Septum Formation in *Escherichia coli*: Characterization of Septal Structure and the Effects of Antibiotics on Cell Division

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Septa can be demonstrated in sections of *Escherichia coli* strains B and B/r after fixation with acrolein and glutaraldehyde. The septum consists of an ingrowth of the cytoplasmic membrane and the mucopeptide layer; the outer membrane is excluded from the septum until the cells begin to separate. Mesosomes have also been observed. The septum is highly labile and, except in the chain-forming strains, *E. coli* D22 env A and CRT 97, not easily preserved by standard procedures. The labile nature of the septum may be due to the presence of autolysin(s) located at the presumptive division site. Blocking division by addition of ampicillin (2 to 5 \( \mu \)g/ml) to cells of *E. coli* B/r produces a bulge at the middle of the cells; bulge formation is stopped by addition of chloramphenicol. Cephalosporins also induce bulge formation but may stop cell elongation as well as division. Bulge formation, due to the presumed action of an autolysin(s), may be an initial step in the septation sequence when the mucopeptide is modified to allow construction of the septum. In a nonseptate filament-forming strain, PAT 84, which ceases to divide at 42 C, bulge formation only occurs in the presence of ampicillin at the time of a shift-down at 30 C or at 42 C in the presence of NaCl (0.25 to 0.34 M). Experiments with chloramphenicol suggest that the filaments are fully compartmentalized but fail to divide owing to the inactivation, rather than loss of synthesis, of an autolysin at 42 C.

Attempts have been made to follow the growth pattern of individual cells of *Escherichia coli* (for reviews, see 7, 17), but it has not been possible to differentiate the principal stages. This is due, in part, to the apparent absence of distinct septa in many strains under the usual conditions of fixation (49). Instead, the dividing cell merely seems to constrict at the middle, and all wall layers, including the cytoplasmic membrane, infold together (49). Only in certain chain-forming strains (30, 34), some thermosensitive mutants (22), and in *E. coli* B held at 45 C (49) has it been possible to demonstrate septa routinely.

Although a relationship exists between chromosome replication and cell division in *E. coli* (5-7, 15, 20, 53), very little is known about the conditions which initiate septation, i.e., entry of the cells into the D period (5). Genetic characterization of conditional thermosensitive mutants (39) indicate that at least seven genes (fts A-fts G) are involved in septation. Complex though the analysis of the septation process is likely to be, it is important to stress that septation must essentially involve some modification of both the cell wall and the cytoplasmic membrane. This fact has been amply demonstrated in studies of *Streptococcus faecalis*, for example (17). In this organism it has been possible to show, by electron microscopy, that wall growth occurs at a specific region of the cell and that the development of the septum follows a definite course. As in bacilli (8), the nascent cross wall is closely associated with the cytoplasmic membrane and with mesosomes.

Growth of the cell wall is likely to be modulated by autolysins (in the case of *S. faecalis*, an N-acetylmuramidase) whose primary target of attack is the mucopeptide (peptidoglycan, murain) moiety of the cell wall. The mucopeptide itself consists of covalently bonded polysaccharide strands cross-linked by peptide side chains (see ref. 3). Cumulative evidence from studies of bacilli (9, 10, 11, 17, 41) and of *S. faecalis* (17) suggest that autolysins may fulfill a number of specific roles in wall growth, such as: (i) cleavage of bonds in mucopeptide to allow for insertion of newly synthesized constituents; (ii) localized hydrolysis of bonds at specific areas of the wall to allow for alteration in cell shape.

accompanying specific events of the cell cycle, such as septum formation; and (iii) a role in cell separation. Autolysins may therefore be involved in the initiation of wall synthesis as well as localized remodelling of the wall. In S. faecalis, autolysis proceeds from the newly synthesized tip of the growing cross wall, whereas the peripheral wall is less susceptible to autolytic attack (16).

In E. coli, the evidence of autolysis action is less direct, but it has been shown that two growth processes, septation and elongation, can be differentiated by the response of the cells to varying doses of penicillin (46). Low doses of penicillin (10 to 50 U/ml) block division and give rise to a bulge located at the middle of the cell at presumptive cross wall sites. Elongation of the cells can continue under these conditions, but ceases on the addition of high concentrations (100 U/ml or more) of penicillin which totally inhibit mucopeptide synthesis. The formation of a bulge is thought to be due to the continued action of hydrolytic enzymes (autolysins) acting on mucopeptide after inhibition of the transpeptidation reaction (46) with penicillin.

The reasons for the common occurrence of "constrictive" modes of division is at present obscure, and one object of this paper is to re-examine the nature of this phenomenon. The results indicate that septa can be preserved in some strains of E. coli, such as B and B/r, and that the labile nature of the septum may be due to the presence of autolysin(s) located at the site of the cross wall. Studies of a thermosensitive mutant, PAT 84, suggest that the autolysin(s) may be involved in modifying the mucopeptide layer at an early stage of septation.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of E. coli examined in the present study are shown in Table 1. The thermosensitive mutants CRT 97, CRT 257, and PAT 84 were grown at 30 C in a reciprocating shaker-water bath; the restrictive temperature chosen was 42 C. All other strains were grown in water baths at 37 C. Flasks five times the volume of the culture were used throughout.

