Characterization of a Conditional Mutant with Altered Envelope Showing pH-dependent Morphology and Temperature-dependent Division

By G. SATTA AND ROBERTA FONTANA
Institute of Microbiology, University of Genoa, 16132 Genoa, Italy

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SUMMARY

The properties of a conditional mutant of Klebsiella pneumoniae (Mir M7) are described. The mutant has a pH-dependent disturbance of morphology and a temperature-dependent disturbance of division. At pH 7 the mutant grows as cocci and at 41 °C (restrictive temperature) division is inhibited. At acid pH and restrictive temperature, filaments are formed. At pH 7 and permissive temperature, polymorphous cocci are obtained. At pH 7 and 41 °C, giant polymorphous cells are formed. The inhibition of division is phenotypically reverted by 0.05 M-Mg²⁺, 0.1 M-Na⁺ or 0.4 M-sucrose. