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Membrane-Murein Attachment at the Leading Edge of the Division Septum: a Second Membrane-Murein Structure Associated with Morphogenesis of the Gram-Negative Bacterial Division Septum

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Electron microscopy of plasmolyzed cells of *Salmonella typhimurium* revealed a continuous zone of membrane-murein attachment at the leading edge of the division septum at all stages of septal invagination. The membrane-murein attachment site had a characteristic ultrastructural appearance and remained as a bacterial birth scar at the new pole of each of the two daughter cells after cell separation. The continuous zone of membrane-murein attachment at the leading septal edge represents the second organelle based on a topologically ordered domain of membrane-murein adhesion to be described at the site of cell division.

Formation of the bacterial division septum occurs by the circumferential ingrowth of the cell envelope at the division site. In gram-negative bacteria, this formation requires the coordinate invagination of the three cell envelope layers, i.e., inner (cytoplasmic) membrane, murein, and outer membrane. The mechanism responsible for this topologically restricted differentiation is unknown.

A new structure that appears at future division sites in gram-negative bacteria before the onset of septal invagination was recently described (4, 8). The structure consists of two circumferential rings, the periseptal annuli, that flank the region of the cell envelope in which septal ingrowth will occur. Each annulus is a continuous zone where inner and outer membranes are closely apposed to the murein layer. It has been suggested (8) that the paired annuli define the periseptal domain and restrict essential elements of the division machinery to this location.

In this paper, we describe a second division-related structure that is associated with ingrowth of the septum and that is also based on a topologically ordered domain of membrane-murein adhesion. The new structure consists of a continuous zone of membrane-murein attachment at the innermost edge of the nascent septum. After septal closure, the membrane-murein attachment site remains as a bacterial birth scar at the newly formed pole of each of the two daughter cells.

MATERIALS AND METHODS

Salmonella typhimurium SA534 (*lkyD*$^+$), Rts34 [*lkyD*(Ts)] (5) and R71 (*lkyD*) (11) were grown in Proteose Peptone-beef extract medium (Difco Laboratories) containing 0.1 M NaCl to mid-exponential growth phase as previously described (5). Cells were harvested by centrifugation at 12,000 × *g* for 15 min at 4°C, washed with 0.1 M sodium cacodylate buffer (pH 7.2), and plasmolyzed by either (i) suspension in 10 or 20% sucrose in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 min at room temperature or (ii) suspension in 0.15 M NaCl–0.1 M sodium cacodylate buffer (pH 7.2) for 30 min at room temperature, followed by centrifugation and suspension in fresh NaCl-sodium cacodylate solution for two additional 30-min periods. The second method allows for a largely selective plasmolysis of the cell at the poles, while the first method results in general plasmolysis over the body of the cell and the poles (7, 8, 12).

Unless otherwise noted, the final cell suspension was mixed with an equal volume of 4% glutaraldehyde in plasmolyzing buffer and immediately centrifuged, and the pellets were allowed to stand, without decanting of the supernatant solution, for 1.5 h at room temperature. The pellets were then washed three times with the plasmolyzing buffer, overlaid with 2% osmium tetroxide in the plasmolyzing buffer, and allowed to stand for an additional 1.5 h at room temperature. The cell pellets were then washed twice with distilled water, dehydrated through ethanol, and embedded in Poly/Bed 812 (Polysciences, Inc.). Preparations for freeze-substitution were plasmolyzed in the appropriate plasmolyzing buffer as described above, and small samples were pelleted by centrifugation for 15 s in 400-μl Microfuge tubes (Beckman Instruments, Inc.). The tips of the tubes, containing the pellets, were cut off and immediately frozen in a Freon slush (E. I. du Pont de Nemours & Co., Inc.) cooled with liquid nitrogen. The samples were transferred to precooled (−70°C) absolute methanol and stored at −70°C for 7 days. The samples were then transferred to 2% OsO_4 prepared in absolute methanol at −70°C and were allowed to warm to room temperature over a period of 6 h. After three 20-min washes with absolute ethanol, the samples were embedded in Poly/Bed 812 as described above.

