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Localization of Ampicillin-Sensitive Sites in *Escherichia coli* by Electron Microscopy

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Growth of *Escherichia coli* B/r ATCC 12407 (doubling time, 65 to 70 min) in the presence of 500 μg of ampicillin per ml for 15 to 20 min induces a sphere alongside the cell. The position was determined with respect to the length axis of the cell by electron microscopy. Although spheres may be found anywhere, some prominent sites do occur. In the shortest cells, which have a length of about 1.5 μm, they are found at the presumed new cell pole. In slightly older cells (length, about 1.8 μm), the position of the sphere is not well defined. Later on spheres occur predominantly at the cell center. In dividing cells (average length, 2.8 μm) a sphere may also occur at about one-quarter of the cell length. The position of the spheres bears resemblance to sites where a pulse of ^{3}H-labeled diaminopimelic acid is incorporated into the peptidoglycan, as has been found by others.

Penicillin interferes with the cross-linking of the side chains of the peptidoglycan of *Escherichia coli* (7). At low concentration of antibiotic (10 to 50 IU/ml), cell division is prevented without cell elongation being inhibited (10). Morphologically, the site of action can be discerned by way of so-called "bulges" occurring in the cell center (1, 2, 10). At high concentration of penicillin (more than 200 IU/ml), cell division and cell elongation are prevented (3, 10). In this instance a small sphere can generally be observed alongside the still rod-shaped bacterium (3).

The distribution of spheres along the length of the cell has been used as a basis to formulate a model of growth of the bacterial cell (3). In particular, it has been emphasized that for *E. coli* cells with a doubling time (τ) of 60 min, the growth zone is located at the pole of the newborn cell; it remains at this site, i.e., at a distance of one unit cell length (1.7 μm for *E. coli* B/r and 15T$^-$JG151) from the oldest cell pole. At division this site is, therefore, located in the cell center (3). The concept that the presumed growth zone (i.e., the penicillin-sensitive site) is located asymmetrically in young cells with τ = 60 min differs from other reports on the determination of the mode of growth of the cell envelope (8). For this reason, and because the localization of small spheres was originally carried out with the light microscope (3), it appeared worthwhile to reinvestigate this matter with electron microscope techniques. We have used ampicillin instead of penicillin (2). Our observations show that topographically the ampicillin-sensitive sites resemble those where ^{3}H-labeled diaminopimelic acid is incorporated into peptidoglycan (11). The electron microscopic data differ from those obtained with the light microscope (3).

MATERIALS AND METHODS

Culture and medium. *E. coli* B/r ATCC 12407 was cultured in the minimal medium used by Helmstetter (4), with 0.1% glycerol as carbon source. The doubling time varied from 65 to 70 min. Growth was measured turbidimetrically at 620 nm. To increase the yield of smallest and largest cells, in some experiments synchronization was carried out according to the membrane elution technique described by Helmstetter (5). The elution rate of the prewarmed medium was 1.5 ml/min.

Treatment with ampicillin. The ampicillin was a gift of Beecham Research Laboratories (Amstelveen, The Netherlands). It was stored, refrigerated, as a stock solution of 10 mg/ml and used at a final concentration of 500 μg/ml. Ampicillin was added when the culture had reached an absorbance of about 0.6 at 620 nm. Ampicillin was added to synchronized cultures immediately after elution from the membrane filter (Millipore Corp.), or after subsequent growth for 30 min. Light microscope observations showed that spheres become visible after 10 min of ampicillin treatment and that a large proportion of the cells lyse after 25 min. For this reason treatment with ampicillin was carried out for 15 or 20 min. Per length class, 30 to 50% of the cells develop spheres.

Agar filtration. Agar filtration was carried out according to the method of Kellenberger (6).

Electron microscopy. Fixation with osmium tetroxide, dehydration in acetone, and embedding in Vestopal W were carried out according to Ryter et

al. (9). Locating of spheres on cells prepared by agar filtration was done on electron micrographs at a final magnification of ×12,000. For each series of photographs the magnification of the electron microscope was calibrated with a line-grating replica (spacing, 1.67 μm). Electron micrographs were taken with a Philips EM 200 or EM 300 electron microscope.

RESULTS

Morphology of ampicillin-treated *E. coli* cells. The spherical structures visible alongside the cell will be termed spheres, so as to distinguish them from the so-called bulges (10). Bulges are visible at the division site after growing the cells with 10 to 15 IU of penicillin per ml (10) or with 5 mg of ampicillin per ml (1, 2). The creation of bulges involves a rather broad zone of the cell envelope (10). This is in contrast to the spheres that arise after treatment with 417 IU of penicillin per ml (3) or 500 mg of ampicillin per ml (this paper).

