Biochemical and Topographical Studies on *Escherichia coli* Cell Surface

IV. Giant Spheroplast Formation from a Filamentous Cell

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Long, nonseptate filamentous cells consisting of 5 to 40 single-cell unit lengths were formed from *Escherichia coli* surface mutant ONT-3 by treatment with a sublethal concentration of sodium dodecyl sulfate. As distinct from several other elongated cells (e.g., thymine-starved filaments), it was found here that stable giant spheroplasts, 5 to 10 μm in diameter, were produced by the action of lysozyme in the presence of bovine serum albumin via the gradual fusion of distinct spheroplasting bulbs.

It is well known that several types of bacteria or their mutants can be converted into filamentous forms by means of antibiotic treatment (5, 20), UV irradiation (17), cultivation at a high temperature (4, 6, 7, 18), or by nutritional deficiency (18). Although defects in septum formation and hence presumably in the mechanisms of cell division have been demonstrated, information on the properties and physiology of such filamentous cells is too scarce to provide a complete understanding of the phenomenon. In an attempt to further elucidate the regulatory mechanism, we have studied the effect of sodium dodecyl sulfate (SDS) on septum formation in the cell surface mutant *Escherichia coli* ONT-3 and found that a sublethal concentration of SDS produced a filamentous form (15) which seemed to lack cross septa and exhibited the "zebra"-like structure observed in heat- and 5% SDS-treated *Bacillus subtilis* (10).

In the present paper, we describe physiological studies on the filamentous cells and show that they can be converted to giant spheroplasts, 5 to 10 μm in diameter, under certain conditions, thus confirming the lack of functional cross septa. Several other morphologically similar filamentous forms did not convert to such a large spheroplast under identical conditions.

Unusually enlarged polyplloid cells of *E. coli* have been reported after repeated exposure to camphor vapor (12, 21), and a large spheroplast was obtained by Bhatti et al. from the lysozyme treatment of filamentous, nonmotile *Pseudomonas aeruginosa* cells grown at 46°C and plasmolyzed (1). The latter was, however, too unstable to withstand processing for electron microscopic examination. This is the first report of a spheroplast of unusually large size from *E. coli* which is sufficiently stable to allow observations of the gradual morphological changes that take place during spheroplast conversion.

**MATERIALS AND METHODS**

Organisms and culture conditions. The strains used in this study include a cell surface mutant, *E. coli* ONT-3, derived from *E. coli* W3110 (13), a thymine auxotroph *E. coli* H-5 (Hfr "Thy" "Met"), and *Bacillus megaterium* DL, a lysine- and dianimopimelic acid-requiring mutant derived from strain IFO 7581, obtained from P. J. White, Sheffield University, United Kingdom. All strains were, unless otherwise stated, grown in 10 ml of nutrient broth plus the specific growth factors at 37°C by shaking-tube cultures.

Filamentous cell formation. *E. coli* ONT-3 was grown in nutrient broth to the early logarithmic phase, and SDS was added to give a final concentration of 0.006%. Growth was continued until middle to late logarithmic phase (approximately 4 h) by which time an almost completely filamentous population of 10 to 40 single-cell unit length (15) had been found. Filamentous *E. coli* H-5 cells were obtained by thymine starvation for several hours in CR medium which contained (per liter): 13.6 g of KH₂PO₄, 2.0 g of (NH₄)₂SO₄, 0.2 g MgSO₄·7H₂O, 0.5 mg of FeSO₄·7H₂O, 3 ml of glycerol, 50 g of Casamino Acids, and trace metal mixture (pH 7.4).

*B. megaterium* DL was grown in a peptone broth containing 0.02 M NH₄Cl, 0.02 M KCl, 0.12 M Tris-hydrochloride (pH 7.0), 0.0016 M MgCl₂, 0.5% protease peptone (Difco), 100 μg of meso-dianimopimelic acid (sodium salt) per ml, and lysine hydrochloride, and 20 μg of biotin per liter. Cultivation for 16 h at 42°C made cells filamentous, whereas multiseptate chains were obtained when cultured at 30°C for 16 h under otherwise identical conditions.
Spheroplast formation. Normal or filamentous cells of *E. coli* made as above were harvested, washed twice with 1.5 M sucrose, and suspended in 0.35 ml of 1.5 M sucrose. To this, were added 0.17 ml of 30% BSA (bovine serum albumin, Fr. V., Sigma), 20 μl of 2-mg/ml lysozyme ( Worthington Biochemical Corp. ), and 40 μl of 4% EDTA. The mixture was incubated at 30°C for 5 to 10 min and then added to 10 ml of Penassay broth (Difco) supplemented with 10 mM MgCl₂. The mixture was again incubated at 30°C without shaking, and the morphological changes were followed by phase-contrast microscopy. Photographs were taken every 30 s. Lysozyme-spheroplasts were usually obtained within 10 min of incubation time.

Alternatively, glycine-spheroplasts and penicillin-spheroplasts were prepared as follows. Filamentous cells of *E. coli* ONT-3 made as above by 2 h of incubation were harvested and suspended in the same volume of fresh nutrient broth containing 0.4% MgSO₄·7H₂O and 5 or 15% sucrose supplemented with either 3% glycine or 100 μg of penicillin G per ml and cultured by shaking (180 strokes/min) at 37°C. Morphological change was followed as above. Under these conditions, spheroplasts of normal single cells were obtained within 2 h of cultivation.

Protoplast formation from filamentous or chained forms of *B. megaterium* was attempted by the following methods. Cells grown in high or low temperature as above were harvested, washed, and suspended in one-tenth volume of phosphate buffer (0.1 M, pH 6.8) containing 10% sucrose, 10 mM MgSO₄, and 10 μg of lysozyme per ml. Incubation for 10 min at 30°C gave single-cell protoplasts of *B. megaterium* under this condition (16) from the chained forms but not from the filaments.

Other methods. β-Galactosidase was assayed spectrophotometrically by a modified method of Lederberg (see 3). The alkaline phosphatase assay followed that of Neu and Heppel (11). DNA was determined by the diphenylamine method (2), and protein was determined by the Lowry method (8). Total cell number was counted under phase-contrast microscopy, whereas colony-forming cell counting was done by the conventional plating method on nutrient agar throughout the experiment for both filamenting or defilamenting cells.

RESULTS AND DISCUSSION

Effect of SDS and filamentous cell formation. The cell surface mutant *E. coli* ONT-3 has defective barrier function of the outer membrane as characterized by loss of tolerance to surfactants, actinomycin D, or gentian violet (13), insensitivity to T4 or T7 phage adsorption (14), nonrequirement for EDTA during spheroplast formation by lysozyme (14), and in situ agglutinability by concanavalin A (9), together with other surface defects such as reduced motility and lower frequency of R plasmid transmission (14). On the other hand, its cytoplasmic membrane has increased stability, since the spheroplast obtained from this by lysozyme treatment was more stable than that from wild-type *E. coli* W3110 (14).

When the strain ONT-3 was cultured in nutrient broth at 37°C with shaking and in the presence of SDS at a concentration higher than 0.05%, it was completely lysed (13). At an SDS concentration of around 0.005%, however, ONT-3 cultures were found to increase in turbidity but not in viable cell number. Figure 1 shows the relationship between turbidity (absorbance at 650 nm [A₆₅₀]) and the cell numbers during growth. The parent strain (W3110) in the presence of 0.005% SDS and ONT-3 in the absence of SDS exhibited a concomitant linear increase in both A₆₅₀ and cell numbers, whereas in the presence of 0.005% SDS, ONT-3 increased in turbidity with time of growth while decreasing in cell numbers and colony-forming units by a factor of 7 to 4 (see also Fig. 3). After 5 h of incubation with 0.005% SDS, cell morphology was examined by a phase-contrast microscope (Fig. 2); we found that ONT-3 consisted exclusively of extremely long filamentous forms (Fig. 2B). The length of the filaments was 40 to 80 times that of an average single cell. The filaments seemed polynuclear and lacked cross septa when examined microscopically after Giemsa staining. The population of penicillin type elongated cells (cross wall, septated) was apparently negligible. Filament formation was not observed with ONT-3 grown in the absence of SDS or with the parental W3110 strain even in the presence of 5% SDS.

Fate of filamentous cells after removal of SDS. Figure 3 shows the effect of SDS addition at early logarithmic phase and its removal after 5 h on the turbidity and colony-forming cell number. During 5 h of treatment with 0.005% SDS, the colony-forming cell number of ONT-3

![Figure 1](http://jb.asm.org/)
was reduced by one-fiftieth in spite of the turbidity increase. Since the total number of filamentous cells did not significantly change in this period, it may be deduced that only 2 out of 100 cells, or 4,000 to 8,000 “single” cell units in total, possessed colony-forming activity in the conventional assay for viable cells.

When SDS was washed out and cells were again incubated in a fresh medium of the same composition and volume, a rapid recovery of the colony-forming activity was seen (Fig. 3, solid circles) as well as a rise in turbidity similar to that observed in the presence of SDS. The distribution of cell length at this period was microscopically determined (Fig. 4). It was found that, after reincubation in SDS-free medium, normalized single cells having colony-forming activity were rapidly produced from the filaments presumably by the completion of defective septa via intermediate-sized cells of 4 to 8 single-cell unit length. It was noted, however, that the recovery phase seemed to proceed in an unsynchronized manner, since the distribution of cell size in the population after SDS wash-out was quite heterogeneous. Even after 3 h of incubation, a minority of filamentous forms still remained among the dominant population of single-sized cells.

**Properties of filamentous cells.** No differences were found between several biochemical properties of filamentous and normal cells. Thus, the ratio of DNA to protein and the specific ratio of incorporation of labeled thymine, uracil, and leucine in the filamentous form were the same as those in SDS-untreated cells calculated on the basis of total protein. No differences were found in the inducibility of β-galactosidase upon the addition of isopropyl-β-thiogalactopyranoside, in its catabolite repression pattern, or in its derepression by cyclic AMP (not shown). Furthermore, the inducible synthesis of alkaline phosphomonoesterase of ONT-3 was not impaired during filament formation, suggesting a difference in the role of the enzyme from that in

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**Figure 2.** Cell morphology of 5-h-incubated ONT-3 cells with (B) or without (A) 0.005% SDS as observed by phase-contrast microscopy using a Nikon SKE/AFM model (×600). As control, W3110 without SDS (C) and with 5% SDS (D) are also illustrated. Bars = 10 μm.
Pseudomonas aeruginosa, where an active participation in septum formation was implicated (1).

These results, together with the fact that fil-

Fig. 3. Growth curves and colony-forming activity of ONT-3 and parent W3110. 0.005% SDS was added at the point shown by arrow and washed out 5 h later. Growth curve (turbidity A\textsubscript{650}) of W3110 (-----) and ONT-3 (-----) and colony-forming ability of W3110 (○) and ONT-3 (●) are shown. For details see text.

Fig. 4. Cell length and number observed microscopically and using bacteria counter after washing out of SDS. Growth curves (turbidity A\textsubscript{650}) of ONT-3 in the presence of SDS and after its washing out are shown (-----). Number of bacteria having a cell length of 1 to 2 times (●); 4 to 8 times (○); 9 to 30 times (□) the mean unit cell length.

Fig. 5. Phase-contrast micrographs of normal ONT-3 rod cells (A) and spheroplasts obtained from SDS-treated filamentous cells with (B) or without (C) BSA (×600). For details see text. Bars = 10 μm.
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Fig. 6. Morphology of B. megaterium DL obtained from IFO 7581. (A) Normal rod in nutrient broth; (B) filamentous form at 42°C in phosphate-deficient medium; (C) multiseptate chained form at 30°C in the same medium; (D) chained protoplasts after lysozyme treatment of chained form; and (E) single separated protoplasts. Bars = 10 μm.

amentous cells contained amounts of DNA and nuclear structure both analytically and microscopically equivalent to their single-cell unit length, indicated strongly that filamentous cells were normal in all respects except for their defect in septum formation and colony-forming ability when tested by the conventional viable counting method. In other words, it is most likely that the filamentous ONT-3 mutant was defective only in terms of the uncoupling of nuclear division from septum formation, which could be the reason for its low plating efficiency.

