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Morphogenesis of the Bacterial Division Septum: Identification of Potential Sites of Division in *lkyD* Mutants of *Salmonella typhimurium*

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It previously has been shown that *lkyD* mutants of *Salmonella typhimurium* form large blebs of outer membrane over the septal and polar regions of dividing cells. To determine whether the outer membrane blebs are formed over potential sites of division even in the absence of septal ingrowth, *lkyD* strains were studied under conditions in which ingrowth of inner membrane and murein was prevented by inactivation of the *envA* gene product. In aseptate filaments of the LkyD EnvA strain, outer membrane blebs occurred with the usual frequency and were preferentially located over regions where new septa were formed when cell division was subsequently permitted to resume. The results indicate that the outer membrane blebs of the LkyD strain are markers for potential sites of cell division, implying that an alteration in association of outer membrane and murein exists in these sites before the initiation of septal ingrowth. This localized change in cell envelope organization is independent of the septation-inducing effects of the *envA* gene product.

Formation of the bacterial division septum requires that the site of cell division be differentiated from the remainder of the cell envelope. This differentiation process appears to be under complex genetic and physiological control since septation can be prevented by mutations at several different genetic loci or by physiological manipulations which prevent DNA synthesis or interfere with other aspects of normal cell physiology (8).

The presence of differentiated sites in the cell envelope before the onset of invagination of the septum is suggested by the rapidity with which septum formation is resumed in certain septation-deficient mutants after the shift from nonpermissive to permissive conditions (2). Experimental support for the idea of preexisting sites also comes from studies of the effects of low levels of penicillin on growing cells. In this case, septation does not occur, and bulges appear over the resulting filaments (3, 7). The bulges are thought to reflect alterations in the continuity of murein in preparation for ingrowth of the division septum, although subsequent septum formation at these sites has not been demonstrated.

Interference with septation also can result from mutations which affect the coordinate invagination of the three layers of the cell envelope during cell division. These mutations (*lkyD* mutations of *Salmonella typhimurium*) are associated with a defect in ingrowth of outer membrane in the presence of apparently normal ingrowth of the cytoplasmic membrane and murein layers of the nascent septum (10). This leads to formation of blebs of outer membrane over defective septa which contain murein and cytoplasmic membrane but lack outer membrane. In addition, outer membrane blebs are present over the poles of some cells, presumably as a result of subsequent cell separation. The abnormality in outer membrane invagination is associated with an apparent defect in formation or maintenance of the normal covalent bond that links the murein-lipoprotein of the outer membrane to the murein layer of the cell envelope.

The fact that outer membrane blebs in *lkyD* mutants form over septal regions could reflect one of two situations. (i) Bleb formation could be due to a failure to form the murein-lipoprotein link at the division site at the time of initiation of ingrowth of the inner membrane and murein layers. In this case, the nascent septum would pull away from the growing but unattached outer membrane; the outer membrane then would bulge outward due to the osmotic effects of the periplasmic contents. Bleb formation would not occur in the absence of ingrowth of murein and cytoplasmic membrane. (ii) Alternatively, bleb formation could reflect local changes in cell envelope structure which precede the septation event itself. In this case, blebs would form at potential division sites even in the absence of ingrowth of cytoplasmic membrane and murein.

We have attempted to distinguish between

these possibilities by studying the expression of *lkyD* mutations under conditions in which septation (i. e., ingrowth of cytoplasmic membrane and murein) is prevented. The results indicate that outer membrane blebs in *lkyD* mutants occur over potential division sites and are markers for these sites even before initiation of septal ingrowth. Several stages of differentiation of potential division sites can be distinguished by this approach.

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MATERIALS AND METHODS

Organisms and growth conditions. Strains of *S. typhimurium* used in this study are shown in Table 1. Strain Rts34 is a temperature-sensitive *lkyD* strain obtained by mutagenesis of strain SA534. Reversion analysis and genetic mapping studies to be published separately indicate that all of the temperature-sensitive characteristics of the LkyD phenotype are due to one mutation. Bacteria were grown in proteose peptone-beef extract medium (6) with shaking.

Construction of *lkyD envA* double mutants. Strain TK484-ts34 [*envA*(Ts) *lkyD*(Ts)] was isolated from a conjugational cross between strains Rts34 [HfrK4, *lkyD*(Ts) *serA cysB*] and TK484 [*envA*(Ts) *his thy*]. After 3 h of mating at 30°C, Cys^+ His^+ recombinants were obtained from selective plates containing serine and thymine. Clones were checked at 30 and 42°C for leakage of RNase, sensitivity to deoxycholate and rifampin, and formation of aseptate filaments. Clone TK484-ts34 showed all of these characteristics when grown at 42°C but not when grown at 30°C, confirming the LkyD(Ts) EnvA(Ts) phenotype.