As can be judged from electron micrographs (Fig. 1 and 2), a sphere arises from a very small area in the cell envelope. The spheres comprise the outer membrane, the damaged peptidoglycan layer, the cell membrane, and some cytoplasmic material. When a local rupture has occurred in the peptidoglycan, the osmotic pressure in the cell will cause an extrusion of the cytoplasmic membrane together with a small portion of the cytoplasm, which thereby expands the outer membrane. The observation that after fixation, i.e., in both agar filtration (Fig. 1) and thin sections (Fig. 2), many spheres appear to be empty suggests that the cytoplasmic membrane, together with cytoplasmic material, retracts again during preparation of the cells. Presumably, this retraction occurs when the osmotic pressure of the cell is abolished during fixation with osmium tetroxide. Occasionally an extreme case of sphere formation is observed (Fig. 2c). In such a case a large part of the cell contents is found in the sphere. With few exceptions (less than 5%), cells possessed only a single sphere after growth in the presence of 500 mg of ampicillin per ml. This may be expected, because sphere formation will relieve the osmotic pressure in the cell (3).

Location of the spheres. The location of the sphere was measured on 2,250 cells prepared by agar filtration (Fig. 1). In Fig. 3a, the distance from the center of the sphere to the nearest cell pole is plotted against the cell length. The lines drawn in Fig. 3a represent spheres located at: (i) the cell pole, (ii) one-quarter of the cell length, and (iii) the cell center. It appears that in very short cells (length, about 1.5 μm) spheres tend to originate at the cell pole. When cells grow older (length, about 1.8 μm), no preferential site for sphere formation is observed. However, later on spheres occur preferentially at the cell center. The positional distribution of spheres in cells of a given average length has been plotted in Fig. 3b. Also drawn is a line that indicates the sphere position during cell growth according to the unit-cell model of Donachie and Begg (3). This model implies that during growth in length the sphere should remain at a fixed distance (the length of the newborn cell) from one cell pole. We cannot confirm their observations. Our results show that spheres tend to arise at the cell center, except in young cells where no preference is observed (Fig. 3b). Note that the averaging procedure (Fig. 3b) obscures the occurrence of polar spheres in very short cells (Fig. 3a).

Fig. 4 shows the location of spheres in dividing cells. The cells were taken from the total population of Fig. 3a. Spheres occur predominantly at the cell center, but some clustering appears along the line representing one-quarter of the cell length.

DISCUSSION

Our observations apply to cells with a doubling time of 65 to 70 min. It has been observed that in the smallest cells spheres appear especially at the cell pole (Fig. 3a). This is probably the pole that arose from the latest division. It is plausible that the youngest cell pole has retained some of the properties of the division site. When cells grow older these properties are lost. Initially, the spheres may arise anywhere, but early in the cell cycle they become prominent at the (future) division site. Our results (Fig. 3b) clearly differ from those predicted by the cell growth model of Donachie and Begg (3). We do not know whether strain differences are important. The cells they used were *E. coli* 15T⁻JG and *E. coli* B/r. What can be said at present is that differences have been found with respect to the localization of the deoxyribonucleic acid replication period in different B/r substrains (C. L. Woldringh, unpublished data). Nevertheless, we obtained the same results with *E. coli* B/r H266 (cf. legend to Fig. 2). A more likely explanation may be the fact that we used the electron microscope instead of the light microscope (3). Perhaps the resolution of the light microscope is not sufficient to make a distinction between the curve (our observations) and the straight line (3) in Fig. 3b.

It is further noteworthy that the location of spheres bears resemblance to sites where ^{3}H-labeled diaminopimelic acid is incorporated into the peptidoglycan layer after application of

