The periseptal annulus: An organelle associated with cell division in Gram-negative bacteria

ABSTRACT
Evidence is presented that the site of cell division in Salmonella typhimurium is flanked by two circumspherical zones of cell envelope differentiation, the periseptal annuli, which separate the division site from the remainder of the cell envelope. Each annulus is composed of a continuous structure in which the membranous elements of the cell envelope are closely associated with the murein cytoskeleton. The paired annuli appear early in the division process and the region between them defines a new cellular domain, the periseptal compartment, within which the division septum is formed.

The process of bacterial cell division occurs by ingrowth of the cell envelope from a narrow circumspherical zone at the mid-point of the cell. This leads to segregation of the cytoplasm into two compartments and finally to separation of the daughter cells. Ingrowth of the new septum at the proper location requires that the molecular organization of the division site differ from the organization of the envelope over the remainder of the cell. The nature of this local differentiation and the cellular mechanisms that initiate and maintain it at the proper location are not known.

In this paper we describe an organelle that is associated with early stages of the division process in Gram-negative bacteria and that is likely to play a role in these events.

METHODS
Salmonella typhimurium SA534 (lkyD^-) and Rts34 (lkyD(Ts)) were grown at 30°C with shaking in proteose peptone-beef extract medium (1) to an optical density of 0.15 at 600 nm. The cultures were incubated for an additional 90 min at 42°C prior to harvesting. When strain Rts34 is grown under these conditions, most cells are present in long chains held together by bridges of outer membrane (see Fig. 1b). The remainder appear as doublets containing an outer membrane bleb over the septal region (1).

Cells were collected by centrifugation at 12,000 × g for 15 min at 4°C and washed once with 0.1 M sodium cacodylate buffer (pH 7.2). Plasmolysis was carried out by one of the following procedures: cacodylate buffer was present in the sucrose and NaCl solutions and the procedures were done at room temperature (approximately 20°C). (i) Cells were resuspended in either 312 mM sucrose for LkyD^- cells or 370 mM sucrose for LkyD^+ cells for 3 min prior to fixation by 2.5% glutaraldehyde. (ii) Cells were resuspended and washed three times with 0.15 M NaCl prior to fixation in 2.5% glutaraldehyde.

Fixation (1.5 hr) and subsequent washing and postfixation with 2.0% OsO4 were carried out in the presence of the respective plasmolyzing solutions. In the experiment described in Fig. 3, the cells were subsequently washed twice more with distilled water and resuspended for 20 min in a saturated aqueous solution of thiocarbohydrazide (Sigma) to enhance contrast in the final sections; excess thiocarbohydrazide was removed by two washes with distilled water and the cells were again exposed to 2% OsO4 prior to embedding. Embedding, sectioning, and counterstaining with lead citrate and uranyl acetate were performed as described (2). Serial sections approximately 60 nm thick were mounted on carbon-backed Formvar-coated slotted (2 × 1 mm) copper grids and examined in a Hitachi HU11E electron microscope at 75 kV accelerating voltage.

RESULTS
The cell envelopes of Gram-negative bacteria contain two membranes which completely surround the cell. Between the inner (cytoplasmic) and outer membranes lies the continuous murein layer, a rigid crosslinked peptidoglycan that provides the only known cytoskeletal structure of these and other bacteria (3). During normal cell division the three layers invaginate coordinately to form the new septum (Fig. 1a).

When cells are plasmolysed by brief exposure to hypertonic solutions, the resulting decrease in cytoplasmic volume causes the inner membrane to shrink away from the rigid murein/outer membrane layer. As originally shown by Bayer (4, 5), the plasmolysis procedure reveals the presence of small zones where the cytoplasmic membrane fails to pull away from the murein/outer membrane layer. These sites of membrane adhesion are thought to represent points of attachment or continuity between inner and outer membranes.

In the present study we examined serial sections of plasmolysed cells to determine the three-dimensional distribution of the zones of membrane adhesion. This revealed that the zones of adhesion are not individual points of membrane attachment, as had generally been believed, but are linear structures that extend for considerable distances along the body of the cell. We do not yet know whether there is any long-range order in the organization of the linear adhesion zones over the body of the cell. At sites of cell division, however, the adhesion zones are organized into two circumferential structures that flank the division site, as described below.

Periseptal Annuli. The association of zones of adhesion with sites of cell division was first noted in lkyD mutants of S. typhimurium in which septal crosswalls can easily be visualized because of a defect in outer membrane invagination during septum formation (1, 6). Because of this, LkyD^- cells grow as long chains in which individual cells are held together by bridges of outer membrane (Fig. 1b). Ingrowth of the remaining layers of the cell envelope appears to occur normally, leading to formation of crosswalls composed of inner membrane and murein (Figs. 1b and 2i). This permits the visualization of multiple septal crosswalls in random or serial electron micrographs.

Electron microscopy of plasmolysed LkyD^- cells showed a site of membrane adhesion at each of the corners of the septal crosswall.
crosswalls (D in Fig. 2j). When individual cells were examined in serial sections (Fig. 2a–h), these adhesion sites were found to extend completely around the cell, forming two continuous rings, one on each side of the septum. Each of the annular rings was composed of a structure in which inner membrane, murein, and outer membrane were closely apposed within an amorphous electron dense matrix (Fig. 2i). Fig. 2k represents a surface projection of these structures reconstructed from the serial sections.

It is relatively easy to demonstrate the annuli in LkyD− cells, in which invagination of inner membrane and murein gives rise to septal crosswalls. This allows two large periplasmic bays to form in the septal region (Fig. 2j), permitting convenient identification of the perисепtal zones of adhesion. A similar situation exists in plasmolyzed cells of enxA mutants of Escherichia coli (7) in which crosswalls and perisепtal bays are present and the perisепtal adhesion zones can be easily identified (data not shown).

It is more difficult to visualize the annular adhesion sites in LkyD+ organisms in which it is usually impossible, by normal techniques, to identify cells whose crosswall has been completed prior to cell separation (8, 9). This limits observations to stages in which the new septum extends only partially into the cell. In these cells the absence of complete crosswalls prevents formation of the characteristic paired perisепtal plasmolysis bays that are seen in Fig. 2. Therefore, because the perisепtal adhesion zones appear to be more resistant to disruption by plasmolysis than are the zones of adhesion over the body of the cell, heavily plasmolyzed cells were examined. The large periplasmic bay over the cell body was used as the major landmark to identify the perisепtal adhesion zones (D in Fig. 3h), which remain intact under these conditions. Serial sections showed that these sites extended completely around the cylinder (Figs. 3a–g and i), confirming their annular distribution. The absence of a visible bay at the division site in these cells may reflect the presence of the partially invaginated septum, which reduces the volume available for formation of a large plasmolysis bay between the two annuli; the pressure differential generated by the exit of water from the cytoplasm during the plasmolysis procedure would be dissipated adequately by the large plasmolysis bay over the body of the cell. However, because retraction of inner membrane cannot be visualized over the partially invaginated crosswall the possibility cannot be excluded that in wild-type cells the annular adhesion zone extends into the septum itself or that the inner membrane is linked to murein at the ingrowing tip of the nascent septum (see Fig. 2d and e).

**Polar Annuli.** If the normal division septum is flanked by the paired annular rings, each daughter cell should inherit a single copy of the same structure. The unpaired annulus should be located at the end of the cell—that is, at the site of the most recent septation event. This in turn predicts that circumferential zones of membrane adhesion should be present at one or both poles of cells from normally growing cultures. The presence of these polar annuli was confirmed when serial sections of plasmolyzed wild-type cells were examined (Fig. 4). The fact that polar annuli were present at both ends of the cells indicates that the polar annulus is conserved after each division event, perhaps permanently. The presence of an annular zone of membrane adhesion at each end of the cell explains previous observations that large plasmolysis bays or polar caps are often seen at the ends of cells when random sections are examined (10–12). As shown in Fig. 4j, these bays are anchored at their periphery by the polar annuli.

**Nascent Annuli.** In addition to the complete perisепtal annuli (D in Fig. 2j and k), each LkyD− cell also contained a pair of incomplete annuli (D'). The incomplete annuli were located at the midpoint of the cell, at sites where the next division event would be anticipated. Serial sections showed that each pair of incomplete annuli extended partway around the cell and there was no evidence of ingrowth of the cell envelope between them (Fig. 2a–h). Because of their location we suspect that the paired incomplete annuli represent intermediate stages in biogenesis of the complete perisепtal annuli.

The presence of incomplete annuli before visible evidence of invagination of inner membrane and murein suggests that annulus formation precedes septal ingrowth. This was supported by the observation (to be published elsewhere) that annular zones of adhesion are present at potential division sites in mutants that are completely blocked in septal ingrowth.

**DISCUSSION**

The striking association of the perisепtal annuli with the site of cell division suggests that the paired annuli play a role in the division process. We speculate that the annuli provide a mechanism to direct essential elements of the division machinery to the proper location in the cell and to maintain them there. In this view the interaction of the inner and outer membranes with the murein cytoskeleton in the annular adhesion zone plays a key role in the localization process.

The murein layer of the cell envelope is a crosslinked macromolecular structure that is responsible for the shape and mechanical resistance of the cell (13). This rigid cytoskeleton is the only known element that can provide a fixed frame of reference for the establishment of spatial relationships within the cell. It seems reasonable to suggest, therefore, that the murein skeleton acts as the measuring rod used by the cell for events that require accurate positional information, such as placement of the division septum or segregation of DNA during cell division. This implies that cellular elements responsible for these events will interact, directly or indirectly, with the murein sacculus. The perisепtal annuli are likely to be such elements because of their topologic relationship to sites of cell division and because they define a zone where the membranous elements of the cell envelope are closely associated with the murein skeleton.
assume that the membrane–murein interaction fixes the annuli in position at the midpoint of the cell, thereby ensuring that subsequent events of the division process are restricted to this location.

How do the annuli participate in the division process itself? 

**Septal Morphogenesis.** Although other models are possible, we speculate that a major function of the annuli may be to segregate periplasmic or membrane components of the division process in a new cellular domain, the periseptal compartment.

