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Proposed mechanism for generation and localization of new cell division sites during the division cycle of *Escherichia coli*

(periseptal annuli/plasmolysis/periplasm/adhesion zones)

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ABSTRACT **The earliest detectable event at future sites of cell division in *Escherichia coli* is the appearance of paired periseptal annuli that flank the site of formation of the division septum. The development and localization of these structures were followed as the cell progressed through the division cycle. The data suggest that (*i*) new periseptal annuli are generated from annuli already in position at the midpoint of the newborn cell; (*ii*) the nascent annuli are then displaced laterally during cell elongation to positions at 1/4 and 3/4 cell length; and (*iii*) the annuli at 1/4 and 3/4 cell length are retained during division, becoming the midpoint annuli of the newborn cells at the sites of the forthcoming division septum. The results indicate that the sites of future divisions can be identified and committed to the division process prior to the division cycle in which these sites are utilized for septum formation, and they suggest a model in which preexisting sites of cell division generate future division sites by a replication/displacement mechanism.**

All cells that are capable of autonomous replication must be able to identify the future site of cell division and to initiate the sequence of biochemical changes at this site that lead to formation of the division septum. The mechanisms responsible for these events are largely unknown. In this paper we describe early events in the bacterial division cycle that lead to the appearance and localization of new sites of cell division.

In *Escherichia coli*, as in most cells that divide by binary fission, cell division normally occurs at the midpoint of the cell. The first detectable differentiation event at this site is the appearance of an organelle composed of two concentric rings—the periseptal annuli—that flank the site of invagination of the septum (1). Each periseptal annulus is a continuous circumferential structure where inner and outer membranes of the cell envelope are tightly apposed to the rigid murein layer. Following cell division each daughter cell inherits a single annulus, which remains at the cell pole (1). Incomplete annuli that do not extend completely around the cell are also visible at nonseptal regions along the cell body (1, 2).

Serial-section electron microscopy has shown that the annuli form the boundaries of localized regions of plasmolysis within the periplasmic space (1, 2). The localized plasmolysis bays are also visible in phase micrographs (Fig. 1), where they provide a convenient means of establishing the locations of periseptal annuli and related structures in large numbers of cells (2).

We have used this technique to follow the formation and localization of the periseptal annuli that define the future sites of division. The experiments described below indicate that the future division site is not formed *in situ* at the midpoint of the cell. Instead, the results support a model in which the periseptal annuli to be used for future sites of division are generated by replication of preexisting annuli during the preceding cell cycle and are subsequently displaced to their final locations.

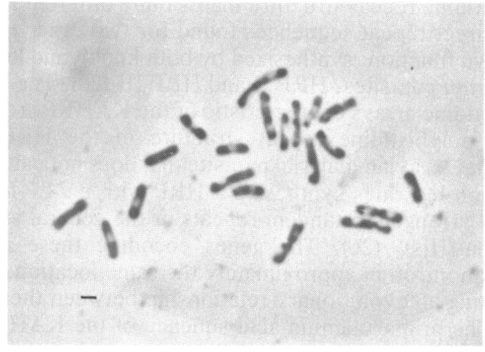

FIG. 1. Phase micrograph of plasmolyzed cells. (Bar = 1 μm.)

EXPERIMENTAL PROCEDURE

E. coli ML30 was grown in minimal salts/glucose/Mops medium (3) and cells were collected by centrifugation for 1.5 min at room temperature. After suspension in 0.25 ml of minimal salts/Mops medium (4), the cells were plasmolyzed in 20% (wt/vol) sucrose and fixed with glutaraldehyde as described (2). The tubes were allowed to stand in ice overnight before examination by phase microscopy. Preliminary experiments established that these conditions gave optimal plasmolysis for this strain under these growth conditions. The cell lengths and locations of plasmolysis bays and septa were determined by computer-assisted analysis of the enlarged phase micrographs (2). All cells that were in sharp focus were analyzed.