Strain TK484-71 was obtained in a similar manner from the cross between strains R71 and TK484 by selection for His^+ Ser^+ recombinants on selective plates containing thiamine, thymine, and adenine. When grown at 37°C, strain TK484-71 leaked RNase, was sensitive to deoxycholate and rifampin, and grew as filaments; when grown at 30°C, the strain did not form filaments but continued to show RNase leakage and drug sensitivities.

Identification and localization of blebs. Cells were examined by phase microscopy as previously described (10). To determine the location of blebs, photographs were taken of every cell containing a visible bleb, and the position of each bleb was determined by measuring the distance of the bleb from the closest end of the cell. The position was expressed as the ratio of the measured distance to the total length of the cell. Blebs were scored as septal or polar if they were localized over visible complete or incomplete septa or over the ends of cells (see Fig. 2); blebs were scored as nonseptal if they were located over aseptate regions of filaments. To determine the frequency of blebs per ml of culture, the ratio of visible blebs to total cells was determined by phase microscopy, and the total number of cells was determined in parallel in a hemacytometer chamber; the number of blebs per milliliter of culture was calculated from these two values. Electron microscopy was performed as previously described (4).

TABLE 1. *Strains of S. typhimurium*

Strain	Genotype	Source
SA534	*serA* HfrK4	K. Sanderson
R71	*lkyD serA pur* HfrK10	R. Weigand et al. (10)
Rts34	*lkyD*(Ts) *serA cysB* HfrK4	Mutagenesis of SA534
TK484	*envA*(Ts) *his BHAFIE thy*	Z. Ciesla et al. (2)
TK484-ts34	*envA*(Ts) *lkyD*(Ts) *serA thy*	Rts34 × TK484 (this paper)
TK484-71	*envA*(Ts) *lkyD pur*	R71 × TK484 (this paper)

RESULTS

Location of outer membrane blebs in dividing cells. As reported previously (10), approximately 20% of the LkyD cells contained outer membrane blebs over the septal and polar regions of the cell (Fig. 1). When 120 bleb-containing cells were examined by phase microscopy, every bleb was located either over a division septum or at the pole. In strain R71, blebs were seen in cells grown at 30, 37, or 42°C; in strain Rts34, the blebs were seen when the cells were grown for 60 min at 42°C but not when grown at 30°C (Fig. 1c).

Prevention of septum formation in *envA lkyD* double mutants. To determine whether expression of the *lkyD* mutation requires ingrowth of the inner two layers of the septum, the temperature-sensitive *lkyD* mutation of strain Rts34 was introduced into a temperature-sensitive septation-defective mutant, TK484 [*envA*(Ts)]. Strain TK484 is unable to initiate septum formation at elevated temperatures and therefore grows as long, multinucleate filaments when incubated at 37°C or higher. Examination of 1,000 randomly selected filaments from a 42°C culture confirmed that the filaments of strain TK484 did not contain outer membrane blebs.

In the double mutant TK484-ts34, the *envA* and *lkyD* mutations are both temperature sensitive. Therefore, when filaments are formed by shifting the cells from 30 to 42°C, blebs that are present over the filaments should represent new blebs formed during the period of blocked septation. As shown in Fig. 2, the filaments that were formed during growth at 42°C contained many blebs, whereas, as expected, cells grown at 30°C showed neither filaments nor blebs. Of 1,860 cells examined by phase microscopy after 90 min at 42°C, 97% were filaments longer than two normal cell lengths, and a total of 160 blebs were seen; nearly all (154/160) of the visible blebs were located over filaments. In contrast, of 4,000 cells examined after growth at 30°C for 5 h only 0.1% contained visible blebs when exam-

