Morphological Mutants of *Escherichia coli.*
Isolation and Ultrastructure of a Chain-forming envC Mutant

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Non-conditional morphological mutant envC derived from *Escherichia coli* K-12 strain R678, isolated after treatment with 1-methyl-3-nitro-1-nitrosoguanidine, produced chains of bacteria on synthetic or rich media at 30 °C and 40 °C. It was sensitive to deoxycholate and less resistant to penicillin and rifampicin than the parent strain. In section under the electron microscope, the mutant showed greatly disorganized septum formation. Preliminary mapping of envC by conjugation located it near *xyl.* The morphological and physiological characteristics of envC+ recombinants and the envC+ parent were identical.

INTRODUCTION

The mechanisms that control bacterial morphology and division are not clearly understood. Most mutations affecting these processes might be expected to be lethal. However, recent reports show that conditional and non-conditional morphological mutants can be obtained and used to learn something about their physiology and genetics. In their extensive study of thermosensitive mutants of *Escherichia coli* K-12, Kohiyama, Cousin, Ryter & Jacob (1966) observed many strains which grew normally at permissive temperatures but presented morphological, cytological and physiological alterations and irregularities in division at 40 °C. Three principal types of alterations were recognized: (i) filament and chain formation, (ii) irregular morphology; and (iii) spherical forms. Several other authors isolated non-conditional or thermosensitive mutants of *E. coli* with characteristic morphological alterations belonging to one of these groups (Normark, Boman & Mattson, 1962; Adler, Terry & Hardigree, 1968; Hirota, Ricard & Shapiro, 1971; Henning et al. 1972; Lazdunski & Shapiro, 1972). Another type of conditional mutant of *E. coli* is osmotically unstable at non-permissive temperature and the bacteria lyse (Kohiyama et al. 1966; Mangiarotti, Apirion & Schlessinger, 1966; Matsuzawa, Matsuhashi, Oka & Sugino, 1969). In most cases lysis can be prevented by osmotic stabilization.

Morphological mutants and mutants defective in peptidoglycan synthesis were also reported for *Bacillus subtilis, B. licheniformis, Staphylococcus aureus and Agrobacterium tumefaciens* (Rogers, McConnell & Burdett, 1968; Boylan & Mendelson, 1969; Chatterjee & Young, 1972; Fujiwara & Fukui, 1972; Good & Tipper, 1972). It appears that the morphological defect is generally accompanied by a chemical and/or structural alteration of wall or cytoplasmic membrane and that the division is also affected in most cases.

During our recent work with morphological mutants we have isolated a chain-forming mutant of *Escherichia coli* K-12 which is not thermosensitive. In this first communication we
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Fig. 1. Growth of the mutant \( \text{pm}61 \) (○), of the parent strain \( \text{p}678 \) (●) and of the recombinant \( \text{pm61} \times \text{p}4x \) (●). DOC (0.1 % final concn) added to \( \text{p}678 \) and to the recombinant (arrows) was without effect on growth rate, but produced a rapid lysis (●) of \( \text{pm}61 \). Temperature, 37 °C.

describe the ultrastructural characteristics of the defective division and some physiological properties of this mutant. The gene mutated, designated \( \text{envC} \), maps in the xylose (xyl) region of bacterial chromosome.

METHODS

Strain. The strain Escherichia coli K-12 \( \text{p}678 \) originally isolated by F. Jacob was obtained from H. I. Adler and has the following genetic constitution: F- thr leu lac gal xyl mal man thi and is resistant to streptomycin.

Mutant isolation. To obtain both thermosensitive and non-conditional mutants, organisms of the strain \( \text{p}678 \) grown in rich medium were mutagenized with 1-methyl-3-nitro-1-nitrosoguanidine (Kohiyama et al. 1966). After mutagenesis bacteria were washed free of mutagen, allowed to grow overnight in rich medium at 30 °C and then plated on solid medium. The colonies were replicated twice on the same medium and incubated at 30 °C and 40 °C, respectively. Selection of morphological mutants was made by observation of organisms in each colony under a phase contrast microscope. Strain \( \text{pm61} \) was one of a number of mutants purified from colonies which grew more slowly after replication and were visible only after 48 h incubation at both temperatures.