The following media were made in glass distilled water, and the concentrations of the ingredients are shown in grams per liter: (i) M9 medium (ref. 29): NH4Cl (1.0); Na2HPO4 (7.0); KH2PO4 (3.0) supplemented with MgSO4, 7H2O and glucose to final concentrations of 0.1% and 0.1%, respectively. (ii) Minimal medium (ref. 15): NH4Cl (2.0); Na2HPO4 (6.0); KH2PO4 (3.0); NaCl (3.0); MgSO4 (0.25) and glucose (1.0). Stock solutions of MgSO4 and glucose were autoclaved separately. In some experiments this medium was supplemented with Casamino Acids and tryptophan to final concentrations of 0.2% and 20 µg/ml, respectively. (iii) LB medium (ref. 2): tryptone (Difco) (10.0); yeast extract (5.0); NaCl (10.0); glucose (1.0). (iv) Complete medium (ref. 22): Beef extract (10.0); bacitryptone (10.0) and NaCl (10.0). In the case of E. coli PAT 84, the NaCl was omitted from the medium or the level was reduced to 5.0 g/liter (see below). In all experiments, complete medium was supplemented with thymine (20 µg/ml, final concentration).

Growth of the cultures was monitored by following the absorbance at 660 nm (A660) in a Beckman DU spectrophotometer, using cells of 1-cm path length. Cell numbers were estimated with a model B Coulter counter (Coulter Electronics, Hialeah, Fla.) using an aperture of 30 µm. Samples (1.0 ml) were removed at intervals from the culture and placed into an equal volume of 0.5% formalin (in 0.9% NaCl) before counting or simply diluted into 0.9% NaCl and counted directly. All fixations were performed with exponentially growing populations having an A660 of 0.2 to 0.35.

Electron microscopy. (a) Fixation: (a) The standard OsO4 fixation schedule of Ryter and Kellenberger (RK; ref. 45) was used. Samples (0.9 ml) were removed from the culture and pipetted into the fixative (1.0 ml), centrifuged at 5,000 × g for 5 min, enrobed in 2% agar, and left for about 16 h in the 1% OsO4 solution. After being washed with 0.5% uranyl acetate dissolved in RK buffer, the pellets were dehydrated in ethanol or acetone and embedded in Epon or Vestopal by standard procedures.

(b) Most samples were fixed with acrolein and glutaraldehyde by adding 1/10 volume of fixative, either directly to the culture flask or by removing a sample of cells. After centrifugation at 5,000 × g for 5 min, the cells were resuspended in 1 to 2 ml of the stock fixative solution containing acrolein (5%), glutaraldehyde (0.25%), and 0.05 M sodium cacodylate buffer, pH 7.5, for 3 to 6 h at room temperature; (1. D. J. Burdett and R. G. E. Murray, submitted for publication). The pellets, previously enrobed in agar and washed overnight in buffer, were postfixed with 1% OsO4 in 0.05 M cacodylate buffer, pH 7.5, for 1 h at room temperature. Occasionally, some samples were postfixed with 1% OsO4 by the RK technique (see above).

Glutaraldehyde and acrolein were obtained as electron microscopy grade products, sealed under N2, from Polysciences Inc. (Warrington, Pa.).

(c) Our most successful preparations were obtained by using acrolein and glutaraldehyde (b, above), but a wide range of fixatives (singly or in combination) have also been used. These include acetaldehyde, crotonaldehyde, picric acid, and tannic acid, in concentrations 0.1 to 5% and in the presence of acrolein or glutaraldehyde or both. These fixatives have also included varying species and concentrations of ions (Li, Zn, Pb, Mg, Mn, Ca, La, and Ni) and inhibitors (azide, cyanide). Pretreatment of the cells at
and cephaloridine ("Loridine") buffer, pH 8.0-9.0, containing ethylenediaminetetraacetic acid at a concentration of 10^{-1} to 10^{-4} M before fixation, was also used.

All sections were cut with a diamond knife on a Reichert ultramicrotome, picked up on 200-mesh copper grids covered with Formvar/carbon, and stained with 1% uranyl magnesium acetate (or uranyl acetate) for 1 to 2 min, and then with lead citrate for 2 min.

(ii) Freeze-etching: Samples were concentrated by centrifugation (5,000 × g for 5 min), and portions of the pellet were deposited on 3-mm copper disks and immediately frozen in Freon 22. When a cryoprotective agent (20% glycerol) was used, the bacterial sample was washed by one cycle of centrifugation and then frozen in Freon. Preparations were stored in liquid nitrogen (for no longer than 1 week) prior to freeze-etching. The method used was essentially that of Moore (25), using a Balzer's apparatus (model BA510 M, Balzers AG, Lichtenstein). Samples were etched for 1.5 min after fracturing and then shadowed with platinum and carbon. The replicas were washed, successively, with distilled water and undiluted H2SO4 (1 h) and distilled water. Following a further rinse in Javex (1 h) and distilled water, the replicas were mounted on 200-mesh copper grids bearing a Formvar film. The micrographs of freeze-etched material have not been reversed; i.e., shadows are white.

Micrographs were taken on 35 mm fine-grain positive film by using a Philips EM200 operated at 60 kV.

**Inhibitors and antibiotics.** Chloramphenicol (CAM) was obtained from Sigma Chemical Co. (St. Louis, Mo.) or from Calbiochem (San Diego, Calif.). Nalidixic acid was obtained as a gift from Winthrop Labs. (Aurora, Ont.) and prepared as a stock solution (1 mg/ml) in 0.1 N NaOH. Ampicillin (Ayerst Labs., Montreal, Que.) and sodium cephalothin ("Keflin") and cephaloridine ("Loridine") from Eli Lilly and Co. (Canada), Toronto, Ont. were received as gifts and used from stocks (1 mg/ml in water). All inhibitors and antibiotics were freshly prepared or refrigerated overnight before use.

