Sphere-Rod Morphogenesis of *Escherichia coli*

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SUMMARY

The morphogenetic capacity of *E. coli* was studied by converting the rod-shaped cells into spheres and then determining whether these spheres could revert to rods. The morphogenesis of cells was followed by immobilizing them in a viscous Methocel-containing medium. Two different types of spheres were prepared: cells which retained a mechanically intact sacculus, and osmotically sensitive sphaeroplasts lacking a sacculus. The sphaeroplasts were not able to revert to rods although they were able to synthesize a new sacculus. In contrast, spheres which had retained an intact sacculus were able to reshape themselves into rods. They were also able to form new ends at (or near) the sites of the ends on the original rods.

INTRODUCTION

Regeneration experiments have been one of the classical approaches used to study the control of morphogenesis in multicellular eukaryotes. Some of these organisms have the capacity to form a complete organism from a small piece of body tissue. Extensive regeneration of this sort is often polarized; the anterior end of the regenerating organism is formed at the anterior end of the fragment. In single-celled prokaryotes such regeneration experiments are not possible since small sections cut from them are not viable. However, it is possible to study the morphogenetic capacity of these cells by artificially altering their shape and then measuring their capacity to return to the original shape: one can convert *Escherichia coli* rods into spheres and determine whether they can reshape themselves into rods. One can also determine whether the polarity of the original rods is retained, by investigating whether new ends are formed at the sites on the envelope where the original ends were.

The growth of spherical *E. coli* was first described by Lederberg (1956). He reported that the spheres themselves were not able to revert to rods; only by forming rod-shaped outgrowths were they occasionally able to produce rods. In these experiments the formation of spheres involved a considerable alteration in wall structure: the spheres were sphaeroplasts formed by disruption of the sacculus, the substructure of the envelope which gives it its mechanical stability and maintains cell shape. Therefore, it remained open whether loss of the sacculus damaged the cell and affected its morphogenetic capacity, or whether spherical *E. coli* were inherently unable to revert to rods.

In this study we have made use of the fact that it is possible to form spherical *E. coli* which retain an intact sacculus with the aid of a new penicillin derivative, penicillin FL-1060 (Lund & Tybring, 1972; Greenwood & O'Grady, 1973). Morphogenetic mutants are also available which become spheres with an intact sacculus after a temperature shift (Henning *et al.* 1972). The growth of cells was followed in Methocel medium (Lin, Hirota & Jacob, 1971). In this viscous liquid they remain in place and do not lose their original orientation. Thus it was possible to determine whether polarity was retained during rod-sphere-rod

morphogenesis. We found that cells which retained their sacculus were able to reshape themselves into rods, and that the areas of the envelope which had been the ends of the original rods again became ends in the newly formed rods.

METHODS

Strains and media. *Escherichia coli* w7 (Ryter, Hirota & Schwarz, 1973), requiring meso-2,6-diaminopimelic acid (DPM) and lysine, was used in most experiments. The temperature-sensitive morphological mutant Lss 12 (Henning *et al.* 1972), derived from w945T3282 (F⁻, *thi*, *leu*, *trp*, *lac*, *gal*, *his*, *thy*, *dpm*, *lys*, *thr*), was obtained from Dr U. Henning. Strain w7 was grown in MC1, a minimal citrate medium (Vogel & Bonner, 1956) supplemented with glucose (2 mg/ml), DPM (20 µg/ml) and Casamino acids (2 mg/ml; Difco). To form sphaeroplasts from w7 by DPM starvation, the cells were grown in MC2, a minimal medium containing glucose (2 mg/ml), Casamino acids (2 mg/ml), sucrose (12 %, w/v) and 0.1 M-MgSO₄. Lss 12 was grown in complete medium (antibiotic medium No. 3, Difco) supplemented with thymine (5 µg/ml), DPM (20 µg/ml), MgSO₄ (0.1 M) and sucrose (12 %, w/v). Unless otherwise stated, the bacteria were grown at 30 °C. Penicillin FL-1060.HCl.2H₂O (a gift from Leo Pharmaceutical Products Ltd, Ballerup, Denmark) was used at a concentration of 1 µg/ml.