Media and growth. Rich medium contained \((\text{g/l distilled water})\): Bacto peptone (Difco, Detroit, Michigan, U.S.A.), 5; Bacto beef extract (Difco), 3; Bacto yeast extract (Difco), 3; NaCl, 5. Rich medium for colony growth and replica plating was made by solidifying this medium with 12 g Bacto agar/l. Tryptose medium contained \((\text{g/l distilled water})\): Bacto tryptose (Difco), 5; NaCl, 10. Tris-buffered synthetic medium was that of Hershey (1955),
Morphological mutants of E. coli

Fig. 2. A section through a part of a chain of pm61, showing arrangement of septum (arrow) enlarged on Fig. 7.

Fig. 3. Oblique septum and infoldings of the wall.

Fig. 4. Anomalous wall constrictions often without achieved septum (arrows). S, septum.

Fig. 5. Chain of bacteria mostly with achieved septa. Arrows indicate ‘abortive’ constrictions.
supplemented with thiamine, threonine and leucine of 50 μg/ml. Growth of agitated cultures was measured in a Jean & Constant spectrophotometer at 450 nm.

Electron microscopy. Cultures on rich medium were centrifuged and fixed by glutaraldehyde and osmium tetroxide according to Ryter & Jacob (1966). Ultrathin sections of bacteria embedded in Araldite were contrasted with potassium permanganate (Thomas, 1972).

Bacterial matings. Conjugation experiments were performed at 37 °C in rich medium using Hfr P4X met thi (λ)− sensitive to streptomycin, as donor. Mating mixtures of exponentially growing bacteria containing about $5 \times 10^8$ Hfr and $10^9$ F− organisms/ml were incubated for 90 min and appropriate dilutions were plated on selective media.

RESULTS

General characteristics of the chain-forming mutant PM 61

Mutant PM61, observed stained or unstained under phase contrast after growth on liquid rich medium at 47°C, formed long chains consisting of individuals of rather irregular length between 1 and 10 μm; their number ranged from 10 to 80 and more per chain. In the minimal medium the chains were generally shorter. Culture at 30 °C or 40 °C did not affect the morphology. The generation time in tryptose medium at 37 °C was 53 min for PM61 and 36 min for the parental strain P678. A striking difference between the mutant and the parent was observed when sodium deoxycholate (DOC) was added to growing cultures of both strains: the parental strain grew normally with 1% DOC but growth of PM61 was inhibited by 0.1% detergent (Fig. 1). In tryptose medium, PM61 was inhibited to 10 μg penicillin/ml and by 0.5 μg rifampicin/ml; for P678, inhibitory concentrations were 50 μg penicillin/ml and 10 μg rifampicin/ml.

Electron microscopic observations

Thin sections in the electron microscope indicated that septum formation by PM61 was either accompanied (Fig. 2) or not (Fig. 12) by a constriction of the dividing organism. The portion of the cytoplasmic membrane invaginating at the division plane formed a simple fold, progressively separating the daughters (Fig. 6, 7, 8). The invagination of the membrane was followed by formation of a thin septum connected with the dark layer surrounded by the outer membrane (Fig. 9). This layer is considered to be the mucopentide containing portion of the wall (De Petris, 1965; 1967).

In most cases the constriction of the outer membrane stopped at an early stage, presumably because the mucopentide layer could not be split in two separate structures. In stationary phase of the growth some individuals were lysed but they were still attached to the

Fig. 6. Early stage of the division. Invaginated cytoplasmic membrane (M) surrounds a monolayered septum (S) growing out of the internal (mucopentide) layer of the wall (W).

Fig. 7 to 9. Two daughter organisms completely separated by a septum. Arrows indicate a fork-like attachment of the central layer of the septum (S) to the internal layer of the wall. This is the stage of division most frequently found in sections of chains. M, cytoplasmic membrane.

Fig. 10. A section of an autolysed organism with invaginated wall (W) including outer membrane. M, cytoplasmic membrane.

Fig. 11. Advanced stage of division showing completed septum with outer membrane.

Fig. 12. Septum without typical constriction.

Fig. 13. Vesicular invagination of cytoplasmic membrane. Note the sparse layer between two membranes (M).