Giant spheroplast formation from filamentous cells. When filamentous cells of ONT-3 obtained by growth in the presence of 0.005% SDS were washed with 1.5 M sucrose, treated with lysozyme-EDTA in 1.0 M sucrose contained 10% BSA at 30°C, and diluted 20-fold with the same medium, a huge spherical cell, 5 to 10 μm in diameter, was produced within 40 min (Fig. 5B). The volume was 120 to 200 times greater than that of an ordinary spheroplast obtained from ONT-3 grown under similar conditions but in the absence of SDS (Fig. 5A). When BSA was omitted from the reaction mixture, the formation of the huge spheroplast was
seen only partially, with most of the filamentous cells remaining in a tangled or aggregated state (Fig. 5C). More than 1% BSA was necessary to exert the protective effect, and this could not be replaced by glycerol, polyethylene glycol, or oleic acid. High concentrations (1 to 5%) of certain amino acids such as glycine, valine, and serine were found to be partially effective in this protection, whereas tryptophan was inactive. Spheroplasting was pH dependent with an optimum pH of 6.0 to 7.5. At more alkaline pH, severe tangleing and aggregation was observed to occur.

Whether formation of giant spheroplasts was specific to filamentous cells derived from SDS-treated ONT-3 was then examined in three different ways. First, SDS-induced filamentous cells from ONT-3 were subjected to further growth in the presence of either glucose or penicillin under the conditions described in Materials and Methods. In both cases only medium-sized spheroplasts were obtained in low frequency (not shown). Secondly, a thymine auxotrophic mutant E. coli H-5 was grown under thymine starvation. The filamentous cells obtained were treated in the same way with lysozyme but failed in spheroplast formation, and a marked cell lysis was observed (not shown). The third approach used a B. megaterium mutant requiring both lysine and diaminopimelic acid derived from strain IFO 7581 (19). This formed a filamentous cell when cultured at 42°C in a phosphate-deficient medium (16) and produced a chained form when grown at 30°C (Fig. 6B and C). Lysozyme treatment of the filamentous cell gave no large protoplast formation regardless of the presence of BSA, resulting in complete lysis. The chained form was converted by lysozyme to a chain of ordinary-sized protoplasts (Fig. 6D) that gradually separated to produce single protoplasts (Fig. 6E). These results strongly suggest that the formation of giant spheroplasts from E. coli ONT-3 SDS-induced filaments by lysozyme in the presence of BSA is a unique phenomenon.

Time course and manner of the giant spheroplast formation. Figure 7 represents the time course of spheroplast formation from a typical filamentous cell in 10-times-diluted Penassay broth-MgCl₂ medium observed by phase-contrast microscopy. At 7 min after lysozyme addition, spheroplasting commenced at three different positions along the single filament including both ends, which converted into two swollen bulbs at 10 min. With time the distance between the two swollen parts became gradually smaller, finally fusing at around 50 min via spindle-shaped, intermediary forms. The series of events observed are schematically summarized in Fig. 8, together with the determined diameter of each intermediary spherical form and the distance between them. A filament approximately 25 μm long was shortened within 7 min to about two-thirds of its initial length during the increase in diameter of three developing spheroplast bulbs. These gradually approached one another and, at around 37 min, fused to make up a giant spheroplast of 4.1 μm in diameter which remained unchanged for 120 min or longer. It is noticeable that the speed at which

![Fig. 7. Time course of spheroplast formation from a typical filamentous cell. (A) time 0, (B) 7 min, (C) 10 min, (D) 20 min, (E) 35 min, (F) 37 min, (G) 37.5 min, (H) 38.5 min, (I) 50 min, and (J) 60 min after (A). Bars = 10 μm. Medium: Diluted Penassay broth-MgCl₂.](http://jb.asm.org/)
SPHEROPLAST FROM E. COLI SURFACE MUTANT

Schematic illustration of time course in spheroplast formation.

<table>
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<th>0</th>
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<td><strong>diameter (μm)</strong></td>
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<td>2.9</td>
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<tr>
<td><strong>distance (μm)</strong></td>
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**FIG. 8.** Schematical representation of time course of spheroplast formation abstracted from Fig. 7.

**FIG. 9.** Electron micrograph of thin section of giant spheroplast of 5.9-μm diameter from a typical filamentous cell. Note that the outer membrane has been lost from half of the surface. Bar = 1 μm.
two bulbs coalesced was rather slow initially but rapid after the distance between them became less than 5 μm.

A typical electron micrograph of a thin-sectioned giant spheroplast is shown in Fig. 9. It represents a round-shaped cell of 5.9 μm in diameter surrounded by a continuous plasma membrane accompanied by partially stripped cell wall. The size of such a spheroplast is quite close to the dimensions of typical animal cells. The dynamics of the cell membrane and cytosol during this fusional spheroplasting, as well as the significance of the spindle-shape formation, remain to be solved.

LITERATURE CITED