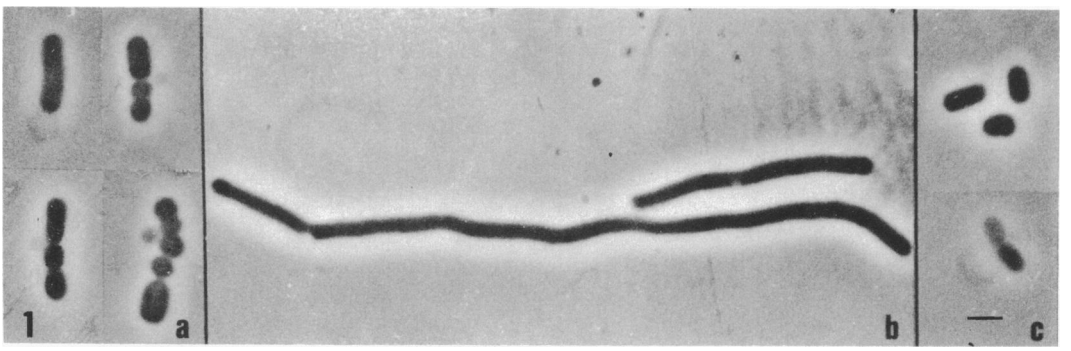

FIG. 1. *Phase micrographs of lkyD and envA strains. (a) R71 (lkyD) grown at 37°C, (b) TK484 [envA(Ts)] grown at 42°C, (c) Rts 34 [lkyD(Ts)] grown at 30 and 42°C. Bar represents 1 μm.*

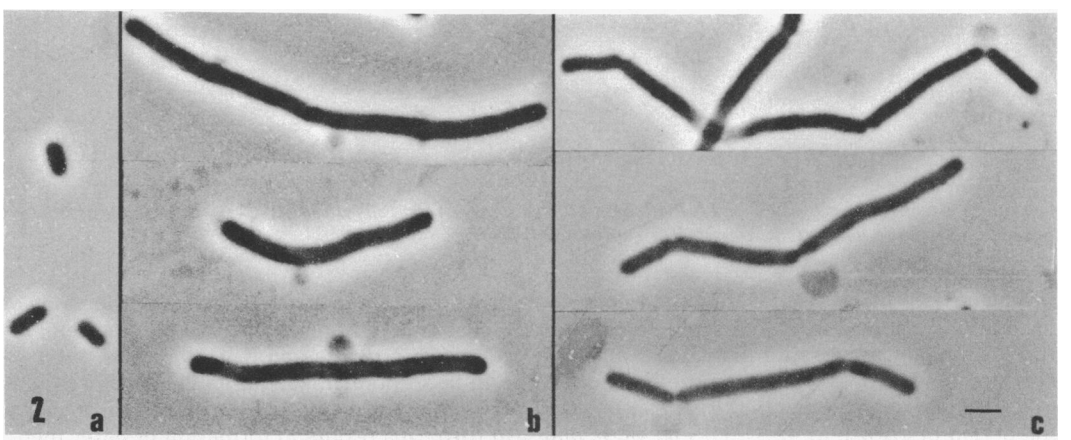

FIG. 2. *Phase micrographs of TK484-ts34 at 30 and 42°C. An actively growing culture of strain TK484-ts34 at 30°C was shifted to 42°C. After 105 min, the culture was shifted back to 30°C for 90 min. (a) After initial growth at 30°C. (b) After 105 min at 42°C. (c) 90 min after shift back to 30°C. Bar represents 1 μm.*

ined by phase microscopy, and only occasional short filaments were seen. The outer membrane nature of the blebs and the absence of septal invagination were confirmed by electron micros copy of 25 randomly selected bleb-containing cells (Fig. 3).

Further evidence that bleb formation did not require ingrowth of inner membrane and murein was obtained from studies of strain TK484-71, in which the *lkyD* mutation originated from the independently isolated mutant strain R71 rather than Rts34. The double mutant TK484-71 [*envA*(Tss *lkyD*] retained the temperature-sensitive septation defect of strain TK484. Phase and electron microscopy showed that the filaments contained many outer membrane blebs located along the length of the cells; there was no visible ingrowth of inner membrane and murein at the sites of the blebs.

These results established that formation of outer membrane blebs in *lkyD* mutants did not require ingrowth of the inner two layers of the nascent septum. This prompted us to ask whether blebs seen over aseptate filaments were randomly distributed or whether they were preferentially found in specific locations, perhaps over potential sites of division. The position of blebs along the length of the filaments was determined by analysis of phase micrographs. This revealed that the blebs were not randomly distributed but rather were clustered in regions corresponding to 0.5, 0.25, and 0.12 to 0.16 cell lengths (Fig. 4a), suggesting a possible relationship to potential sites of cell division.

