cycle of *Escherichia coli*

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Cells of *Escherichia coli* were plasmolyzed with sucrose. They were classified according to length by way of electron micrographs taken from samples prepared by agar filtration. The percentage of plasmolyzed cells increased about two- and threefold between mean cell sizes of newborn and separating cells. However, dividing cells were less frequently plasmolyzed than nondividing cells of the same length class. Analysis of cell halves (prospective daughters) in dividing cells showed that they behaved as independent cellular units with respect to plasmolysis. The results indicate that compressibility of the protoplast (given a certain plasmolysis space) is inversely related to cell size. That a dividing cell does not react as one osmotic compartment to osmotic stress may suggest that cell size-dependent strength of the cell membrane-cell wall association, rather than variation in turgor, plays a role during the cell division cycle.

It has been hypothesized that the internal osmotic pressure would fall at the end of the cell cycle and that this would allow the initiation of cell division (11). Once initiated the turgor could rise again. This is in line with the behavior of some temperature-sensitive division mutants of *Escherichia coli*. Filaments are able to divide at the restrictive temperature when, by the addition of salts or sucrose, the osmolarity of the medium is increased (12, 13, 16, 20). For this reason we would like to know whether the internal osmotic pressure changes during the division cycle.

Early experiments on bacteria have been mentioned by Mitchell and Moyle (10). However, to our knowledge it is not known whether the turgor changes when cells elongate and divide.

An indirect measure of osmotic pressure is plasmolysis. As early as 1885, de Vries (4) used plasmolysis to study the osmotic pressure in plants. Cells that have a low internal osmotic pressure are apt to plasmolyze, the more so upon further increase of the osmolarity of the culture medium.

In our case exponentially growing cultures were plasmolyzed with sucrose. The cells were then collected by agar filtration (23) and classified according to length. The percentages of plasmolyzed cells were determined in the successive length classes of nondividing cells, dividing cells, and prospective daughter cells.

The percentages increased with increasing cell length for nondividing cells. In dividing cells the fraction of plasmolyzed cells was decreased as compared with that of nondividing cells of the same respective length classes. However, when

plasmolysis of the prospective daughters (in the dividing cells) was scored separately, it was found that they behaved in the same fashion as their nondividing counterparts of the same size.

This suggests to us that the increase in the fraction of plasmolyzed cells with increasing cell length is not related to a change in internal osmotic pressure during the division cycle, but rather to an alteration in compressibility of the protoplast or strength of cell membrane-cell wall attachment (or both).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* B/r A (ATCC 12407) was obtained from C. E. Helmstetter. Cells were grown in a minimal salts medium (7) supplemented with L-alanine (0.04%) and L-proline (0.04%); the doubling time (T_D) was about 60 min. *E. coli* W7 (*dap lys*) was obtained from U. Schwarz. It was grown in a minimal salts medium (5) with 0.2% sodium succinate as a carbon source; the T_D was about 65 min. *E. coli* B/r F26 (*thy his*) was obtained from C. E. Helmstetter and grown in a medium (7) supplemented with 50 μ g of thymidine per ml, 50 μ g of histidine per ml, and 0.2% glucose as a carbon source; the T_D was about 43 min. All cultures were grown with shaking at 37°C.

Conditions for plasmolysis. *E. coli* B/r A or W7 was grown to a density of 1.25×10^8 cell per ml. To prevent deplasmolysis KCN was added at a final concentration of 10 mM at room temperature (about 20°C). Sucrose was added to induce plasmolysis. The sucrose was dissolved in the relevant growth medium to a final concentration of 1 M. Various amounts of sucrose were added to the cell suspension to obtain the desired concentrations (0.15 to 0.40 M). Incubation was carried out for 2 min at room temperature. Plasmolysis was stabilized by fixation with formaldehyde

at a final concentration of 4% during 30 min at room temperature. For an initial assessment the above steps were followed by phase-contrast light microscopy.