For curve-fitting analysis the experimental data set was sorted into eight consecutive data groups, each composed of a unique cell-length range. The data in each group were then fitted to one, two, or three Gaussian distribution curves, using the nonlinear regression routine of Johnson *et al.* (5). Computations were simplified by assuming that any lateral peaks, if present, were symmetrically distant from the cell center. This method uses a least-squares analysis to estimate the parameters of the component curves (position, standard deviation, and number of bays in each peak) by iteratively converging on a solution in which the sum of the squared residuals is minimized. To test the *in situ* biogenesis model (see *Results*), the positions of the component curves were fixed at 1/4, 1/2, and 3/4 of cell length (i.e., standard deviation and number of bays in each peak were used as the variables in the analysis).

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RESULTS

Examination of large numbers of cells from exponentially growing cultures of *Salmonella typhimurium* has shown that periseptal annuli are located predominantly at the midpoint of the cell (2). This is consistent with their suggested role as components of the division apparatus and with electron microscopic evidence that the division septum is formed between the two central annuli.

In addition to the annuli at midcell, previous studies showed plasmolysis bays clustered at 1/4 and 3/4 cell length. In the present study this was shown also to be true of *E. coli* ML30 and several strains of *E. coli* K-12 (data not shown). It was previously suggested (2) that the bays at 1/4 and 3/4 cell length might represent nascent annular structures that were precursors of the central annuli that are the sites of septum formation during the next cell cycle. In this view (*i*) nascent annuli at 1/4 and 3/4 cell length are formed during the elongation and maturation of newborn cells and (*ii*) these annuli are retained during the division process so that newborn cells already contain a pair of annuli at midcell.

To test these predictions we compared the distribution of annular structures in cells at different stages of development (Fig. 2). Cell length was used as an index of progression through the division cycle, based on the assumption that the shortest cells in the population represent the youngest cells.

The results of this analysis were consistent with the predictions of the model. The shortest (youngest) cells in the population contained plasmolysis bays only at the midpoint (Fig. 2*B*), whereas the longest (oldest) cells also contained localized periplasmic bays that were clustered at 1/4 and 3/4 cell length (Fig. 2*F*). These results are consistent with the view that the annuli at 1/4 and 3/4 cell length are formed during one division cycle and are retained after septation and cell separation to become the central annuli of newborn cells.

If these conclusions are correct, the mechanism responsible for formation of the structures at 1/4 and 3/4 cell length accounts for the localization of the septum at the midpoint of the cell during the next division cycle. This excludes a model in which mechanical stress is the trigger for the initial events in differentiation of the division site, since this would initiate the differentiation process only at the midpoint, where the stress is expected to be the greatest.

Biogenetic Models. We consider two general biogenetic models, *in situ* biogenesis and replicative biogenesis, to explain the formation of new annuli at 1/4 and 3/4 cell length. (*i*) In the *in situ* biogenesis model, new annuli are formed *de novo* at 1/4 and 3/4 cell length (Fig. 3*A*). (*ii*) In the replicative biogenesis model, new annuli are generated from annular structures that are already in place in the newborn cell and are subsequently localized by a displacement mechanism; two variations of the model are illustrated in Fig. 3 *B* and *C*.

In the first variation (bidirectional biogenesis, Fig. 3*B*), each polar annulus and each of the two central annuli generate a new annulus or annulus precursor. The final localization of the paired annuli is achieved by displacement of the new structures until they meet, thereby generating the paired annuli at 1/4 and 3/4 cell length. The distribution of plasmolysis bays expected from the bidirectional mode of biogenesis will depend on the nature of the precursor structures. If the preexisting annuli each generated an unpaired annulus precursor as illustrated in Fig. 3*B*, broad plasmolysis bays might initially be seen at 1/4 and 3/4 cell length, which then become progressively narrower as the new annuli approach each other. Other patterns would also be possible. If, for example, the polar and midcell annuli each generated an annular structure that sequestered a portion of the periplasm, new plasmolysis bays would first appear adjacent to the preexisting polar and midcell annuli. The new bays would then be progressively displaced until they met and fused at 1/4 and 3/4 cell length.