Thin sections were cut with a diamond knife and mounted on 300-mesh copper grids. Serial sections were mounted on Formvar-coated, single-hole slotted grids (2 by 1 mm; Polysciences). Counterstaining with uranyl acetate and lead citrate was performed as previously described (4), and sections were examined in a Hitachi HU11-E electron microscope with an accelerating voltage of 75 kV.

RESULTS

Membrane-murein attachments in nascent septa of wild-type cells. As originally shown by Bayer (1), zones of membrane-murein adhesion can be visualized after exposure of gram-negative bacteria to hypertonic solutions of solutes that do not readily cross the cytoplasmic membrane. The resulting decrease in cytoplasmic volume causes inner membrane to retract from the rigid murein-outer membrane layer

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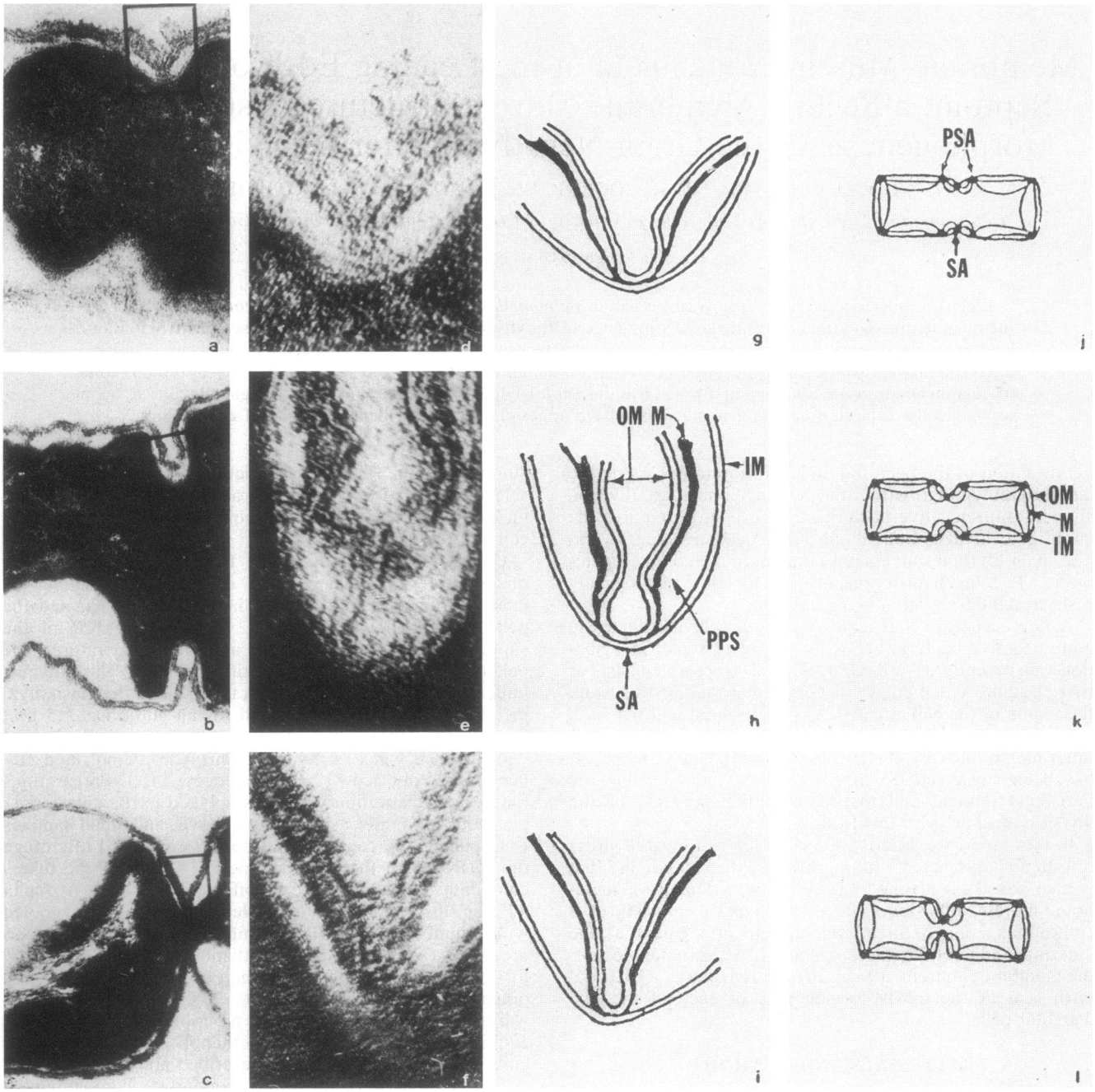