For *E. coli* B/r F26 a somewhat different approach was followed. Cells were grown to a density of 2.2×10^8 cells per ml and then osmotically shocked with different sucrose concentrations as above. However, instead of formaldehyde, glutaraldehyde (final concentration 0.2%) was used as a fixative. Glutaraldehyde was more effective than formaldehyde in preserving plasmolysis spaces (data not shown), and it enabled us to avoid the use of an agent to block deplasmolysis.

Electron microscopy. Samples of cells were further fixed and contrasted with a final concentration of 0.1% osmium tetroxide. Next, they were spread by agar filtration (23) and in some cases contrasted by Pt-Pd shadowing. The micrographs were taken with a Philips EM300 electron microscope.

Cell lengths were measured from micrographs or from projections of negatives with a data tablet digitizer (Summagraphics, Fairfield, Conn.) which was connected to a Hewlett Packard 9825 B calculator (Hewlett-Packard, Loveland, Colo.). With the latter instrument the results were statistically analyzed.

RESULTS

Morphology of plasmolyzed cells. Upon plasmolysis the protoplast retracts from the cell wall, presumably at the site where association of cell membrane and cell wall is weakest (3). The morphology of plasmolysis has been described by Cota-Robles (3), by Scheie and Dalen (17, 18), and by Woldringh (21). So-called plasmolysis spaces occur mostly at the cell pole and, to a lesser degree, laterally. In the latter case the location was predominantly between the cell pole and cell center, not in the cell center (cf. references 3 and 21). With increasing sucrose concentration, the size and number of plasmolysis spaces, in particular lateral ones (17), tended to increase. A light microscopic example is shown in Fig. 1.

After agar filtration for electron microscopy cells lay flattened on the perforated parlodion film (Fig. 2). Generally, the shrunken protoplast was retracted from the cell wall, which then appeared thinner. Small plasmolysis spaces hardly visible in the light microscope could still be observed in the electron microscope. For instance, a preparation which showed 25% plasmolyzed cells in the light microscope showed 74% plasmolyzed cells in the electron microscope. This difference might be explained by an enlargement of already existing plasmolysis spaces due to dehydration of samples prepared for electron microscopy. However, it is more likely that the difference is due to the superior resolution of the electron microscope. It should be added that the electron microscopic procedure did not cause plasmolysis.

Because we wished to follow the course of plasmolysis during the cell cycle by looking at

FIG. 1. Light micrograph of extensively plasmolyzed B/r A cells (T_D , 60 min). Cells were photographed after 3 min in the presence of 0.7 M sucrose. Fixation with formaldehyde at room temperature (final concentration, 4%). Magnification, $\times 6,500$.

different length classes it was relevant to compare the length distribution of exponentially growing cells before and after plasmolysis. These length distributions were transformed into cumulative ones, and the two were then compared with the Kolmogorov-Smirnov goodness-of-fit test (19; $\alpha = 0.05$). No significant difference between the two distributions was found, which means that under the above mentioned plasmolysis conditions cell length does not change. The same test was carried out for length distributions of constricted cells, which gave the same result. Also, plasmolysis did not affect the percentage of constricted cells within the total length distribution. It should be noted that, in our hands, the first visible sign of the division process was the onset of constriction. This also applies when we use the acrolein-glutaraldehyde fixation of Burdett and Murray (2) (our unpublished observations).

Plasmolysis during cell elongation. To follow plasmolysis as a function of cell length, cells were classified according to length. For each

FIG. 2. Electron micrograph of agar-filtered *E. coli* F26 cells (T_D , 43 min). Arrows indicate (a) polar and (b) lateral plasmolysis spaces. Bar, 1 μ m.

length class the fraction of plasmolyzed cells was determined. The results from measurements on cells of *E. coli* B/r F26 (T_D , 45 min) are shown in Fig. 3. The percentage of plasmolyzed cells increased with increasing length. From the mean length of the newborn cell ($\bar{L}_0$) to the mean length of the separating cell ($\bar{L}_s$) the percentage of plasmolyzed cells increased about threefold. The mean percentage of all plasmolyzed dividing cells (Fig. 3) was smaller than that of nondividing cells of the same length class. Deeply constricted cells (Fig. 3) seemed to plasmolyze less readily than the average constricting cell (cf. Fig. 3 and Fig. 6).