**RESULTS**

**Fine structure of septa.** When fixed by the RK technique, dividing cells of *E. coli* B, B/r, and CRT 257 all showed constrictive divisions (Fig. 1A, B). The invaginated area contained the cytoplasmic membrane, the mucopeptide layer, and also the outer membrane (for identification of layers, see ref. 13, 26, 27, 32); these layers were usually closely apposed, even at the apex of the V-shaped constriction. This apparently simultaneous invagination of the wall and membrane at the site of division will be referred to as "constriction" in the subsequent discussion. The appearance of the constricted cell (Fig. 1A, B) suggested that the infolded wall and membrane did not merely invaginate from the mid-point of the cell but also from an area (about 0.05 to 0.1 μm wide) each side of the middle, forming a deep trough. Constrictive divisions were also seen after prefixation with glutaraldehyde (Fig. 1C) and indeed with a wide range of other treatments. These treatments included prefixation with acetaldehyde, crotonaldehyde, picric acid, tannic acid, and combinations of these fixatives. Various ions (e.g., Mg, Ca, Zn, or Ni, in varying concentrations) or pretreatment of the cells with ethylenediaminetetraacetic acid had little modifying effect on the appearance of constrictive division. Even freeze-etching of unfixed cells only revealed constrictive division.

The only fixative providing consistent demonstration of septa in the common strains of *E. coli* was the acrolein/glutaraldehyde mixture described in Materials and Methods. It had the further advantage in that cell form and internal structures were preserved with persuasive regularity. Dividing cells of *E. coli* B and B/r, when fixed in this manner (see Fig. 2A-D), contained a distinct septum composed of the cytoplasmic membrane and the mucopeptide layer. In all cases, the outer membrane did not appear to take part in the initial stages of septation. Vesicles and sheets of membrane, presumably formed by blebbing of the outer membrane, were observed at the site of septation (Fig. 2A, B). *E. coli* B could also be fixed successfully solely with 5% acrolein (in 0.05 M cacodylate buffer, pH 7.5), but the outer membrane of *E. coli* B/r appeared to be damaged by this treatment, forming many small vesicles along the cell. Mesosome-like membranes were also occasionally seen (Fig. 3B) in *E. coli* B and B/r, particularly if the basal glucose medium was supplemented with Casamino Acids and tryptophan. Under the latter conditions, some cells also contained a septum apparently composed solely of the cytoplasmic membrane (Fig. 3D, 4A); asymmetric divisions, involving the ingrowth of the septum from one side of the cell, were also noted. *E. coli* B completed septa

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**Table 1. Strains of *E. coli* examined**

<table>
<thead>
<tr>
<th>Strain</th>
<th>UWO* collection no.</th>
<th>Medium</th>
<th>Source and reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>301</td>
<td>M9</td>
<td>Laboratory collection</td>
</tr>
<tr>
<td>B/r</td>
<td>828</td>
<td>MM</td>
<td>C. E. Helmstetter (15)</td>
</tr>
<tr>
<td>D21 eno A</td>
<td>839</td>
<td>LB</td>
<td>H. Boman (30)</td>
</tr>
<tr>
<td>D22 eno A</td>
<td>840</td>
<td>LB</td>
<td>H. Boman (30)</td>
</tr>
<tr>
<td>CRT 97</td>
<td>925</td>
<td>CM</td>
<td>F. Jacob (22)</td>
</tr>
<tr>
<td>CRT 257</td>
<td>926</td>
<td>CM</td>
<td>F. Jacob (22)</td>
</tr>
<tr>
<td>PAT 84</td>
<td>942</td>
<td>CM</td>
<td>F. Jacob (18)</td>
</tr>
</tbody>
</table>

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FIG. 1. Examples of constrictive division in sections of E. coli B/r (A), E. coli CRT 257 (B), and E. coli B (C); both A and B were fixed by RK technique; C was fixed with glutaraldehyde-osmium. Note deep cleft formed by invaginated layers of closely apposed wall and membrane layers; cytoplasmic membrane (cm), outer membrane (om), and mucoprotein (mp). Unless otherwise stated, magnification bar equals 0.1 μm.

FIG. 2. Septa in sections of E. coli B in M9 medium (A, C) and B/r in glucose minimal medium (B) or glucose-Casamino Acids (D); fixed with acrolein-glutaraldehyde. Abbreviations as in Fig. 1. Unlabeled arrows show mucoprotein portion of septum; note blebs or sheets of outer membrane (A, B) and double lamellae of mucoprotein in septum.
FIG. 3

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before any distinct ingrowth of the outer membrane rounded the profiles of the newly formed poles of the cells thus formed. However, E. coli B/r (Fig. 3A, 3D) cells were seen in which the rounding of the poles accompanied, or closely followed, the ingrowth of the septum, but the mucopeptide portion of the septum still preceded the invagination of the outer membrane. Similar results were obtained from examination of sections of E. coli D21 env A, the parent strain of the chain-forming mutant D22 env A discussed below.

The initial stages of septation were the most difficult to preserve. Also, relatively few cells could be detected in batch cultures which showed this stage. The basic structure of the septum (e.g., Fig. 2B, 2C) showed that it was composed of an invaginated portion of the cytoplasmic membrane containing two distinct, but separate, lamellae of mucopeptide. Study of synchronous cultures of E. coli B and B/r (Burdett and Murray, submitted for publication) has fully confirmed this picture.

Our most successful preparations have been of cells prefixed at the growth temperature with a one-tenth volume of fixative for 1 to 3 min and then resuspended at room temperature (23 to 25°C) in full-strength acrolein/glutaraldehyde. During the prefixation step, the fixative was simply added to the flasks, allowing the culture to shake meanwhile. If the pH of the fixative was less than 7.0, septa were seen infrequently, and the cytoplasm was aggregated into clumps of amorphous material. Attempts to confirm the presence of septa in E. coli B or B/r, previously fixed with acrolein/glutaraldehyde and then freeze-etched, have been only partially successful, owing to technical difficulties in obtaining clean replicas. However, prefixation of E. coli B with OsO₄, by exposing a lawn of cells on nutrient agar to vapors of the fixative immediately before freezing, suggest that septa are present in many dividing cells (R. G. E. Murray, unpublished data).

In E. coli D22 env A and E. coli CRT 97 (Fig. 5C, 5D), prominent septa were seen without difficulty by using any of the standard fixatives, and Fig. 4C–4E show a simulated division sequence. Both these organisms tended to grow in chains, with 2 to 3 cells per chain (Fig. 4B; refs. 22, 30). Although septa were easily preserved in sectioned material of E. coli D22 env A, very few septa were seen in unfixed, freeze-etched cells. In all cases of septation (Fig. 4C–4E), only the mucopeptide portion of the wall, plus the cytoplasmic membrane or, more rarely, the cytoplasmic membrane alone, formed the septum. As in E. coli B or B/r, the mucopeptide in the septum was composed of two lamellae (Fig. 4C–4D). In a few cells of E. coli D22 env A (Fig. 4E), the septum was apparently composed of a single dense layer of mucopeptide, about twice the thickness (4 to 6 nm) of the mucopeptide at the peripheral wall. Splitting of the septum in these cells apparently started at the center (Fig. 4D). Attempts to stain this gap within the septum with phosphotungstic acid or to see whether extracellular tracers (e.g., lanthanum or ruthenium red) would penetrate the septal arms, have been unsuccessful. Mesosome-like structures were also seen occasionally, associated with the initial stages of septation or with the completed septum (Fig. 5B). Both in thin sections and in freeze-etched material (Fig. 5A, 5B), the outer membrane was closely apposed to the outer wall components, although, as in E. coli B and B/r, it was excluded from the septum until the onset of cell separation.

Antibiotics and septation. Because low doses of penicillin can block division selectively (see Introduction), we have examined further the relationship between septum formation and lysis of E. coli. In these experiments, ampicillin, rather than penicillin, was used, because one strain (E. coli PAT 84; see below) was found to be susceptible to ampicillin but indifferently so to penicillin (see also ref. 39).

When ampicillin (2 to 5 μg/ml, final concentration) was added to cultures of E. coli B/r growing in glucose minimal medium, absorbance (A₅₉₅) continued to rise for about 1.5 h (Fig. 6A). As monitored by phase-contrast microscopy and decrease in absorbance (Fig. 6A), the cells rapidly lysed after this time. A prominent bulge was observed in cells treated with ampicillin (Fig. 7B, 7C) as previously shown for penicillin-treated cultures of E. coli (46). The bulge was found at or near the middle of the cells (Fig. 7B, C) or, more rarely, towards one pole. All layers of the wall plus the cytoplasmic membrane were found at the site of the bulge. In lysing cells (Fig. 7G), dense granules were observed in the cytoplasm, and, on rupture of

Fig. 3. Septation in E. coli B/r in glucose-Casamino Acids (A, C, D) and E. coli B (B). Septum is formed by ingrowth of cytoplasmic membrane and mucopeptide (mp; A) or by cytoplasmic membrane across the cell (D). An intermediate layer (i; A) between mucopeptide and outer membrane is excluded from the septum. Unlabeled arrows show mesosome-like structures (B, C); nucleoid (n). Acrolein-glutaraldehyde fixation was used. Bar in Fig. 3B equals 0.5 μm.
FIG. 4. Septation in E. coli B/r in glucose-Casamino Acids (A) and E. coli D22 env A (B–E). Septum is formed by cytoplasmic membrane (A) or by growth of the membrane and mucoprotein layer (C–E). Note double lamellae of mucoprotein (arrows; D) or single structure (E); chain formation in cells of D22 env A is shown in B. Acrolein-glutaraldehyde fixation was used. Bar in Fig. 4B equals 1 μm.
Fig. 5. Freeze-etch (with glycerol) micrographs of septa (s) and mesosome-like membranes (m) in E. coli D22 env A (A, B); arrows at top left indicate direction of shadowing. Septation in E. coli CRT 97 showing partial (C) and complete (D) septa; RK fixation was used. Abbreviations as in Fig. 1. Bar in Fig. 5A equals 0.5 μm.
The addition of cephapathin (or cephaloridine) in low concentrations (1 to 5 μg/ml, final concentration) resulted in lysis of the cells after approximately one-half of a generation time, i.e., about 25 min (Fig. 6A). Very few distinct bulges were noted in cells treated with very low doses of cephapathin (0.1 to 1.0 μg/ml). Instead, the septal area merely appeared as a furrow (Fig. 7E). Cells treated with higher concentrations of cephapathin (5 μg/ml or greater) showed more extreme central enlargement and bulge formation prior to lysis. Control cultures (no additions) were septate, as described above. The concentrations of ampicillin and cephapathin used in these studies stopped division immediately (unpublished data).

Bulge formation was suppressed by the addition of chloramphenicol if added (at a final concentration of 2 to 40 μg/ml) at the same time as ampicillin (5 μg/ml) or cephapathin (5 μg/ml). The absorbance of the cultures also remained level (Fig. 6B). In thin section, the outer membrane of cells treated with ampicillin and low levels of CAM (2 μg/ml, final concentration) was often extended and displaced (Fig. 7F); multiple layers of the outer membrane were also seen (Fig. 7F). A slow increase in the A660 was noted for cultures treated solely with CAM (2 μg/ml; Fig. 6B), whereas increase in absorbance ceased after approximately 30 min in cultures exposed to CAM at a final concentration of 40 μg/ml (see Fig. 6B). On the other hand, if CAM (2 to 4 μg/ml) was added to a culture of E. coli B/r previously treated with ampicillin (5.0 μg/ml), lysis could not be prevented unless the CAM was added within 0.1 to 0.20 of a generation time. Control cultures (no additions) showed no evidence of bulge formation (Fig. 7A).

Ampicillin and cephapathin in low concentrations also induced bulge formation in E. coli CRT 97, although lysis of the cultures extended over 1 to 3 h. The env A mutants, both producers of penicillinase (30), continued to grow and divide in the presence of the ampicillin and cephapathin.