Cultivation of w7 in Methocel media. Cells were grown in either MC1 or MC2 containing 3 % (w/v) Methocel (Roth Laborchemikalien, Karlsruhe, Germany) and the medium was spread on glass slides as described by Lin *et al.* (1971). Instead of paraffin oil, a cover slip was placed over the Methocel. A thin strip of stopcock grease around the edge of the cover slip prevented it from touching the Methocel and served as a seal. To prevent fogging, the cover slip was coated with a 0.1 % solution of Triton X-100 (Serva).

Penicillinase (Calbiochem, B grade), if needed, was added to the Methocel medium on the glass slides with a small wire loop. The penicillinase was dissolved in MC1 (180 000 units/ml) containing 0.013 % Triton X-100 to reduce the surface tension. Fifteen minutes before the addition of the penicillinase, the slide and the penicillinase were placed at 4 °C. At this temperature the viscosity of the Methocel-containing medium was higher and the cells were held more firmly in place while the penicillinase solution was added. The wire loop (7 mm diam, made from 0.01 mm diam wire) was dipped into the penicillinase solution, causing a film to form across the loop. Excess solution was removed from the loop and it was then touched to the Methocel medium. A ring of excess solution often remained on the medium where the wire itself had touched it; this was carefully removed. The slide was left at 4 °C for another 15 min and then returned to 30 °C.

DPM was applied to the Methocel medium in the same manner. The DPM (20 µg/ml) was dissolved in MC2 medium containing 0.013 % Triton X-100.

Cultivation of Lss 12 in Methocel medium. The cells were grown in complete medium containing 0.7 % Methocel. (The 3 % Methocel medium used above was too viscous at 42 °C to allow the cells to grow in chains.) The medium was placed between a glass slide and a cover slip and thinned to a thickness of 1 to 2 µm.

Photography. Phase contrast micrographs were taken with a Wild compound microscope equipped with an automatic camera. To measure cell dimensions and orientation, the images were projected onto a screen (total magnification ×4000).

Preparation of sacculi. Sacculi were isolated by boiling the cells in 4 % (w/v) sodium dodecyl sulphate for 15 min (Ryter *et al.* 1973) and then treating with α-amylase (Merck) to remove glycogen trapped inside the sacculi (Leutgeb & Weidel, 1963). The sacculi were

Fig. 1. Rod-sphere-rod morphogenesis of *E. coli* in liquid medium. Penicillin FL-1060 was added to an exponentially growing culture of *E. coli* w7. After the cells had become spherical (3.5 h; arrow), they were washed twice and inoculated into a penicillin-free medium. Samples were periodically removed and the bacteria photographed. From the photographs the length (□) and width (○) of 100 bacteria from each sample were measured. Bacteria in the process of division were not measured. Cell titre (●) was measured with a Coulter counter. To allow continued exponential growth, the culture was diluted tenfold after 3.5, 7.5, 9.5 and 11.5 h.

resuspended in 0.1 % sodium dodecyl sulfate (Ryter *et al.* 1973) and prepared for electron microscopy as described by Kellenberger & Arber (1957).

RESULTS

Formation of spheres with penicillin FL-1060

In minimal medium, *E. coli* w7 were rods with a diameter of 0.6 μm and an average length of 2.0 μm. When penicillin FL-1060 was added to the medium, they gradually lost their rod form (Fig. 1). The length decreased and the width increased, until after 3 to 4 h they were spherical with a diameter of 1.2 μm. They continued to divide both during (Fig. 1) and after this rod-sphere transition.

The spheres did not require osmotic protection, and physically intact sacculi could be isolated from them (Fig. 2). In general, penicillins cause breakdown of the sacculus; they inhibit the synthesis of murein [the polymer from which the sacculus is made (Weidel & Pelzer, 1964)], specifically the formation of peptide cross-links in the murein (Strominger *et al.* 1971). This is not so with penicillin FL-1060 (Park & Burman, 1973). Under the conditions used in our study (1 μg penicillin/ml minimal medium), penicillin FL-1060 did not

Fig. 2. Sacculi from penicillin FL-1060-treated cells. *Escherichia coli* w7 were grown for 4 h in medium containing penicillin FL-1060. The sacculi were isolated by boiling the cells for 15 min in 4 % sodium dodecyl sulphate (Ryter *et al.* 1973).

affect the degree of peptide cross-linking, nor did it inhibit the rate of murein synthesis (A. Asmus and U. Schwarz, unpublished).

Sphere-rod morphogenesis after removal of penicillin FL-1060

After 3.5 h growth in penicillin FL-1060, *E. coli* w7 had lost their original shape and dimensions. Electron micrographs of these cells, or of isolated sacculi (Fig. 2), indicated that they were almost perfectly spherical.

To determine if these cells could reshape themselves into rods with the proper dimensions, they were washed twice in minimal medium and inoculated into either fresh penicillin-free liquid medium (Fig. 1), or into solid Methocel-containing medium (Fig. 3) to follow the growth of single cells. In both media cells transformed themselves into relatively straight rods with differentiated ends and middles within one to two generations. Many of the rods were bent, but the angle of the bend was usually small; of 850 cells measured (liquid medium) 90 min after removal of the penicillin, 80 % had a bend of less than 30°. (Untreated w7 had no detectable bend.) A small proportion (7 %) of the cells were Y-shaped, with three ends.

The cells also reduced their diameter (Figs. 1 and 3). In liquid medium they started to

Fig. 3. Sphere-rod morphogenesis in solid Methocel medium after removal of penicillin FL-1060. *Escherichia coli* W7 were grown for 3.5 h in penicillin FL-1060-containing medium and then transferred to Methocel medium which lacked penicillin. Pictures were taken after (a) 1 h, (b) 2 h, (c) 2.75 h, (d) 3.25 h and (e) 3.75 h.

reduce their diameter within 30 min of the removal of penicillin FL-1060. They readjusted their diameter to that of the untreated rods; thereafter their diameter remained constant (Fig. 1). Changes in length were more complex; after 90 min many were longer than normal rods (Fig. 1). These 'filaments' did not disappear from the medium until 6 h after removal of the penicillin.

Normally, rod-sphere-rod morphogenesis required several generations to complete (Fig. 1). However, single cells in principle were able to undergo the complete rod-sphere-rod transition without dividing. This was shown by growing cells in Methocel medium and treating them with nalidixic acid (50 µg/ml) which inhibits DNA synthesis and cell division (Goss, Deitz, & Cook, 1964). They were able to form spheres and to revert to rods; they became long filaments. However, protein synthesis was apparently necessary. Spherical cells did not revert to rods when chloramphenicol (100 µg/ml) was added to the medium or when lysine was deleted from the medium (W7 is a lysine auxotroph).

Polarity of end formation

We attempted to determine whether the spheres were forming new ends at random sites on their surface or specifically at those sites which originally had been ends in the rods before penicillin treatment. To do this, the rod-sphere-rod morphogenesis of cells was followed in Methocel medium.

When normal *E. coli* are grown in Methocel medium, they form chains of cells aligned end to end; the long axis of each cell lies along the axis of the chain (Lin *et al.* 1971). If these cells are converted to spheres and then allowed to become rods again, they should re-form themselves into a chain of rods aligned end to end if they are able to re-form the ends of the original rods. If, however, the reverting cells formed ends at random sites on their surface, then the newly formed rods should not lie end to end, nor should the long axis of the rods lie along the chain axis.

During the complete rod-sphere-rod morphogenesis, the cells divided three or more times. The resulting chains were long and often became disorganized. To overcome this problem, the chain length was reduced to a minimum (i.e. to two cells). This was done by growing the cells for one h in liquid medium in the presence of penicillin FL-1060 before transferring

Fig. 4. Polarity of end formation. *Escherichia coli* w7 were grown for 1 h in liquid penicillin FL-1060-containing medium and then transferred to Methocel medium containing penicillin FL-1060. Pictures were taken (a) 1.5 h, (b) 2.5 h and (c) 3.5 h later. Penicillinase was added and pictures were taken after (d) 1 h and (e) 2 h. The cells in the upper microcolony became spherical after 2.5 h growth in penicillin (b); after addition of penicillinase they reformed themselves into rod-like cells in the end to end fashion (d).

Fig. 5. Morphogenesis of *E. coli* w7 sphaeroplasts. Cells were grown for 2 h in liquid DPM-free medium and then transferred to DPM-free Methocel medium. Pictures were taken (a) 0.5 h, (b) 1.5 h and (c) 2.5 h later. DPM was restored to the medium and pictures were taken after (d) 1 h, (e) 2 h and (f) 3 h.

them to Methocel medium containing penicillin FL-1060. These cells were lens-shaped and thus still had ends (Fig. 4a). After being placed in the Methocel medium some of them divided once and produced two spherical daughter cells (Fig. 4b, c). These were selected for measuring the location of the re-formed ends and were treated with penicillinase (see Methods). The reverting daughters shown in Fig. 4d re-formed themselves into rods aligned end to end. This is the orientation which normal rods have in a chain; thus the spheres in Fig. 4 appear to have formed new ends at the sites where ends would have been if the cells had remained rods. To determine whether this was generally the case, the reversion of 105 pairs of spherical daughter cells was observed: 92 % re-formed themselves into rods aligned end to end. Similar results were obtained with chains of four spheres. Thus the reverting cells usually retained the polarity of the original rods.

Spheres stored at 4 °C for 24 h before being allowed to revert also formed their ends at or near the sites of the original ends. Of 23 daughter cells observed, 22 re-formed themselves into rods in the end-to-end fashion. Spheres could also be stored for 24 h at 37 °C in growth medium containing chloramphenicol (100 µg/ml) without affecting their capacity to revert to rods after the chloramphenicol was removed. The capacity of the spheres to revert was thus retained for long periods of time even though they were not growing.

The above experiments did not exclude the possibility that it was the contact itself between the cells which was actually determining the site of end formation. This possibility seems unlikely, since lens-shaped cells (still showing polarity) occasionally became spheres without dividing. These formed short chains of rods with the same orientation in the Methocel as the original lens-shaped cells.

Reversion of the temperature-sensitive morphological mutant Lss 12

To demonstrate that the above results were neither peculiar to *E. coli* w7 nor specifically caused by penicillin FL-1060, the experiments were repeated with a temperature-sensitive morphological mutant, Lss 12, which grows as a sphere at 42 °C and as a normal rod at 30 °C (Henning *et al.* 1972). This mutant has an intact sacculus at both temperatures.

When grown at 30 °C these cells had a diameter of 0.6 µm and an average length of 2.2 µm. When transferred to 42 °C they became spherical after 2 h, with a diameter of 1.2 µm. Upon being returned to 30 °C they reverted to rods, albeit somewhat slowly; after 13 h they regained the dimensions of the original rods. The reverting cells showed the same retention of polarity as the penicillin FL-1060-treated cells; of 50 pairs tested, 84 % re-formed chains of rods orientated end to end.

The morphogenesis of spheres with an intact sacculus was very different from that of the sphaeroplasts described by Lederberg (1956). Since our results were obtained using a different solid medium and other strains of *E. coli*, these differences could be explained by assuming that Methocel affected morphogenesis or by assuming that the differences observed were strain-specific. To demonstrate that this was not so, the morphogenesis of *E. coli* w7 sphaeroplasts was followed in Methocel medium.