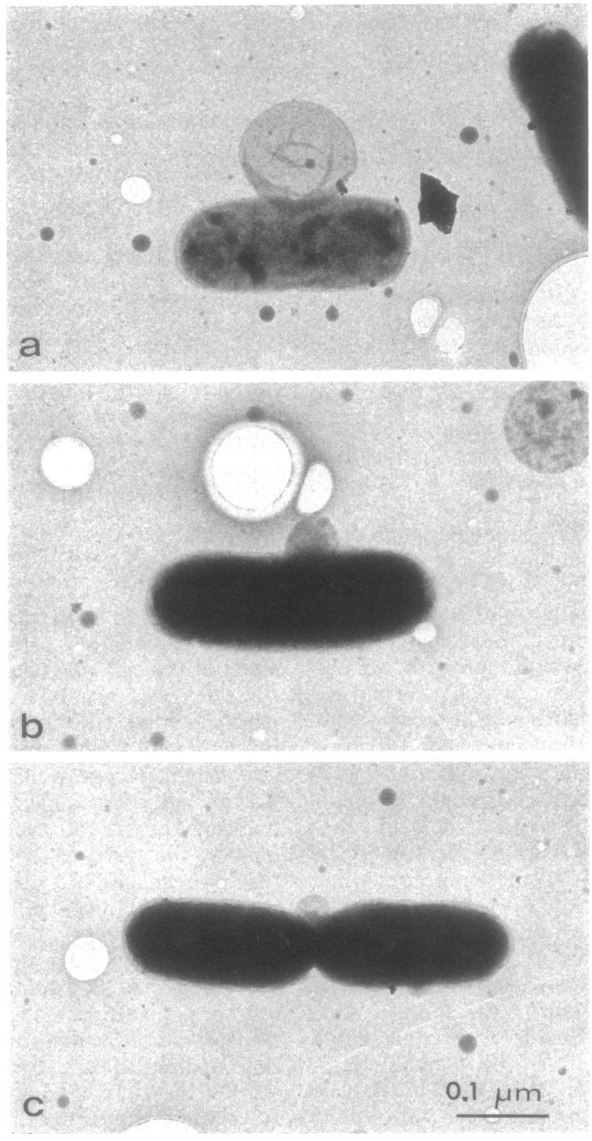

FIG. 1. *E. coli B/r ATCC 12407 treated with ampicillin. The progression of sphere formation is easily visible in these pictures. Before agar filtration, cells were fixed with osmium tetroxide.*

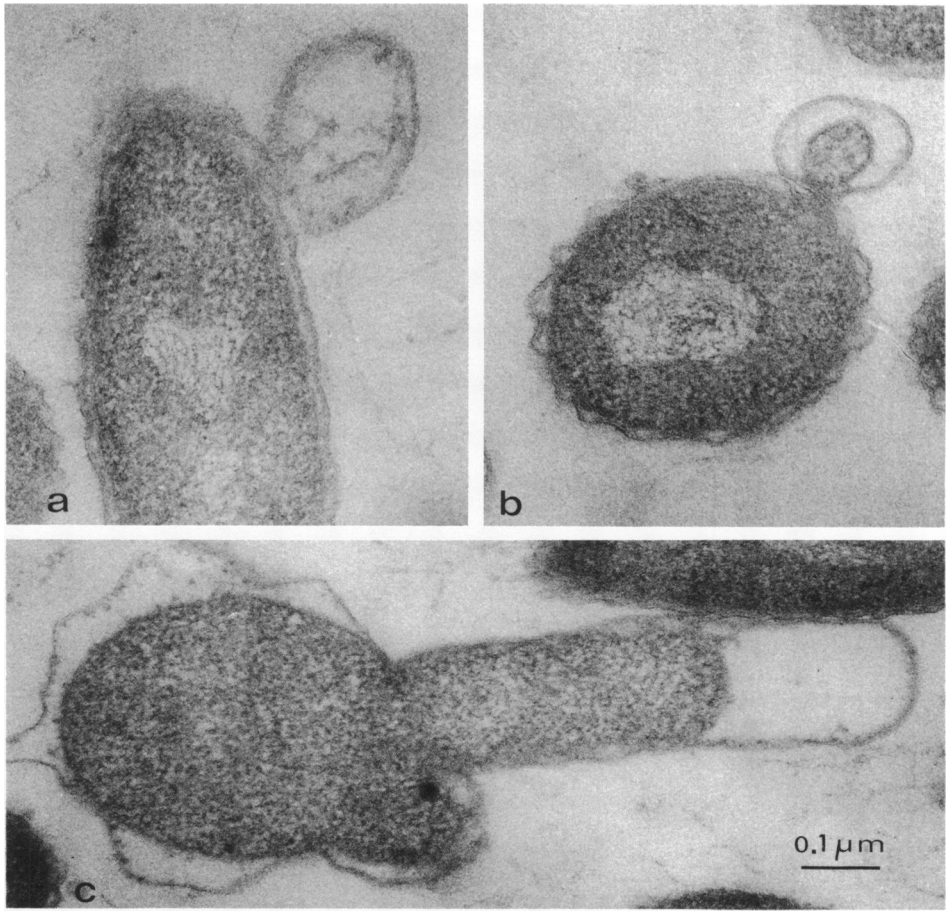

FIG. 2. *Thin sections of cells with spheres after treatment with ampicillin. An extreme case is visible in Fig. 2c. The electron micrographs apply to E. coli B/r strain H266 (Laboratory of Microbiology, Utrecht, The Netherlands). Initial experiments were carried out with this strain. The same results have been obtained as with E. coli B/r ATTC 12407. The latter strain has been preferred in subsequent experiments because it has been more widely referred to in the literature.*