Studies on E. coli PAT 84. Some of the basic properties of this mutant have been described (18, 38). When grown at 30°C, the cells divided normally (Fig. 10A), but on a shift to 42°C (restrictive temperature) division ceased and the cells began to elongate (Fig. 8). There was no apparent increase in the growth rate following the shift to 42°C, as measured by increase of absorbance (Fig. 9). As far as we could determine, the cessation of division at 42°C was abrupt, suggesting that even divisions in progression were halted. The filaments at 42°C contained nucleoids spaced at intervals, as seen by electron microscopy (Fig. 10B) or by a nucleoid staining technique (18). When the cultures were held at 42°C for 2 to 2.5 h and then returned to 30°C, the filaments became compartmentalized, and a rapid increase in cell numbers followed, sequentially but not synchronously, after a period of 15 to 25 min (Fig. 8). Prolonged culture of PAT 84 at 42°C resulted in loss of viability and decrease in cell numbers (Fig. 8).

Division at 42°C and effects of CAM. Division of filaments at 42°C occurred if NaCl was added to a final concentration of 0.25 to 0.34 M if cells were initially grown in complete medium either without NaCl or at low levels (0.08 M; i.e., 5 g/liter). An increase of cell numbers, after a lag of 15 to 20 min, occurred after addition of NaCl (Fig. 11); in no case was the number of cells as great as in a control culture left at 30°C. Also, if NaCl (0.25 to 0.34 M) was added at the time of a shift to 42°C, the cells continued to divide (Fig. 11).

If CAM (40 to 100 μg/ml, final concentration) was added to cells at 42°C and at the time of a
shift-down to 30 °C, a proportion of the cells divided (Fig. 12). Both the A_{400} and cell numbers showed only slight increases if CAM was added 10 min (or longer) before the shift-down (Fig. 12, 13). If CAM (40 μg/ml) and NaCl (0.25 M) were added to cultures at 42 °C at the same time, then a limited increase in cell numbers also was detectable (Fig. 11).

**Bulge formation and lysis.** We tested PAT 84 further to determine whether the nonseptate filaments would form bulges if cultures at 42 °C were treated with ampicillin. The response of the cells was found to be related to the NaCl content of the medium. If NaCl was omitted from the medium, no bulges were formed, but in media containing 0.08 M NaCl low concentrations of ampicillin (2.5 μg/ml) added at the time of a shift to 42 °C induced bulge formation in very few cells (Fig. 10D). These cells may have possessed partially completed septa or were otherwise "committed" to septation at the time of the shift-up. Intracellular membranes were also seen in these cells (Fig. 10D). The concentration of ampicillin used (2.5 μg/ml) induced bulge formation in cultures at 30 °C; these cultures, like those at 42 °C containing ampicillin (2.5 μg/ml) lysed after about 1 to 1.5 h (Fig. 14A). Very low concentrations of ampicillin (1 μg/ml) permitted growth of the cells at 42 °C without lysis (Fig. 14A).

If NaCl (0.25 M, final concentration) was added to a culture of PAT 84 growing in the presence of ampicillin (1 μg/ml, added at time of shift-up), lysis of the culture ensued after a delay of about 20 min (Fig. 15A); bulges were also observed (Fig. 10E). Similarly, if ampicillin (2.5 μg/ml) was added to a culture on a shift-up to 42 °C and the flask was returned to 30 °C, lysis also occurred (Fig. 14B). Conversely, if ampicillin (2.5 μg/ml), lysed after about 1 to 1.5 h (Fig. shift-down to 30 °C, lysis occurred (Fig. 14B). These experiments, like those described below, used complete medium containing 0.08 M NaCl. In most cases, lysis and bulge formation were found to occur at one or two sites along the filaments or occasionally at the poles of the cell (Fig. 16A). Completely lysed cells, viewed in thin sections, showed a profile where no obvious layer of mucopeptide was found in the vicinity of the bulge or at the presumed septal area (Fig. 16B, C; 17A); the remainder of the wall appeared to be intact. It should be noted that high concentrations of ampicillin (50 μg/ml), when added to cells at 42 °C, promoted rapid lysis (Fig. 14A). Our initial studies using penicillin G produced variable results in experiments of this type.

With the exception of the experiment described below, we have not been able to preserve a septum in PAT 84 grown at 30 or 42 °C (cf. Fig. 10A). Freeze-etch studies also showed constrictive divisions, but suggested that differences in the distribution of particles on the cytoplasmic membrane existed between cells grown at 30 and 42 °C. A netlike array of particles on the convex membrane face was seen on replicas of cells grown at 30 °C, whereas cultures at 42 °C appeared to have randomly grouped particles (Fig. 17C–D). The surface of freeze-etched cells, transferred to 42 °C in the presence of ampicillin (2.5 μg/ml) and then shifted down to 30 °C, appeared to possess fewer particles (Fig. 17E) than the control samples at 30 or 42 °C.

When cells of PAT 84 were diluted into nalidixic acid (10 μg/ml, final concentration) and then shifted to 42 °C, elongation occurred. If ampicillin (1 μg/ml, final concentration) was also added, then bulge formation, as noted above, did not occur. Increase in A_{400} (Fig. 15B) continued to increase in both cases but levelled off after about 1 to 1.5 h at 42 °C. When NaCl (0.25 M, final concentration) was added to cultures containing nalidixic acid and ampicillin at 42 °C, lysis eventually followed (Fig. 15B). Examination of the cells under the light microscope suggested that the division site was, in some cells, displaced towards a pole of the filament. In thin section, the septum was observed to consist solely of the cytoplasmic membrane, and intracellular membranes, perhaps erratic incursions of the cytoplasmic membrane, were also present (Fig. 17B). Rupture of the cells was evident at sites of contact of these membranes with the wall (Fig. 17B).