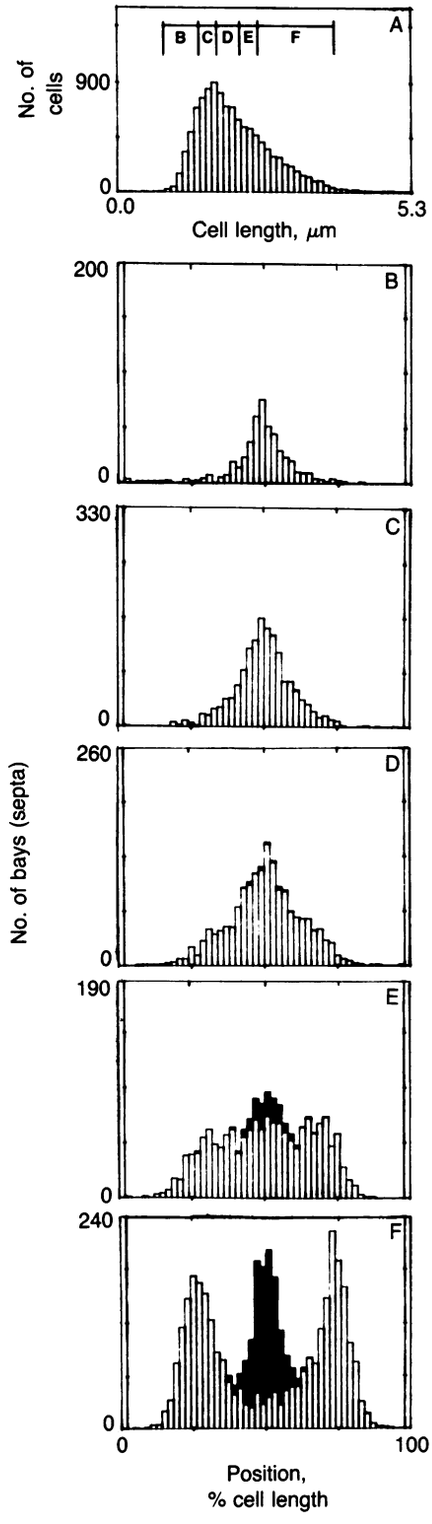

FIG. 2. Location of plasmolysis bays and septa as a function of cell elongation. A random population of cells was analyzed as described (2) and was divided into five size classes. (*A*) Cell length distribution of the total population. Letters above the bar graph indicate the subpopulations used for the analyses shown in *B–F*. (*B–F*) Location of plasmolysis bays and septa in each subpopulation. Horizontal axis represents position along the length of the cell. Vertical axis represents the number of plasmolysis bays or septa at each position. Open bars represent plasmolysis bays. Solid segments represent septa. Numbers of cells in each of the size classes were 1756 (*B*), 2972 (*C*), 2395 (*D*), 1708 (*E*), and 2173 (*F*).

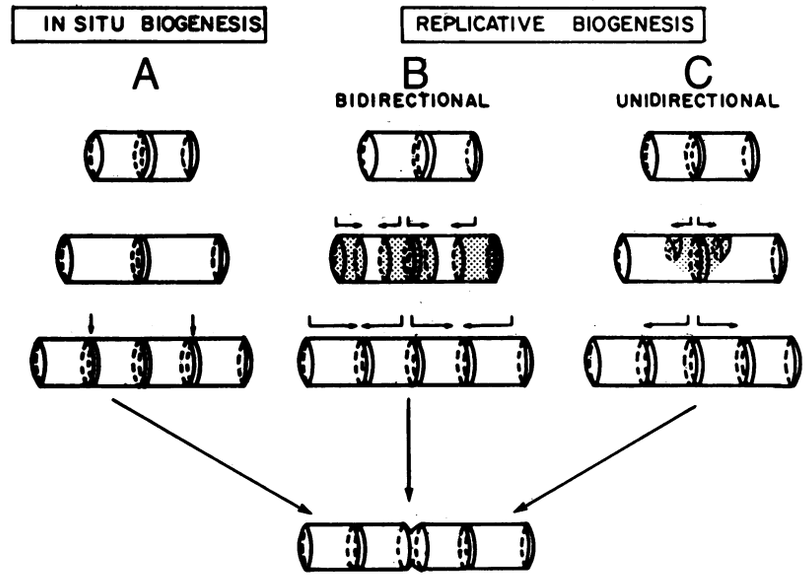

FIG. 3. Hypothetical models of biogenesis of the periseptal annuli at 1/4 and 3/4 cell length. (*A*) *In situ* biogenesis model. New periseptal annuli are generated *de novo* at 1/4 and 3/4 cell length. (*B* and *C*) Replicative biogenesis models. In both examples, precursors of the new annuli at 1/4 and 3/4 cell length are generated from the preexisting annuli, and the new structures are then displaced to their final positions as indicated by the arrows above each cell. See text for details.