At first sight it would seem that the osmotic pressure decreases with cell size and that lowering of the turgor would allow the initiation of cell division. However, one arrives at a completely different interpretation if prospective daughters (cell halves) are treated separately. We calculated (Fig. 4) the fraction of plasmolyzed prospective daughters with the assumption that they behave independently with respect to osmotic stress. The calculated value (Fig. 3) fit very well on a least-squares regression line drawn through

the observed percentages for nondividing cells. In other words, it can be assumed that prospective daughters, although belonging to the same osmotic compartment, react independently on a hypertonic medium. The same conclusion can be drawn when the fraction of plasmolyzed daughter cells is plotted as in Fig. 5b. The regression line through the latter data points has a quite similar position and slope as the one drawn in Fig. 5a for the nondividing cells.

DISCUSSION

The osmotic pressure (biopolymers and electrolytes) of a cell causes the cytoplasmic membrane to push against the wall. During the division cycle the osmotic value has to increase to continuously provide cell wall stretching for the incorporation of new cell wall material (15). It might be envisaged that variation in turgor causes changes in the topological relations of murein hydrolases and their substrates, thus causing, for instance, acceleration of growth or division (or both). Similarly, cell density fluctuation has been postulated to have a signaling function in the timing of cell division (14). However, recent measurements of the buoyant densities of *E. coli* cells (9, 22) showed the variation in

FIG. 3. Percentages of plasmolyzed cells as a function of cell length in *E. coli* B/r F26 (T_D , 43 min). Symbols: $\circ$, percent plasmolysis in 1,615 nondividing cells; $\blacksquare$, percent plasmolysis in deeply constricted cells; $\times$, mean percentage of all plasmolyzed dividing cells ($n = 338$); $\square$, calculated percentage of plasmolyzed cell halves (prospective daughters). The latter was calculated from the percentage of all plasmolyzed dividing cells ($\times$), as indicated in Fig. 4. The drawn line is a linear regression line fitted to the data points. The arrows indicate the mean cell length of newborn ($\bar{L}_0$) and separating cells ($\bar{L}_s$), respectively (6). The size of the data points ($\circ$, $\circ$) reflects the number of observations.

FIG. 4. Statistical probabilities of the different possibilities for plasmolysis of a dividing cell. In this representation the percentage of plasmolyzed cells is 38.8%. The numbers represent the statistical probabilities that no plasmolysis has occurred (0.612), that one of the cell halves is plasmolyzed (0.341), and that both cell halves are plasmolyzed (0.047). The assumption is that cell halves react like individual cells. The above-mentioned values are the calculated percentages for the plasmolyzed cell halves from the measured percentages of plasmolysis in dividing cells (Fig. 3). Let P be the chance that at least one of the cell halves in a dividing cell will be plasmolyzed, then $(1 - P)$ is the chance that a dividing cell will be not plasmolyzed. From the data (Fig. 3) it follows that 38.82% of the deeply constricted cells were plasmolyzed, so $P = 0.388$ and $(1 - P) = 0.612$. If we look at the cell halves separately we can define p as the chance that a given cell half will be plasmolyzed. Assuming independence of cell halves with respect to plasmolysis, we now can calculate the chance that neither of the cell halves in a dividing cells will plasmolyze, i.e., the chance that the dividing cell as a whole will not be plasmolyzed, to be $(1 - p) \cdot (1 - p) = 1 - P = 0.612$. It follows that $p = 1 - \sqrt{0.612} = 0.218$. This means that the percentage of plasmolyzed cell halves of dividing cells would be 21.8% if cell halves reacted independently on plasmolyzing conditions. Fig. 3 shows that this value fits nicely on a least-squares regression line drawn through the data points for nondividing cells; thus, the assumption proved to be correct. For the dividing cells we can also calculate the chance that a cell will be plasmolyzed in one cell half or in both. The chance that one of the cell halves was plasmolyzed equals $p(1 - p) = 0.1704$. Because either of the two cell halves can be plasmolyzed the total chance equals $p(1 - p) + p(1 - p) = 0.341$. The chance that both cell halves will be plasmolyzed in one cell is $p \times p = 0.047$.