**DISCUSSION**

Constrictive division in *E. coli* is an artifact; division of *E. coli* B and B/r is accomplished by septum formation and, in common with the majority of bacteria, the septum is formed by an ingrowth of the cytoplasmic membrane plus the mucopeptide of the wall. Septa of similar structure were also observed in *E. coli* D22 env A and in *E. coli* CRT 97. Our current model of the structure of the septum is shown in Fig. 18. The outer membrane does not appear to participate in septation until the cells begin to separate, but preparatory activity is indicated by the presence of a bleb, or groups of vesicles, at the site of division. This activity was particularly marked in fixed and sectioned cells of *E. coli* B and B/r, although it was not noted in freeze-etched material or in sections of *E. coli* D22 env A or CRT 97. The mucopeptide portion of the
septum in E. coli is essentially a double structure, formed by two lamellae separated by a gap (Fig. 2D, 5C). Electron microscopy of synchronised culture of E. coli B and B/r (Burdett and Murray, submitted or publication) have fully confirmed this observation. Similar structures were reported in cells of E. coli B held at 45°C (49) and are visible in micrographs of E. coli 15T (50). The nature of the material present in the "gap" (Fig. 18) is not known. As we have suggested elsewhere (Burdett and Murray, submitted for publication), it is possible that at least a portion of the lipoprotein (4) covalently linked to muropeptide may be located here, because there is continuity between the site of this component of the peripheral wall and the central portion of the septum (Fig. 18). Septa of Spirillum serpens (49) are formed of a single dense line, and this organism has been reported to lack the covalently bound lipoprotein (24).

Mesosomes of the type reported here have also been observed by others in E. coli (33, 44) and in Pseudomonas aeruginosa (19); mesosomes associated with the septum have been reported in a gram-variable coccus (12). The latter organism, like some cells of E. coli B/r (Fig. 3D, 4A), appears to initiate septation by forming a membrane across the cell prior to ingrowth of the wall.

Septa in E. coli are difficult to preserve. Even when the cells were rapidly frozen in Freon, prior to freeze-etching, it was very seldom possible to demonstrate septa. The necessity to concentrate the suspension of cells prior to freezing may well provide conditions that would trigger the disruption of the septum, or disruption may occur during the freezing process through concentration of solutes. This disruption is hard to prevent with fixatives. However, it is by no means clear that disruption of the septum will necessarily result in constrictive divisions. This is illustrated in Fig. 19, which emphasizes two features: (i) even in constrictive cells, the muropeptide layer appears continuous; (ii) the wall adjacent to the nascent septum (arrow 1) might also infold to yield the cleft seen in constrictive cells (arrows 2, 3). Thus, "collapse" of the septum may occur when the material in the "gap" (Fig. 19) is broken down and the septum can no longer maintain its integrity. Enzymatic attack at this location could conceivably occur through proteases or enzymes breaking the linkage of the lipoprotein to muropeptide. Some cells of E. coli D22 env A were observed to possess a septum consisting of a single dense lamella of muropeptide (see also ref. 30). This may indicate that this mutant is deficient in the synthesis of "gap" substances.

![Figure 8](http://jb.asm.org/)

**Fig. 8.** Effect of temperature shift on division of E. coli PAT 84. Cells at 30°C (O) were shifted to 42°C (□), and division ceased abruptly. A portion of this sample was returned to 30°C (●), and increase in cell number was followed with the Coulter counter.

![Figure 9](http://jb.asm.org/)

**Fig. 9.** Growth of E. coli PAT 84 following a shift from 30 to 42°C and then back to 30°C, measured by change in absorbance at \(A_{540}\).
FIG. 10
However, the tendency to grow in chains may also indicate an impairment of the separation mechanism, a reflection perhaps of an altered autolysin. In *B. subtilis* (9, 10) and *S. faecalis* (48), isolated autolysins and also lysozyme (10) have been used to induce fragmentation of chains of cells. Lysozyme disrupts the septum of the D22 env A mutant (30). Chain formation in *Bacillus subtilis* and *S. faecalis* appears to result from a lowered cell content of autolysin rather than from changes in the type of autolysin or alterations in wall structure. It is possible that constrictive divisions arise from two sources, related to the stage of septation reached by individual cells: (i) rupture of partially complete septa by dissolution of the "gap" substance, and (ii) autolysis of septum "initials" by hydrolytic enzymes associated with the early ingrowth of the septum.

Recent autoradiographic studies of *E. coli* W7 suggest that the center of the cell is a site of incorporation of newly synthesized mucopolypeptide (43). This site is also likely to be the location of hydrolytic enzymes (46). Both penicillin and ampicillin block division by inhibition of the transpeptidation reaction (21) normally cross-linking D-alanine to diaminopimelic acid on adjacent peptide chains (51). There is some evidence showing that the transpeptidase is at least one of the penicillin-sensitive targets involved in septation (14). Although penicillin and ampicillin both block transpeptidation, it is likely that ampicillin may enter cells of *E. coli* more easily than penicillin (51). The effects of cephalosporins, which may block both septation and elongation, are difficult to understand (for discussion, see ref. 23, 31). As in *Staphylococcus aureus* (40), lysis may be prevented by addition of CAM in the presence of ampicillin, possibly due to the inhibition of synthesis of hydrolytic enzymes.

**Fig. 10.** Sections of cells of *E. coli* PAT 84 grown at 30 C (A), at 42 C (B), or at 42 C in the presence of ampicillin (2.5 μg/ml for 45 min; C, D; arrows in D show an intracellular membrane). A portion of the filaments is shown in B and C. Cells grown in complete medium plus 0.08 M NaCl at 30 C and shifted to 42 C in the presence of ampicillin (2.5 μg/ml), to which was added further NaCl to a final concentration of 0.25 M, show bulges (arrow: E); nucleoid (n). Acrolein-glutaraldehyde fixation was used. Bars equal 0.1 μm (D), 0.2 μm (E), 0.5 μm (A), and 1 μm (B, C).