Fig. 14. Oblique invagination of cytoplasmic membrane (M).
Table 1. Analyses for unselected markers in selected recombinants from a cross between P4X and PM61

<table>
<thead>
<tr>
<th>Selected recombinants</th>
<th>Number tested</th>
<th>Thr+</th>
<th>Leu+</th>
<th>Xyl+</th>
<th>Mal+</th>
<th>envC+</th>
</tr>
</thead>
<tbody>
<tr>
<td>thr+ leu+</td>
<td>153</td>
<td>25</td>
<td>5</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xyl+</td>
<td>116</td>
<td>29</td>
<td></td>
<td>26</td>
<td></td>
<td>90</td>
</tr>
</tbody>
</table>

The symbol envC+ designates recombinants having normal morphology. Streptomycin was used as a contraselector.

chain by uncompleted septa (Fig. 10). A complete septum was found only exceptionally (Fig. 11).

Another interesting phenomenon occurring rather frequently was the presence of several constrictions of the envelope in the proximity of incomplete septa, as if there were several unsuccessful attempts at division (Fig. 4, 5). These constrictions were sometimes asymmetrical or only on one side of the organism (Fig. 3). In some, the cytoplasmic membrane formed intracytoplasmic invaginations (Fig. 13, 14), but these structures could be seen only occasionally.

It seems that the phenotypic expression of this mutation concerns septum formation and the organization of the envelope. Thus our mutant PM61 appears to belong to the group of env mutants described by Normark et al. (1969).

Genetic localization of the envC locus

The envA mutation-mediating chain formation in the strain D22 of Escherichia coli is located between leu and azi at 1.5 min (Normark, 1970). Another mutant producing irregular sphere-like organisms was designated envB by the same author and it maps near streptomycin resistance. Since the physiological and cytological properties of PM61 are closely similar to D22 (envA), it was important to locate the env mutation-mediating chain formation of our strain.

We performed a number of mating experiments with Hfr P4X (Wollman strain, met thi (λ+)) which transfers in the order pro-leu-thr-xyl-mal-str. All selected recombinant were observed microscopically. An analysis of the various recombinants obtained in one cross is shown in Table 1. Conjugation experiments indicate a strong linkage (90%) between envC and xyl; thus the mutated gene appears to be located in xyl region and to be distinct from envA. It is not identical with envB whose phenotypic expression (amorphous organisms) and chromosomal location near str are different. Therefore we designate the mutation of PM61 as envC. A more detailed mapping of envC by transduction is in progress.

The one envC+ xyl+ mal gal lac met man man thi str-r recombinant tested was resistant to DOC and less sensitive to penicillin and rifampicin than PM61. Its growth rate was exactly the same as that of the parental strain P678 (Fig. 1).

DISCUSSION

Genetically determined morphological changes in bacteria have been observed mainly with conditional mutants. This is not surprising, since most non-conditional mutations affecting production or activity of wall-synthesizing enzymes, or other processes involved in division or leading to unbalanced formation of wall or cytoplasmic membrane, are expected
to be lethal. The few non-conditional mutants of *Escherichia coli* of which we are aware belong to two morphological types: (i) irregular sphere-like organisms of *mon* mutant (Adler *et al.* 1968) and of *envB* mutant (Normark, 1969); and (ii) chain-forming mutants *envA* (Normark *et al.* 1969) and *envC* described in the present paper.

The mutants *mon* and *envB* are similar in several respects, as pointed out by Normark (1969). The *envB* gene maps near streptomycin resistance and preliminary experiments performed in our laboratory (L. A. Genta, unpublished), indicate that the *mon* gene is also located in this region.

Although *envA* and *envC* mutants are phenotypically similar, our conjugation experiments indicate that *envC* is not identical with *envA*. A more detailed comparison of these mutants is necessary.

The alteration of shape of a number of *Escherichia coli* mutants appears to be a phenotypic expression of mutations in several different genes *mon*, *rod*, *env*, but in no case has the primary effect of the mutation been identified. It is not clear whether the morphology is determined by the cytoplasmic membrane or by some other structure of the envelope. Although the role of the cytoplasmic membrane seems to be of special importance (Henning *et al.* 1972; Ingram, Van Baalen & Fisher, 1972), the role of the remaining layers cannot be eliminated.

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**REFERENCES**