FIG. 1. Membrane attachment at the leading edge of the nascent septum. Cells of strain SA534 were plasmolyzed in 20% sucrose before preparation for electron microscopy (see Materials and Methods). (a through c) Septal region of cells at different stages of septal invagination. (Magnification, ×110,400) (d through f) Enlargements of the septal regions of panels a, b, and c, respectively. (Magnification, ×400,000.) SAS is seen as a characteristic bulbous enlargement of murein-outer membrane that lies in close apposition to the inner membrane. (g through i) Line drawings of the electron micrographs in panels d through f to indicate the relationships of the various cell envelope layers. (j through l) Diagrammatic representations of the electron micrographs. OM, Outer membrane; M, murein; IM, inner membrane; PSA, periseptal annuli; PPS, periplasmic space.

along the cell length, except for regions where the attachment of inner membrane to the murein-outer membrane layer is strong enough to resist the inward pull. These zones of membrane-murein adhesion can then be visualized by electron microscopy.

When septating cells of wild-type *S. typhimurium* SA534 were plasmolyzed with 20% sucrose and examined by this method, a zone of interaction between inner membrane and the murein-outer membrane layer was seen at the innermost edge of the nascent septum in thin sections that passed through the sagittal plane of the cell (Fig. 1a through c). The periplasmic space was visible on either side of this zone because of the retraction of inner membrane from murein-outer membrane along the remainder of the nascent septum. When viewed under higher magnification (Fig. 1d through f), the new structure (Septal attachment site [SAS]) was seen to

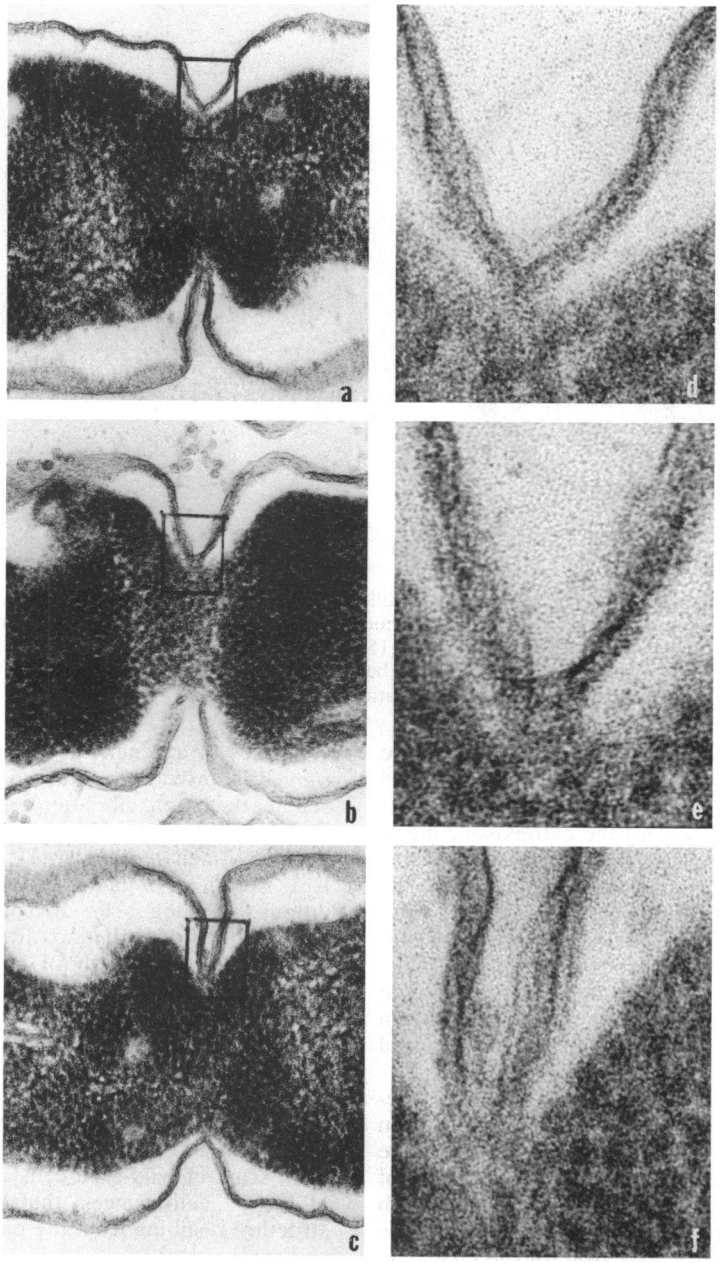

FIG. 2. Septal attachment site visualized in freeze-substituted specimens. Cells of strain SA534 were plasmolyzed in 20% sucrose and prepared for electron microscopy by the freeze-substitution procedure. Panels a through c (magnification, ×75,000) and d through f (magnification, ×380,000) represent low- and high-power views of septa that correspond to those shown in Fig. 1.

consist of a characteristic bulbous enlargement of the murein-outer membrane layer, closely apposed to the inner membrane at its point of deepest penetration. These features were present at all stages of septal development (Fig. 1a through c).

The attachment site at the leading edge of the septum was easily distinguished from the periseptal annuli that flank the division site (4, 8) because of its different location and ultrastructural appearance.

Cells prepared by freeze-substitution showed a similar zone of membrane-murein interaction at the leading edge of the developing septum (Fig. 2), indicating that the attachment was not an artifact of the standard glutaraldehyde fixation procedures.

Fate of SAS. Two possibilities exist for the fate of SAS after cell separation: (i) disappearance of the membrane-murein attachment before cell separation and (ii) maintenance of the membrane-murein attachment as a birth scar at the point of septal closure, i.e., at the poles of the two newborn cells.

Evidence that the membrane-murein attachment remained as a birth scar after cell separation was obtained from studies of the polar regions of plasmolyzed cells. Examination of small cells plasmolyzed by exposure to 10% sucrose for 3 min revealed a discrete 30-nm site at the center of the cell pole where inner membrane appeared attached to the continuous murein-outer membrane layer (Fig. 3a). When plasmolysis was carried out for longer periods by suspension of

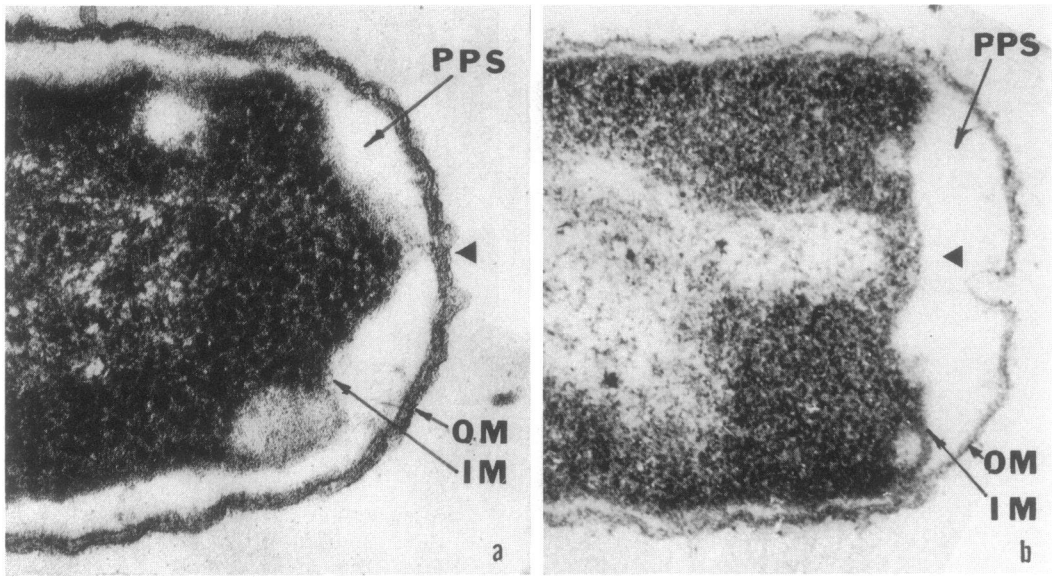

FIG. 3. Membrane-murein attachment sites at cell poles. (a) Cells of strain SA534 were plasmolyzed in 10% sucrose before preparation for electron microscopy. A discrete attachment site between inner membrane and the murein-outer membrane layer is present at the cell pole (◀). (b) Cells of strain SA534 were plasmolyzed by exposure to 0.15 M NaCl for 90 min. At the cell pole, a sharply circumscribed region is present (◀) in which the murein-outer membrane layer appears to be pulled inward toward the inner membrane. IM, Inner membrane; OM, outer membrane-murein layer; PPS, periplasmic space. (Magnification, ×38,000).

the cells in 0.15 M NaCl for 90 min, a central polar structure was also visible as a sharply circumscribed region 25 to 35 nm in diameter in which the murein-outer membrane layer appeared to be pulled inward toward the cytoplasmic membrane (Fig. 3b). In serial sections, the polar structure was visible only in the central section of the series (Fig. 4). The localized region of differentiation was never seen at both poles, suggesting that it was later remodeled, but we can not exclude the possibility that a similar structure existed at the opposite pole but was not seen because it was located between two sections. Similar results were obtained in approximately two-thirds of small cells examined by serial sectioning.

The central polar adhesion zone in the short-term sucrose-plasmolyzed cells and the central polar structure present in cells plasmolyzed for longer periods in 0.15 M NaCl were both located at the same site, corresponding to the site of final septal closure during preceding divisions, and both appeared to involve the attachment of inner membrane to the murein-outer membrane layer. The reason for the change in appearance associated with the longer plasmolysis is unknown (see Discussion).

SAS in *lkyD* mutants. Further evidence regarding the membrane-murein attachment sites at the leading septal edge and at the cell pole came from studies of *lkyD* mutants of *S. typhimurium*. LkyD$^-$ cells are characterized by a defect in invagination of the outer membrane during septum formation. As a result, septal cross walls are formed that contain inner membrane and murein but lack outer membrane (11).

Serial sections (Fig. 5) provided evidence that the murein-inner membrane attachment at the leading septal edge was retained after completion of the murein-inner membrane cross wall. The attachment in the cross wall (Fig. 5f) was limited to the central sections of the series (Fig. 5b through d), establishing its location at the center of the new septum. Since the LkyD$^-$ cross walls do not contain outer membrane (Fig. 5), we conclude that outer membrane is not an obligatory component of SAS.

Studies of a genetically leaky *lkyD* mutant, *S. typhimurium* R71, provided additional evidence for persistence of a septal attachment site after cell separation. R71 cells grown in the presence of 0.1 M NaCl are capable of completing the process of cell separation (11; K. Ishidate, J. C. Fung, and L. I. Rothfield, unpublished observation). Under these conditions, dividing cells are often held together by outer membrane bridges that are attached by narrow stalks to the central region of each of the new cell poles (Fig. 6a). Subsequent cell separation apparently occurs by the mechanical breaking and resealing of the outer membrane bridges, leaving blebs still attached to the poles of individual vcells by their narrow stalks (Fig. 6b). The attachments of the stalks to the cell poles were quite uniform, corresponding in size and location to the birth scars seen in wild-type cells (Fig. 3b and 4). The similar locations and dimensions of the birth scars of wild-type cells and the bleb attachment sites of LkyD$^-$ cells suggest that both reflect a localized polar structure resulting from the preceding septal closure event.

DISCUSSION

On the basis of these and previously reported results, it appears that the division cycle in gram-negative bacteria is associated with formation of two distinct structures, i.e., periseptal annuli (4, 8) and SAS, that are characterized by extended zones of attachment of inner and outer membranes to the murein skeleton. The locations of these structures and the proposed temporal sequence of their participation in the septation process are shown in Fig. 7.

The evidence suggesting that SAS represents a specific division-related organelle includes its unique shape in thin sections, where it is clearly distinguishable from other membrane-murein adhesion sites (Fig. 1d through f), and its location at the leading edge of the nascent septum at all stages of septal invagination. SAS appears to develop after

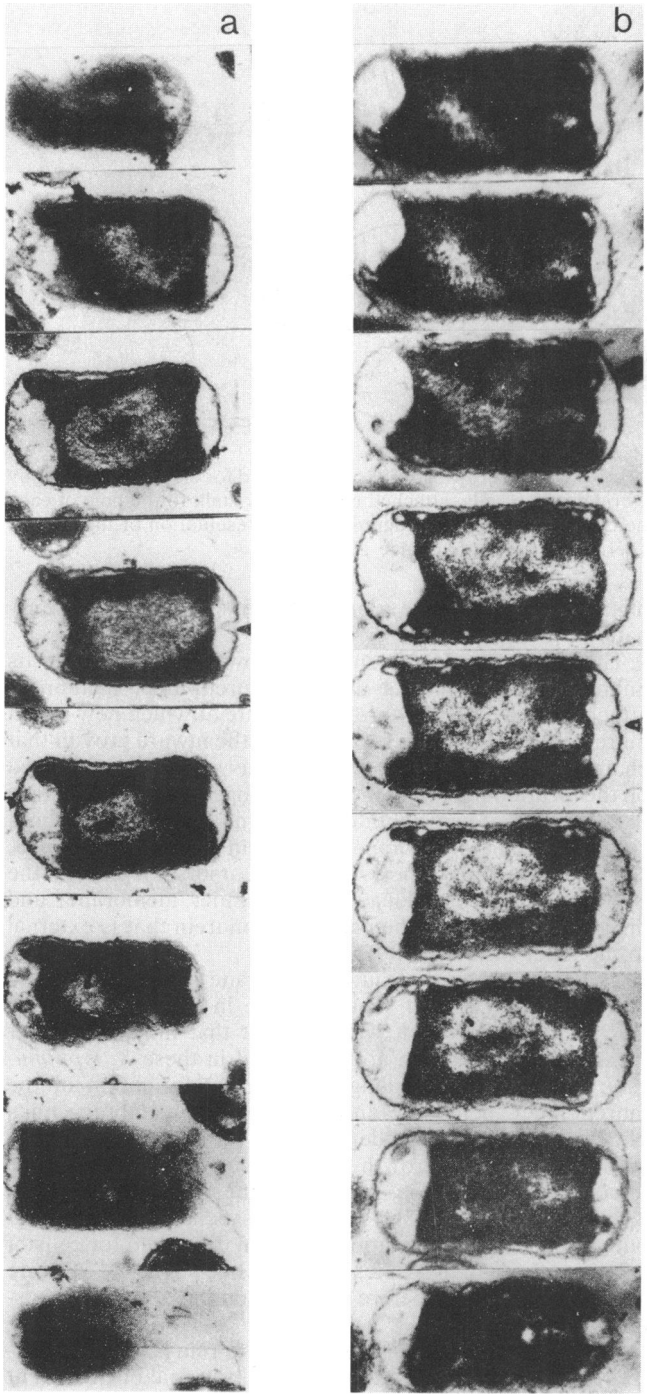

FIG. 4. Bacterial birth scars in serial sections of wild-type cells. Cells of strain SA534 were serially sectioned after plasmolysis in 0.15 M NaCl. Two cells (a and b) are shown. In each cell, large plasmolysis bays are present at each pole, bounded by polar annuli as previously described (8). The central section of each series shows a differentiated structure at one pole (◀) corresponding to the bacterial birth scar seen in random sections (Fig. 3b). (Magnification, ×14,400.)

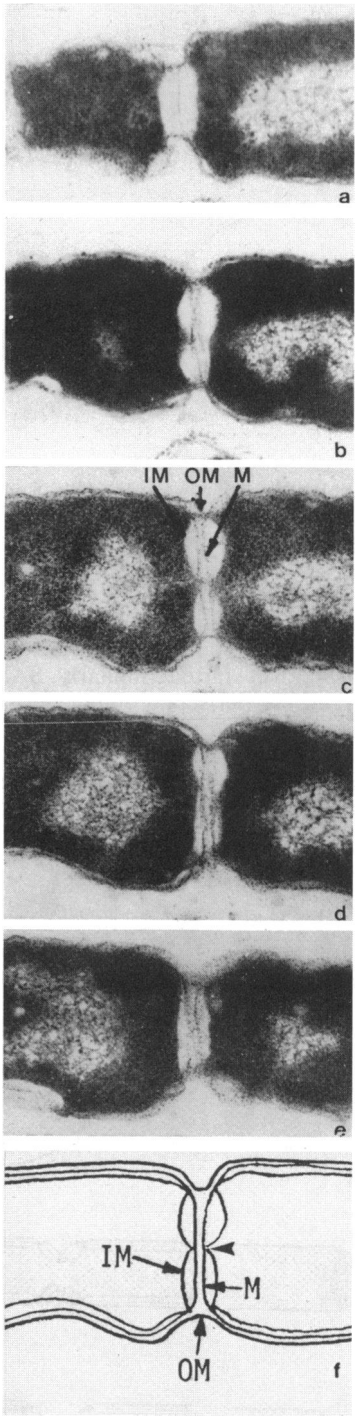

FIG. 5. Membrane-murein attachments in LkyD⁻ cells. (a through e) Serial sections prepared from cells of Rts34 plasmolyzed in 10% sucrose after growth at 42°C for 90 min. The central sections show a zone where inner membrane appears to be attached to the murein cross wall. (Magnification, ×17,000.) (f) Diagrammatic representation of panel c. The murein-inner membrane attachment site is indicated (◀). OM, Outer membrane; M, murein; IM, inner membrane.

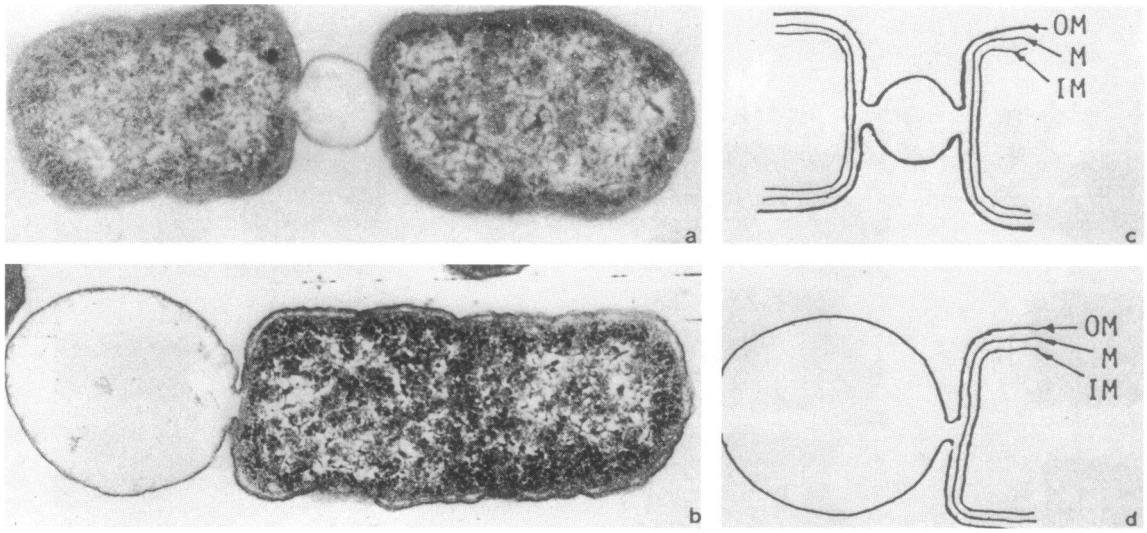

FIG. 6. Polar bleb formation in LkyD$^-$ cells. Cells of *S. typhimurium* R71 (*lkyD*) were grown at 37°C and prepared for electron microscopy without plasmolysis. (Magnification, ×18,500.) (a) Outer membrane bleb, bridging two cells, attached by a narrow stalk to the pole of each daughter cell. (b) Outer membrane bleb attached by a narrow stalk to the cell pole. (c and d) Diagrammatic representation of the sites of attachment to the cell poles. Electron micrographs are reproduced, with permission, from reference 11.

formation of the two periseptal annuli since it has not been seen between the periseptal annuli of cells in which septal invagination has not yet started. Similarly, SAS has not been observed between the periseptal annuli that are present at potential division sites in certain thermosensitive cell division mutants that are blocked before septal invagination (4; unpublished observations). Because it is present at the earliest detectable stages of septal ingrowth (Fig. 1a), formation of SAS may be a necessary first step in the invagination process.