density to be less than 1% during the division cycle. Our plasmolysis experiments also indicate that the osmotic pressure changes little (see below). This suggests that physical factors such

as density and turgor play a very subtle role (if any) in regulating the onset of division.

Osmotic pressure and the division cycle. An increase in the fraction of plasmolyzed cells with length could mean that the turgor is falling when cells grow. However, there is a major point which should not be overlooked. The method we employed to classify cells as plasmolyzed involves the visibility of plasmolysis gaps irrespective of their size. According to the criterion of visibility of a gap there is a certain minimal gap size, which is produced at less osmotic

FIG. 5. Percentages of plasmolyzed cells as a function of cell length in *E. coli* W7 (T_D , 65 min); a, 563 nondividing cells ($\circ$); b, 349 dividing cells ($+$) and 698 cell halves taken from the dividing population ($\square$). The drawn lines are linear regression lines fitted to the data points. The average length of the newborn cell ($\bar{L}_o$) and the average length of the separating cell ($\bar{L}_s$) have been determined by the method of Harvey et al. (6).

FIG. 6. Schematic representation of the results. (i) The percentage of plasmolyzed cells increases with cell length. (ii) Dividing cells are less frequently plasmolyzed than nondividing cells of the same cell length. (iii) Prospective daughters (cell halves) react as individual cells. Within the division cycle plasmolysis frequency thus shows a periodic behavior.

pressure difference in large cells relative to small cells, because the relative volume change of small cells is bigger. Phrased differently, given a certain gap size the protoplast of large cells is easier to compress than that of small cells. Thus, if cells grew from volume x to volume $2x$ in a division cycle and the turgor remained the same, we would expect a twofold increase in the fraction of plasmolyzed cells (provided the attachment of cytoplasmic membrane to cell wall [1] is not a limiting factor). In fact we found an increase between 2.5- and 3-fold (Fig. 5 and 3, respectively); this strengthens the above interpretation, and it also indicates that something extra is happening. The extra part could represent a slight decrease of turgor with increasing cell length, a decrease in the strength of the cell membrane-cell wall attachment sites, or both. Our data indicating an independence of cell halves with respect to hypertonic environment clearly suggest that dividing cells do not have a higher turgor than nondividing cells of the same size class. The independence of plasmolysis behavior of cell halves belonging to the same osmotic compartment resembles the behavior of filaments when subjected to osmotic stress. Penicillin-induced filaments which show no signs of constriction or septation reveal, upon plasmolysis, a periodic distribution of gaps, the periodicity of which agrees with the number of cell equivalents (C. L. Woldringh, unpublished observations).

The approximately equal reaction to plasmolysis of prospective daughters as compared with newborn cells of the same size stresses the cyclic behavior of the plasmolysis parameter (Fig. 6). This is a major criterion in the evaluation of our results, for if the percentage of plasmolyzed cells is increasing during a large part of the cell cycle it necessarily has to decrease somewhere in the same cycle, too.

In summary we feel that our data mean that cells are really more prone to plasmolysis toward the end of their cycle, probably due to altered membrane-wall interactions. These changes could be linked to the process of DNA segregation or activation of murein hydrolases during cell division. A constant osmotic pressure over the cell cycle would also be in accord with a recently proposed model for growth of rod-shaped bacteria in which the turgor is the main driving force for growth of the cell wall (8).

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