**Fig. 11.** Effect of NaCl on division of *E. coli* PAT 84. Control (in complete medium + 0.08 M NaCl, ○); cells at 42 C in same medium (arrow 1, Δ) or with NaCl (to 0.25 M) at time of shift to 42 C (□); addition of NaCl (to 0.25 M) to cells at 42 C (arrow 2, □); with NaCl (to 0.25 M) plus CAM (40 μg/ml) at 42 C (arrow 3, □).

**Fig. 12.** Effect of CAM (40 μg/ml) on division of *E. coli* PAT 84 returned to 30 C after growth at 42 C. Control culture returned to 30 C (no additions, ○); plus CAM at time of transfer to 30 C (△); with CAM added 5 min (△) or 10 min (▲) prior to transfer to 30 C; control left at 30 C (□) or shifted to 42 C without CAM (已完成)
prior to PAT 84 treated with CAM

50 shift to and back (2.5 µg/ml of E. coli)

0)

Bacteriol.

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enzymes. Alternatively, if transport of the active hydrolase(s) from the cytoplasm requires protein synthesis, bulge formation might still be prevented by addition of CAM. In bacilli, protein synthesis must be inhibited before addition of wall inhibitors (vancomycin, d-cycloserine) in order to arrest lysis (42).

Bulge formation in E. coli occurs at, or close to, the sites of division. The potential for the expression of bulge formation appears to be correlated with the deoxyribonucleic acid (DNA) replication cycle and it has been shown that the maximum occurs at or near the time coincident with the end of a round of replication (Burdeett and Murray, submitted for publication; 20). If bulge formation is due to the action of mucopptide hydrolases, then there would appear to be a significant increase in hydrolytic activity close to the point where the cells are entering the septation sequence, i.e., the D period (5). An essential prelude to septation is likely to be a structural modification and, since both septation and bulge formation involved primarily the mucopeptide, the likely candidate

Fig. 13. Absorbance of cultures (A_{660}) of E. coli PAT 84 treated with CAM (40 µg/ml) at various times prior to shift-down from 42 to 30 C.

FIG. 14. Effect of ampicillin on growth of cultures of E. coli PAT 84 monitored by change in absorbance (A_{660}). (A) Control at 30 C (no additions, □); cells at 42 C (no additions, ◆) or with ampicillin (2.5 µg/ml, ▲); cells at 42 C (no additions, ○) or with ampicillin (1 µg/ml, △), 2.5 µg/ml (▼), or 50 µg/ml (○). Arrows indicate time of transfer to 42 C. (B) Control (no additions, ○) shifted from 30 to 42 C and back to 30 C; cells shifted to 42 C plus ampicillin (2.5 µg/ml, at time of shift to 42 C), and then returned to 30 C (□) or ampicillin (2.5 µg/ml) added at time of shift down to 30 C (▲).

Fig. 15. Effect of NaCl on lysis of E. coli PAT 84 at 42 C measured by change in absorbance (A_{660}). Control at 42 C (no additions, ◆) or with NaCl (to 0.25 M) from time of shift up to 42 C (△); cells at 42 C in presence of ampicillin (1 µg/ml) added at time of shift-up to 42 C (□) and treated by addition of NaCl (to 0.25 M). (B) Portions of same culture as in (A) treated with nalidixic acid (10 µg/ml) at shift to 42 C (O) or with nalidixic acid and ampicillin (1 µg/ml, ▼); NaCl was added (arrow) to 0.25 M to a portion of the latter culture.

Fig. 16. Sections of cells of E. coli PAT 84 grown at 42 C in presence of ampicillin (2.5 µg/ml) and then shifted down to 30 C. (A) Spheroplast emerging from a filament (arrow); (C) portions of lysed cells showing bulge formation. The mucopeptide layer (arrows, B) appears to be absent from the site of cell lysis (C). Acrolein-glutaraldehyde fixation was used. Bar in Fig. 16A equals 0.5 µm.
is the cross-linked and covalently bonded network of the mucopeptide layer. It is claimed that the rate of mucopeptide synthesis fluctuates throughout the cell cycle of E. coli (20) and rises at about the start of the D period. One possibility, therefore, is that local hydrolase activity at the center of the cell modifies the existing mucopeptide network to allow for the insertion of the newly synthesized septum. The visible events of the D period appear to occur in the latter half of the septation sequence (Burdett and Murray, submitted for publication), implying that synthesis and assembly occur prior to the initial ingrowth of the cross wall.

This interpretation of events leading to septation is also supported by our observations on E. coli PAT 84. This mutant, like BUG-6 (35-37), retains the capacity to divide after a shift-down to 30 C. The cells can also divide at 42 C by adding high concentrations of NaCl (or other solutes, including sucrose; see ref. 38). It has been suggested that PAT 84 is a missense mutant and that the lesion can be corrected by the addition of NaCl (39).

Our experiments indicate that, although the cells cannot normally form septa at 42 C, all the necessary materials are made at 42 C but cannot be assembled. On a shift-down to 30 C, and following a lag of 15 to 20 min, the cells divided at a rate initially faster than that of a control culture left at 30 C. A proportion of the cells also divided in the presence of CAM and, as shown elsewhere (Burdett and Murray, submitted for publication; 6), it is likely that protein synthesis may not be required in the terminal stages of division. The cells that actually divided, therefore, may have already entered the late stages of septation. Does the apparent delay before the emergence of new cells at 30 C (Fig. 8), or at 42 C in the presence of NaCl (Fig. 11), reflect an assembly of the septation mechanism or is some other process taking place as a preliminary step to septation?