If the blebs in the aseptate filaments were located over potential sites of division, resumption of normal septum formation should result in the preferential localization of new septa at the sites of the preexisting blebs. This was tested by shifting the cultures of strain TK484-ts34 from 42 to 30°C and incubating for 90 min at 30°C, permitting septum formation to resume because of the reversible nature of the *envA* defect. Examination of the culture at this time

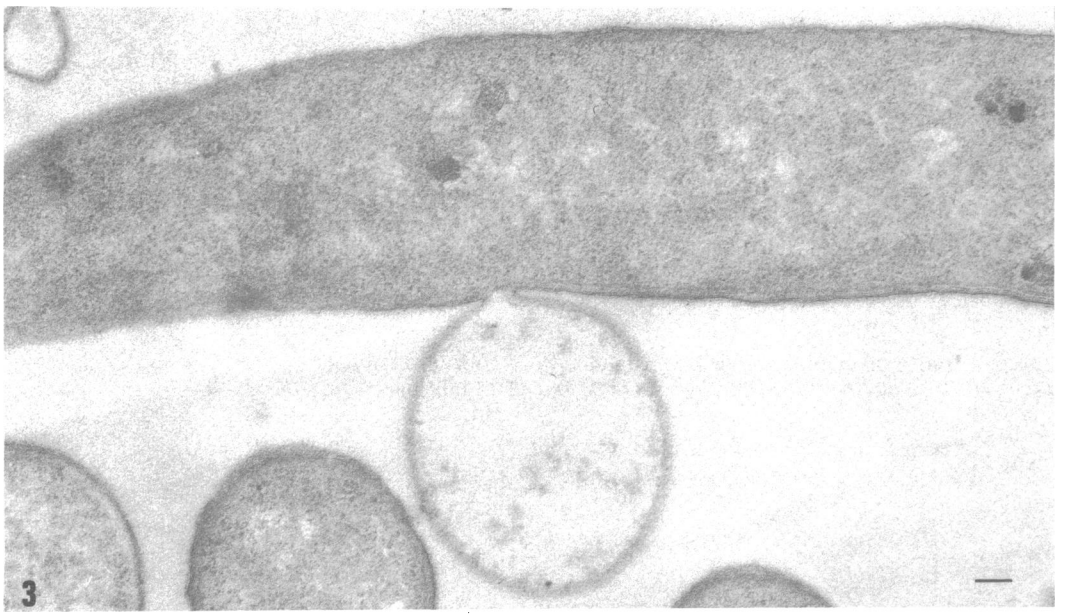

FIG. 3. *Electron micrograph of TK484-ts34. Cells were grown for 120 min at 42°C. An outer membrane bleb is seen over a long aseptate filament. Bar represents 0.1 μm.*

revealed that blebs now were predominantly located over septa (Fig. 2 and Table 2) and that the locations of new septa and of septal blebs corresponded almost exactly to the location of blebs formed over filaments during the preceding period of growth at 42°C (cf. Fig. 4a, b, and c).

The total number of blebs did not change after the shift to 30°C. This indicated that no new blebs were formed at the lower temperature and confirmed that the septal blebs seen after the shift to 30°C represented blebs that had been formed during the preceding period of growth at 42°C.

DISCUSSION

The present experiments establish that outer membrane blebs in *lkyD* mutants are located at potential sites of division. The following evidence supports this conclusion: (i) in dividing cells, blebs were only seen over septa and over the poles of cells; (ii) in nonseptating cells produced by inactivation of EnvA function, blebs were not randomly distributed over the resulting filaments but were present in discrete clusters; (iii) the positions of these clusters corresponded to sites at which septation occurred when cell division resumed.

Thus, the blebs of the *lkyD* strain act as markers for regions in which the cell envelope has differentiated in preparation for ingrowth of the new septum. The results suggest that this differentiation includes a change in the mode of association of outer membrane and murein, resulting in an increased dependence on the murein-lipoprotein link in potential division sites as compared with other regions of the cell envelope; in turn, this results in formation of outer membrane blebs in these locations when formation of murein-bound lipoprotein is inhibited by inactivation of LkyD function, as shown in the present study. Further evidence of a similar nature came from studies of the *lpo* mutant of *Escherichia coli* studied by Suzuki et al., in which lipoprotein is not synthesized (9). In this lipoprotein-deficient strain, outer membrane blebs occur with high frequency in low-Mg^{2+} medium (4, 9). These blebs are predominantly located over septal and polar regions; 93% of blebs seen in an actively growing culture of the *lpo* stain JE5505 in low-Mg^{2+} medium were located over septa or poles when 100 consecutive bleb-containing cells were examined by phase microscopy (Fung, unpublished data). It is striking that in both the *lkyD* and *lpo* mutants, outer membrane blebbing occurs predominantly over actual or potential division sites, implying a difference in the association of outer membrane with murein in these regions.