In the case of the periplasm, each annulus would act as a gasket in which the close apposition of inner membrane, murein, and outer membrane provided a physical barrier to prevent movement of molecules between the periplasm of the periseptal region and the periplasmic space along the body of the cell. Because each annulus forms a complete ring around the cell, components of the periplasm between the annuli (i.e., at division sites) would be prevented from mixing with components in the remainder of the periplasmic space. A similar mechanism

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**Fig. 2.** Periseptal annuli in LkyD− cells. Strain Rtu34 was grown, plasmolyzed with 312 mM sucrose, and prepared for electron microscopy. (a−h) Serial sections through a chain of unseparated cells. (×11,500.) (i) Higher magnification of the crosswall region between two cells of the chain to show the relationship of inner membrane, outer membrane, and murein to the annular adhesion zones (indicated by the arrowhead). (×70,400.) (j) Diagram of d. The cytoplasm is indicated by the shaded area; the clear areas indicate the periplasmic space. Large plasmolysis bays (P) are present on either side of the septal crosswalls and at the midpoints of the cells. In d and e an attachment between inner membrane and murein is present in the center of the crosswall between the two leftmost cells of the chain. This appears to represent the point of completion of ingrowth of the murein-inner membrane crosswall. (k) Surface view of the chain of cells, reconstructed from the serial sections shown in a−h. Abbreviations: IM, inner membrane; M, murein; OM, outer membrane; P, plasmolysis bays; D, zones of membrane–murein adhesion that form the periseptal annuli; D′, zones of adhesion that form incomplete annuli at the midpoints of the cells; S, septal cleft.
Fig. 3. Periseptal annuli in LkyD' cells. Strain SA534 was grown, plasmolyzed with 370 mM sucrose, and prepared for electron microscopy. (a–g) Serial sections of a cell containing a nascent septum. (×14,400) (h) Diagram of c. (i) Surface view reconstructed from the serial sections in a–g, showing the nascent septal cleft (S) bounded on either side by a continuous annular zone of membrane adhesion (D).

could exist for membrane components, with the annular zones of adhesion providing a barrier to lateral diffusion that prevents macromolecules at the division site from freely mixing with membrane molecules along the length of the cell. [A functionally similar situation may exist in epithelial cell layers where it

Fig. 4. Polar annuli in LkyD' cells. Strain SA534 was grown, plasmolyzed with 150 mM NaCl, and prepared for electron microscopy. (a–i and l–o) Serial sections of representative cells. (×14,400) (j) Diagram of e. Large plasmolysis bays are present at both ends of the cell, bounded by membrane–murein adhesion zones (D) that form the polar annuli. (a) Surface view reconstructed from the serial sections in a–i, showing the continuous zones of membrane adhesion that form the polar annuli.
has been suggested that tight junctions provide a barrier to lateral movement of molecules within the cytoplasmic membrane (14).]

As a result of this compartmentalization of membrane or periplasm, or both, the composition of the division zone (i.e., the region between the periseptal annuli) could be independently regulated, permitting molecules required for septal ingrowth to achieve and to maintain critical concentrations in this region. One might further speculate that this is accomplished by direct secretion into the periseptal compartment, perhaps from the annular adhesion zones themselves.

Alternatively, the annular adhesion zones could themselves contain essential structural or catalytic elements of the division machinery—for example, enzymes involved in synthesis of septal murein (13). In this case, isolation of the annular membrane would permit the isolation of components directly involved in the morphogenetic process.

**Localization of the New Division Site.** Prior to the events of septal morphogenesis the organism must be able to identify the midpoint of the cell and to establish it as the next division site. It is possible that the two polar annuli play a role in the initial localization process.

As a result of the division cycle each daughter cell inherits one of the two periseptal annuli. The resulting polar annulus is apparently conserved during subsequent rounds of division because growing cells contain two annuli, one at each end of the cell. These may be vestigial remnants of previous division events and may serve no further function. On the other hand, they are the only known structures that maintain a fixed topological relationship to the next division site, which will be formed equidistant from the two poles. The polar annulus therefore could be used by the cell as fixed points of reference to identify the midpoint and to initiate the division process at this site. Two general models can be considered.

(i) The two polar annuli could secrete a diffusible inhibitor of septation, either soluble or membranous. Simple diffusion would result in a concentration gradient of the inhibitor with a minimum at the center of the cell. Cell enlargement would cause a progressive decrease in concentration at midcell until a critical level was reached, thereby triggering formation of the new division site. (ii) Each polar annulus could elaborate a structural element (for example, a forerunner of a new annulus) that moved in a directed manner toward the midpoint of the cell. When the elements met at midcell, subsequent events of the division process would occur.

Other models involving the periseptal annuli in the division process can also be imagined and it should be noted that schemes for identifying the midpoint of the cell can be proposed that do not require a fixed reference point at the ends of the cell. At this time the available evidence does not permit a choice among these possibilities.

Finally, it is not known whether there is any structural, functional, or biogenetic relationship between the periseptal annuli and the zones of membrane adhesion that are present over the body of the cell (4, 5). Careful three-dimensional reconstructions of adhesion zones in synchronized cells may give information on this important point.

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