When treated with ampicillin (1 to 2.5 μg/ml) at the time of a shift-up, very few cells actually formed bulges. But when shifted down to 30 C, or when NaCl (to 0.25 M) was added at 42 C, the cells lysed after a delay of some 20 min. This could be interpreted to mean that the essential lesion in the mutant is the lack of mucopeptide hydrolase activity at 42 C. Electron microscopy confirmed that rupture of the cells involved the formation of distinct bulges; discontinuities in the mucopeptide layer at sites of bulge formation were also noted. Experiments by Shannon et al. (47) on a temperature-sensitive DNA initiation mutant of Salmonella typhimurium also suggest that division sites can exist in filaments and that septation is preceded by an "initiation" step. PAT 84 there-

![Diagram of septum structure](image1)

**Fig. 18.** Diagram of septum structure (see text). Symbols: OM (B), bleb formed by outer membrane (OM); mucopeptide (MP); cytoplasmic membrane (CM). Mucopeptide lamellae in septum, MP(S); gap is electron-transparent space between lamellae. The position of the lipoprotein covalently bound to mucopeptide is indicated (●). Note that outer membrane is excluded from septum (see text).

![Diagram illustrating a possible mechanism accounting for constrictive divisions](image2)

**Fig. 19.** Diagram illustrating a possible mechanism accounting for constrictive divisions. The septum (arrow 1; A) might be pulled apart and collapse into a wide cleft (arrows 2, 3; B). The profiles of a septate cell (A) and a constricted cell (B) are also shown. See text for discussion.

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**Fig. 17.** (A) Same as Fig. 16A-C; note mucopeptide layer (arrows) is absent in septal area (arrow a); (B) section of E. coli PAT 84 treated with nalidixic acid (10 μg/ml), ampicillin (1 μg/ml), and NaCl (to 0.25 M); see Fig. 15B. Septum(a) is composed of cytoplasmic membrane; incursions of the membrane (arrow) are also associated with sites of cell lysis (nucleoid, n). (C-E) Freeze-etched cells (with glycerol) of E. coli PAT 84 at 30 C (C) showing netlike array of particles on convex layer of cytoplasmic membrane, at 42 C (D) where particles are more aggregated or as netlike arrays after shift-down to 30 C in presence of ampicillin (2.5 μg/ml; E). Arrows at top left indicate direction of shadowing. Bar in Fig. 17D equals 0.5 μm.
Therefore appears to be fully compartmentalized at 42 C, in the sense that DNA synthesis can continue and the nucleoids can be segregated (18), but the filaments cannot divide.

For the mutant BUG-6, Reeve et al. (35-37) have proposed that a protein ("division potential") is synthesized throughout the cell cycle. Shortly after the completion of a round of DNA replication, this protein is assumed to become involved in the division process. Our studies of PAT 84 suggest that "division potential" might be, in fact, an impaired mucoprotein hydrolase(s). This implies that PAT 84 (and perhaps BUG-6) are mutants which are blocked in an early stage when the wall is modified prior to the assembly of the septation apparatus. All the necessary precursors or requirements for division (DNA, ribonucleic acid, and protein synthesis) appear to be synthesized during the preceding C period (Burdett and Murray, submitted for publication; 6). Both PAT 84 and BUG-6 can divide in the presence of CAM, suggesting that the septum or its components are actually synthesized at 42 C but cannot be assembled. We are assuming that the mucopptide substrate itself is not modified at the restrictive temperature. Because division ceases abruptly on a shift-up to 42 C, it is possible that the impaired hydrolase(s) is in fact used throughout septation and cell separation.

Ahmed and Rowbury (1) have performed similar experiments on a temperature-sensitive mutant of S. typhimurium. This mutant, unlike PAT 84, cannot be induced to divide at 42 C by addition of NaCl. On addition of penicillin (25 U/ml) to cells at 42 C, no bulges were observed in the filaments, implying, as these authors suggest, the impairment of an autolysin at 42 C. Other mutants of E. coli (28) can only divide at 42 C provided they are placed initially at 30 C for a brief period. This mutant may possess an impairment in the initiation of septation, which may include an inactivation, or loss of synthesis, of an autolysin. The requirement for a brief period of protein synthesis at 30 C in temperature-sensitive E. coli 20 (ref. 28), if division is to occur in the presence of CAM, suggests that one or more proteins are not synthesized at 42 C. Detection and quantitative measurement of the component proteins are clearly needed to resolve these alternatives. Using envelopes of PAT 84, we have obtained preliminary data, by means of sodium dodecyl sulfate gel electrophoresis, indicating that the components of the wall and membrane are essentially the same at 30 and 42 C. But the preparations are of such relative crudity, indicating no more than the presence of a spectrum of proteins, that the results may be misleading and are therefore not included in this paper. Other types of lesion in these mutants are also possible. If, for example, the hydrolase(s) were synthesized in a latent, perhaps cytoplasmic, form, transport of the active enzyme through the cytoplasmic membrane might be affected. Whether the distribution of membrane particles noted in freeze-etched material (see Fig. 17C-E) reflect conformational alteration of proteins in the membrane is not clear (cf. ref. 52).

In summary, then, we envisage the septation sequence in E. coli to involve: (i) cleavage of the existing mucopeptide framework by a hydrolytic enzyme(s) prior to synthesis of the septum, and (ii) assembly of the septum utilizing the components synthesized during the preceding round of DNA replication. The labile nature of the septum, especially at the initial stages, may be due to the close association of the autolysin(s) with the septation site. The preservation of the dynamic structural components of cell division for electron microscopy to provide satisfactory correlation of structural and biochemical events in the septation process will require new methods of fixation that inhibit the enzymes involved in autolysis. The fixatives we have used, although effective for E. coli B and B/r, are demonstrably ineffective for some other bacteria showing "constrictive division" with RK fixation, including PAT 84.

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