a pulse (8, 11). It is not unexpected that sites sensitive to ampicillin can coincide with sites possessing strong incorporation of peptidoglycan subunits. In fact, the coordinated action of hydrolytic and synthetic activities at one and the same site is an essential part of the concept of cell wall growth (10, 12). The sites of one-quarter cell length (Fig. 2) may represent cell elongation, as suggested by Schwarz et al. (11). They become visible towards the end of the cell cycle. However, these sites are not as well defined in young cells with an average length of 1.8 μm. This could mean that after cell separation cell wall growth starts at several points along the cell wall. Alternatively, and perhaps more likely, our technique does not allow the accurate localization of spheres in short cells.

The distance between the average length of the newborn cell (l_n) and the average length of the dividing cell (l_d), as indicated in Fig. 3b, equals the doubling time (65 min). In such cells the division site becomes visible in the electron microscope about 10 min before cell separation (13). The average cell length at the time of division is 2.8 μm (Fig. 3b). However, when cells grow longer than 1.8 μm the cell center is already marked, long before a sign of septum formation can be observed in cells not treated with ampicillin. Initially, the cell center may be involved with cell elongation; in a later stage of the cell cycle septum formation may take over.

Finally, we wish to stress the different morphology of the bulges formed in the presence of

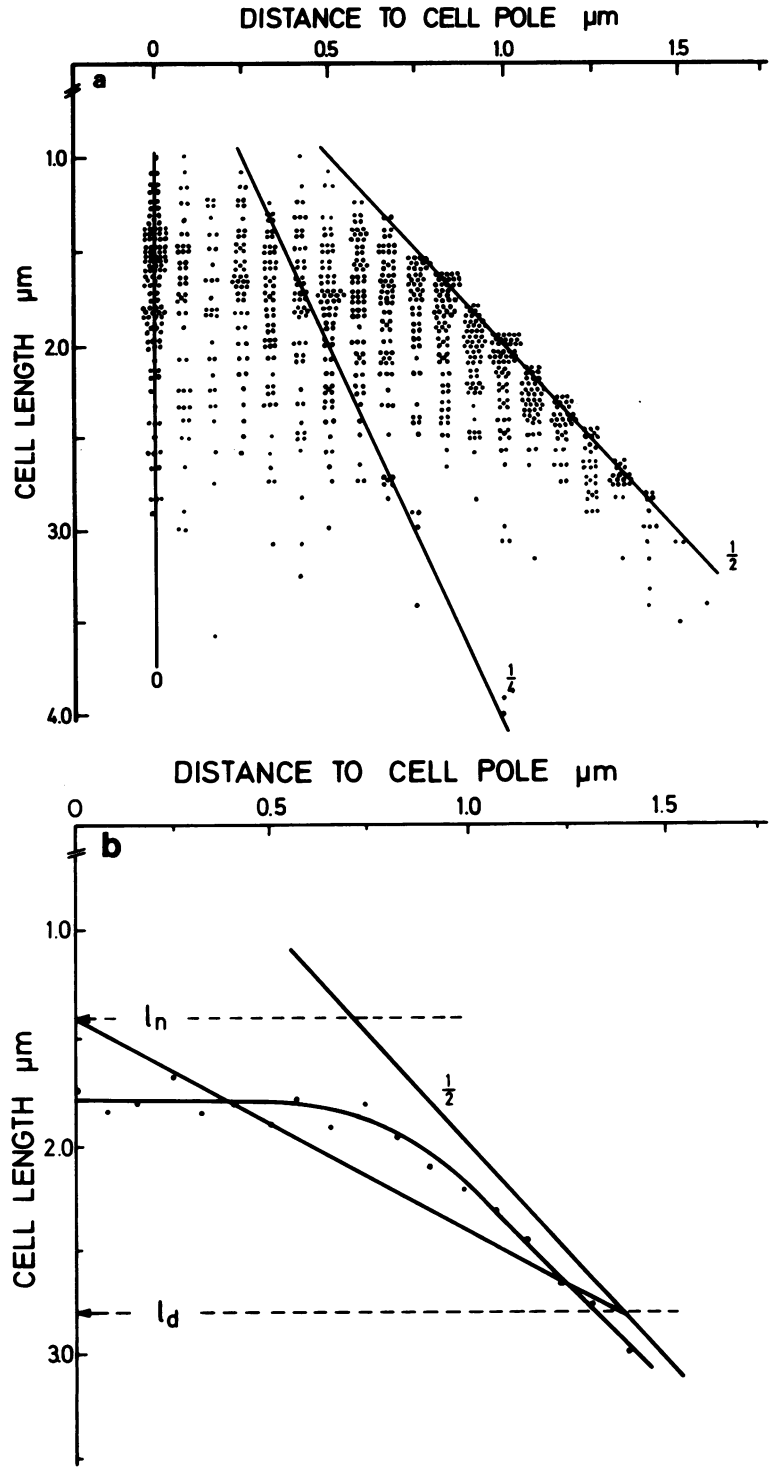

FIG. 3. *(a) Position of spheres with respect to the cell pole in cells of different length. Lines refer to spheres located at the cell pole (0), the cell center (1/2), and at one-quarter of the cell length (1/4). A total of 2,250 cells were measured. (b) Position of the sphere with respect to the average length of the cell. Data were taken from (a). An additional line has been drawn to indicate the cell center (1/2). On the vertical axis the average length of the newborn cell (l_n) is indicated. This length (1.4 µm) has been deduced from a length distribution (not shown) from cells prepared by agar filtration (13). Cells divide at an average length of 2.8 µm (l_d). The sphere position, as predicted by the unit-cell model (3), is represented by the line drawn from l_n to the center of the cell at cell length l_d. Note that in cells longer than about 1.8 µm the position of the sphere is not exactly in the middle of the cell. This is caused by the fact that sphere position has been measured with respect to the nearest cell pole.*

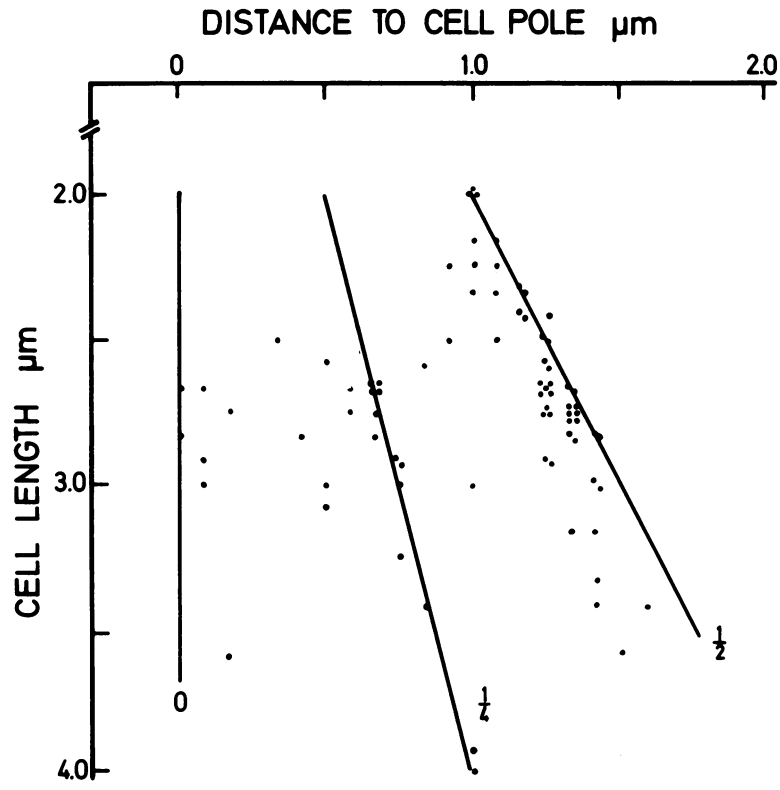

FIG. 4. *Position of spheres in dividing cells. The dividing cells were taken from the total population of Fig. 3.*

low concentrations of penicillin and spheres. In the former a large zone at the (future) division site is involved (1, 2, 10). By contrast, spheres arise at a restricted area (3; this paper), and they are filled only partly or not at all with cytoplasmic material.

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