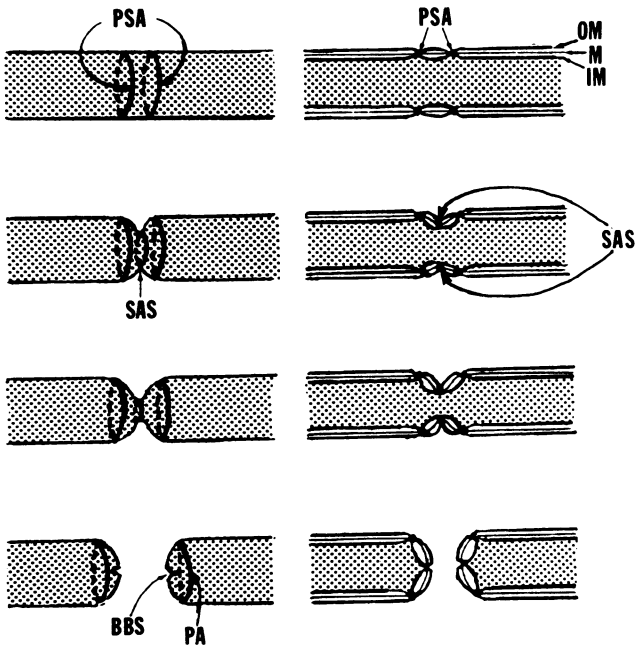

FIG. 7. Proposed sequence of events in formation of periseptal annuli and SAS during the division cycle. Diagrammatic representation of surface view (left) and cross sections (right) of locations of periseptal annuli and SAS at various stages of the division cycle. PSA, Periseptal annuli; PA, polar annuli; BBS, bacterial birth scar; IM, inner membrane; M, murein; OM, outer membrane.

The function of SAS during ingrowth of the septum is unknown. We speculate that the murein-inner membrane attachment in SAS may provide the site at which new septal murein is inserted, thereby leading to the inward growth that characterizes the nascent septum. This speculation is based on the location of SAS at the leading edge and on the premise that insertion of new peptidoglycan units is likely to occur at sites where inner membrane and murein are closely apposed, since murein biosynthesis uses precursors located in the cytoplasm, enzymes located in the inner membrane, and final acceptor sites in the preexisting murein that is external to the inner membrane (9).

The observation that outer membrane is not attached to murein at the point of septal closure in the cross walls of *lkyD* cells is consistent with the fact that inner and outer membrane invagination are uncoupled in these *S. typhimurium* mutants. This finding implies that formation of the murein-inner membrane and murein-outer membrane links of SAS occur as independent events. It has previously been pointed out that ingrowth of the two membranes is also uncoupled during the normal process of septation in *Zymomonas mobilis* (2). A similar dissociation of inner and outer membrane invagination has been described for cells of other gram-negative species that have been exposed to dilute solutions of fixatives before final fixation for electron microscopy (3, 6).

After cell separation, SAS appears to remain as a specialized region of differentiation, i.e., the bacterial birth scar, at the new poles of the daughter cells. When cells were subjected to prolonged plasmolysis, the site of the birth scar was visualized as a sharply circumscribed region where the murein-outer membrane layer appeared to be pulled toward the inner membrane (Fig. 3b and 4). This localized inward displacement suggests a relative weakness of murein in this region, perhaps enhanced by local activation of murein hydrolases during the plasmolysis procedure.

The fact that birth scars were not seen at both poles is consistent with the idea that the visible scar arises from the

most recent division event and that subsequent remodeling restores the normal cell envelope structure in this region. If this formulation is correct, the polar birth scar from the most recent division event marks the new pole. This difference in structure could be used by the cell to distinguish between new and old poles, thereby providing a mechanism to localize structures, such as polar flagella in *Caulobacter crescentus* (10), that are formed exclusively at the newest pole during the cellular division cycle.

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