In the second variation of the replicative biogenesis model (unidirectional biogenesis, Fig. 3*C*), only the central annuli participate in formation of the new structures. The new annuli on either side of the central annuli are subsequently displaced toward the nearest pole until they reach their proper locations at 1/4 and 3/4 cell length. This model predicts that plasmolysis bays will be located at intermediate positions between midcell and 1/4 and 3/4 cell length during the course of cell elongation and will be found at their final locations only late in the division cycle.

Developmental Pattern of Periseptal Annuli. To discriminate between these alternative biogenetic models, we examined cells at intermediate stages of progression through the division cycle (Fig. 2 *B–F*). In the shortest cells, plasmolysis bays were present in a single peak at midcell (Fig. 2*B*). As progressively longer cells were examined, additional bays appeared on either side of the central zone (Fig. 2 *C* and *D*). In the longest cells, septal constrictions were present at midcell and bays were clustered in two clearly defined peaks at 1/4 and 3/4 cell length (Fig. 2*F*).

The continuous nature of the progressive change in localization is shown in Fig. 4*A*, in which data from 9345 cells are displayed as a function of cell length and bay position. This reveals that bays appeared at intermediate locations between the midpoint and 1/4 and 3/4 cell length prior to the appearance of the clusters at 1/4 and 3/4 cell length. Localization of the paired annuli at 1/4 and 3/4 cell length was achieved during the last 40% of the division cycle, at approximately the same time that visible septa appeared at midcell (Fig. 4*B*).

The data were further analyzed to determine whether the clusters of plasmolysis bays could be mathematically resolved into one or more Gaussian distribution curves and to determine the locations of these curves. In the shortest cells, the data fit best to a single Gaussian distribution at the center of the cell. In cells of intermediate length, the data were best described by a set of three Gaussian distributions, one located at the center of the cell and two others lying laterally (Fig. 5). The lateral Gaussian peaks were initially positioned near the center of the cell, and as longer cells were examined, the lateral peaks were progressively displaced away from the cell center, toward final positions that approached 1/4 and 3/4 cell length (Table 1). In the longest cells, central bays could not be distinguished from septa, and the experimental data were described by a central peak, containing both bays and septa, and two lateral peaks, containing only bays and located close to 1/4 and 3/4 cell length.

These results are consistent with the predictions of the replication/displacement model shown in Fig. 3*C*, in which new annuli are generated by the preexisting periseptal annuli at midcell and are subsequently displaced toward the cell poles until they reach their final locations.

To compare these results with those predicted by the *in situ* biogenesis model, the data were also analyzed by arbitrarily fixing the positions of the lateral peaks at 1/4 and 3/4 cell length during the iterative curve-fitting procedure. χ^2 goodness-of-fit tests (6) rejected the *in situ* model ($P < 0.001$) in all cell-length groups except for the shortest (group 1) and longest (groups 7 and 8) cell-length classes, where *in situ* and replication/displacement models predict similar results.

DISCUSSION

In the present study structures resembling periseptal annuli were present at 1/4 and 3/4 cell length in predivision cells, and newborn cells contained similar structures at their midpoint. Based on the locations of these structures and their resemblance to the periseptal annuli that flank the division septum in dividing cells (2), it seems reasonable to conclude that (*i*) the structures at 1/4 and 3/4 cell length are the precursors of the annuli at 1/2 cell length in the newborn cells; (*ii*) the annuli at midcell in the newborn cells later become the periseptal annuli that flank the division septum; and (*iii*) therefore the cell can identify the proper location of future division sites and initiate the differentiation of these sites during the cell cycle that precedes the cycle in which they are utilized for septum formation. The factors responsible for this early commitment event remain to be defined.