Reversion of sphaeroplasts

Escherichia coli w7 is a diaminopimelic acid (DPM) auxotroph. DPM is found only in the sacculus; when these cells are starved of DPM under osmotic protection (12 %, w/v, sucrose) they become sphaeroplasts (Lederberg & St Clair, 1958; McQuillen, 1958; Schwarz & Leutgeb, 1971). The reversion of sphaeroplasts was initiated by restoring DPM to the medium; the spheres enlarged, but, unlike penicillin FL-1060-treated cells, they remained spherical (Fig. 5). The growth of 124 sphaeroplasts was followed in Methocel medium for 8 h: 108 were still spherical, 12 had lysed, and three appeared to have reverted to rods. The spheres occasionally formed rod-shaped outgrowths, and rods were often produced from these outgrowths. These rods usually grew and divided normally; the different fate of the spheres and rods shows that it is not sufficient to follow the reversion of a cell population in liquid medium.

Though the spheres could not reshape themselves into rods, it was possible that their growth was not random. If the rod-shaped outgrowths were being formed at specific sites on the surface of the spheres, such as the sites of the original ends, then one could conclude that the cells had retained the polarity of the original rods. Therefore, the location of the outgrowths on the sphaeroplasts was determined. Figure 6 shows that the outgrowths were not formed preferentially at the sites of the original ends but apparently at random around the cell, suggesting that the sphaeroplasts had lost the polarity of the original rods.

It should be noted that the sphaeroplast growth medium contained 12 % sucrose and 0.1 M-MgSO₄, whereas the penicillin FL-1060 medium did not. These two compounds did not, however, affect the rod-sphere-rod morphogenesis of penicillin FL-1060-treated cells.

DISCUSSION

The present studies show that spherical *E. coli* have the capacity to reshape themselves into rods. The morphogenetic capacity of these cells showed three characteristics which imply that within the cell (possibly within the envelope) there exists some kind of stable

Fig. 6. Location of rod-shaped outgrowths on reverting sphaeroplasts. Cells were grown for 2 h in liquid DPM-free medium and then transferred to DPM-free Methocel medium as described in Fig. 5. Most of the cells divided once in the Methocel medium before becoming sphaeroplasts. The contact point between the two sister cells was assumed to be the site of one of the original ends of each cell and was defined as 0° ; 180° was assumed to be the site of the other end. DPM was added to the medium and the site of formation of outgrowths on these cells was determined. The formation of 127 outgrowths was observed.

order directing morphogenesis: (i) they retained the potential to revert to rods for long periods of time; (ii) they preserved their polarity and re-formed their ends at (or near) the sites of the original cell poles; (iii) when reverting to rods, they were able to reshape their envelope over most (if not all) of its surface.

Sphaeroplasts did not have these capacities. *Escherichia coli* w7 sphaeroplasts synthesize a new (spherical) sacculus within 20 to 30 min of DPM being restored to the medium (Schwarz & Leutgeb, 1971); therefore it is not the lack of the sacculus which prevented them from reshaping themselves into rods. Loss of the original sacculus appears to have resulted, either directly or indirectly, in loss of the organization within the cell which enables it to revert to a rod and to retain its polarity. Thus these observations suggest that the sacculus plays a role in the morphogenesis of the cell in addition to its well-known role in maintaining cell shape (Weidel & Pelzer, 1964). One can only speculate about the nature of this role: within the original sacculus itself there may be specific regions which determine where the ends will be formed during sphere-rod morphogenesis. One other possibility is that the morphogenetic apparatus of *E. coli* is part of a protein matrix within the envelope as suggested by Henning, Rehn & Hoehn (1973), and that this matrix must be stabilized and held in place by the sacculus.

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