The local change in cell envelope structure occurs in the absence of EnvA function and thus defines a stage in maturation of division sites that is distinct from the septation-inducing ef-

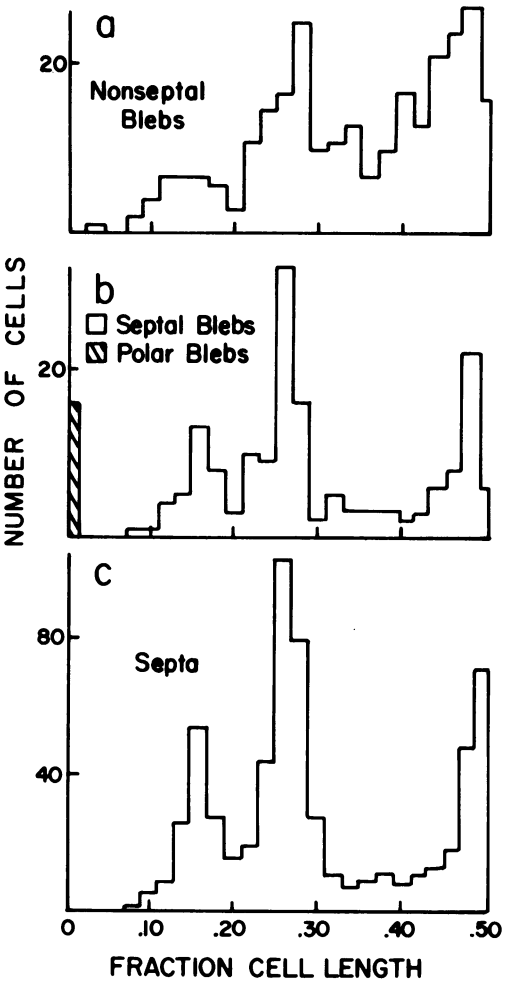

FIG. 4. *Location of blebs and septa in TK484-ts34. Experimental details are given in the legend to Fig. 2. Cells were examined by phase microscopy at 0 and 90 min after the shift to 30°C, and the positions of blebs and septa were determined as described in the text. The appearance of the cells and the numbers of septal and nonseptal blebs are shown in Fig. 2 and Table 2. All blebs seen at 0 and 90 min are included in the figure to give a valid representation of the total population of blebs that were formed during growth at 42°C. (a) Blebs over nonseptate filaments, (b) blebs over septa and poles, (c) septa.*

fects of EnvA. Since the blebs represent the outer membrane from these sites, isolation and characterization of the bleb membrane may provide direct information about changes in organization of the outer membrane that may be related to the division process.

The molecular basis for the morphological change is not known, but there are several possibilities. (i) Murein-lipoprotein may not be distributed randomly throughout the cell envelope but may be concentrated in division sites; in this case, interference with the murein-lipoprotein bond would be expected to cause blebbing of outer membrane only at division sites. Although possible, we do not consider this highly likely because of the large number of molecules of lipoprotein in the cell (1) and the demonstration in *E. coli.* that loss of the lipoprotein can lead to loosening of outer membrane over the body of the cell (9). (ii) Division sites may be characterized by a local alteration in outer membrane composition. This could involve a localized deficiency in one or more of the outer membrane proteins that normally maintain the outer membrane in close apposition to murein. These include the lipoprotein (responsible for the covalent outer membrane-murein link) and other proteins, such as the matrix protein of Rosenbusch (5), which appear to be involved in noncovalent interactions with murein. A relative deficiency in one or more of these proteins at potential division sites would make the outer membrane-murein association in these regions more vulnerable to factors which interfere with the murein-lipoprotein link. (iii) Division sites may be characterized by local changes in murein which reduce the number of accessible attachment sites for lipoprotein and matrix protein(s). This, too, would result in localized blebbing when the murein-lipoprotein link was further compromised. Such an alteration in murein could result from the localized murein hydrolase activity in potential division sites suggested by the studies of Schwarz et al. (7). At this time, the available evidence does not permit a choice among the above possibilities, which are not mutually exclusive.

TABLE 2. *Location of blebs in TK484-ts34 after shift from 42 to 30°C*[a]

Time after shift (min)	No. of nonseptal blebs	No. of septal or polar blebs	Total blebs per ml of culture
0	0.92	0.08	6.2×10^6
90	0.38	0.62	5.9×10^6

[a] Experimental details are described in the legend to Fig. 2. The number of nonseptal and septal or polar blebs is expressed relative to the total number of blebs seen at each time point.

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