The observation that the new structures were present at intermediate locations prior to their final localization does not support the *in situ* biogenesis model (Fig. 3*A*), in which the new structures should be formed *de novo* at 1/4 and 3/4 cell length. The distribution pattern also did not resemble any of those predicted by the bidirectional replication/displacement model (Fig. 3*B*), in which new annuli are generated simultaneously by the polar and midcell annuli. Instead, the results

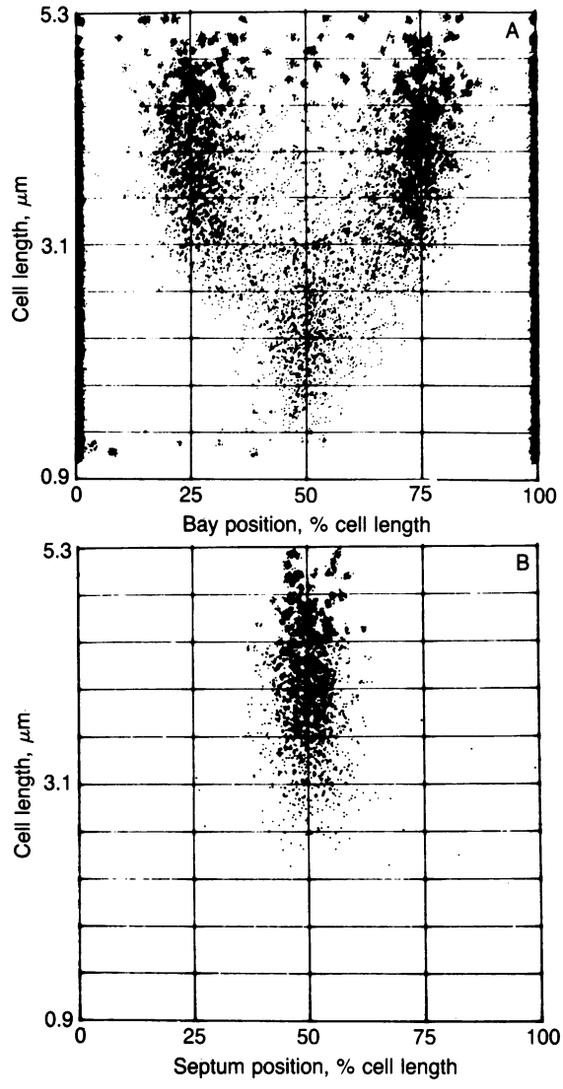

FIG. 4. Locations of plasmolysis bays and septa as a function of cell length. Cells (9345) were analyzed as described in the legend to Fig. 2. Horizontal axis indicates bay (*A*) or septum (*B*) position as percent of cell length. Vertical axis indicates cell length in μm. Each length class corresponds to a cell-length increment of 0.04 μm. To normalize for the varying numbers of cells in the different length classes, data are expressed as bays (or septa) per 100 cells; data from length classes containing fewer than 5 cells were ignored.

are consistent with the replication/displacement model shown in Fig. 3*C*, in which new annuli are generated from the central pair that are already in place at the midpoint of the newborn cell.

Nothing is known about the mechanism of formation of the new structures adjacent to the central annuli or the mechanism responsible for their lateral displacement. The present experiments also provide no information to explain the apparent arrest of the lateral displacement when the precursor annuli reach their final locations at 1/4 and 3/4 cell length.

For convenience, the diagrams (Fig. 3) show the nascent annuli at intermediate positions as discrete structures. It should be noted that the bays at intermediate positions could also be part of a more complex structure that has not yet been visualized. Similarly, the adhesion zones that delimit the plasmolysis bays at intermediate positions may not be oriented at right angles to the long axis of the cell, as shown in the diagrams, but may instead be oriented in an oblique or partially helical pattern as suggested by some of the phase and electron micrographs. Serial electron micrographs (1, 2)

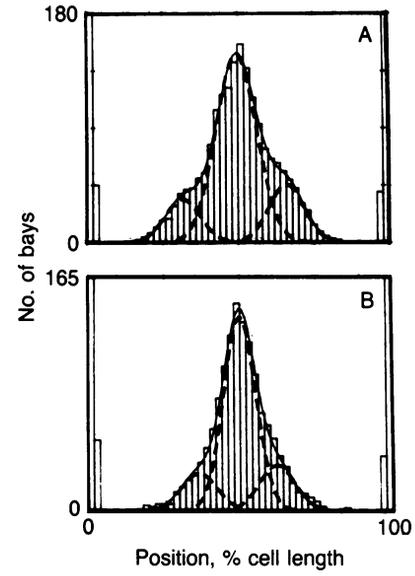

FIG. 5. Curve-fitting analysis. Best-fit solutions were calculated for data from two representative intermediate cell-length classes (*A*, 1.78–2.21 μm; *B*, 2.22–2.66 μm) from the experiment described in Fig. 4. Results are superimposed on the experimental data (histogram). Broken lines represent the component Gaussian curves. The composite curve (sum of the three component curves) is shown as a solid line that closely approximates the experimental data bars.

have also suggested that the nascent annuli may continue to mature during the stage of lateral displacement. Three-dimensional reconstructions of serial electron micrographs of cells at different stages of development should provide further information on these points, and a more complete description of the process must await these studies.

In the suggested replication/displacement model, two new division-related sites are generated by replication (using the term in a general sense) of a central site, and the new sites are subsequently displaced toward the two poles. In this sense the model recalls that proposed by Jacob *et al.* (7) in which

Table 1. Analysis of curve-fitting data

Class	No. of cells	No. of bays	Position, cell length: Central peak	Position, cell length: Lateral peak
1	1308	361	0.550 ± 0.006	—
2	2497	1435	0.507 ± 0.006	0.372 ± 0.025
3	2072	1670	0.507 ± 0.006	0.328 ± 0.026
4	1471	1561	0.525 ± 0.011	0.330 ± 0.021
5	968	1341	0.490 ± 0.048	0.273 ± 0.007
6	564	1292	0.508 ± 0.009	0.261 ± 0.007
7	311	788	0.505 ± 0.011	0.251 ± 0.009
8	107	300	0.512 ± 0.012	0.256 ± 0.015

The experimental data set described in Fig. 4 was resolved into component curves as described in *Experimental Procedure*, except that polar bays were omitted. The following cell-length classes were analyzed (probability values obtained from χ^2 goodness-of-fit tests for the best-fit solution are indicated in parentheses): class 1, 1.33–1.77 μm ($P > 0.3$); class 2, 1.78–2.21 μm ($P > 0.2$); class 3, 2.22–2.66 μm ($P > 0.3$); class 4, 2.67–3.10 μm ($P > 0.5$); class 5, 3.11–3.55 μm ($P > 0.4$); class 6, 3.56–3.99 μm ($P > 0.1$); class 7, 4.00–4.44 μm ($P > 0.6$); class 8, 4.45–4.88 μm ($P > 0.5$). Length classes containing septa (groups 6–8) were analyzed after combining septa and bays. For the best-fit solutions, the positions of the lateral and central peaks are given in fractional cell length ± 67% confidence limits. In the shortest cells a single central Gaussian curve best approximated the data. In other length classes, three peaks were obtained. Since lateral peaks were symmetrically distant from the cell center, only the position of the lateral peak between the cell center and the near pole is shown.

it was suggested that nascent daughter chromosomes are attached to cell envelope sites that originate at the midpoint of the cell and are subsequently pushed apart by central zones of cell envelope growth. There is no evidence from the present study that the lateral displacement of the two new pairs of nascent annuli reflects the presence of localized zones of cell envelope growth between them, nor is there evidence that daughter chromosomes are linked to the annuli at any stage of their development. Nevertheless, it is striking that each of the newly generated structures is destined for one of the two daughter cells from the moment of its genesis from the central annuli. The newly generated annuli therefore would be reasonable candidates to provide the DNA attachment sites that are presumably needed to explain the high fidelity of chromosome segregation during the division process.

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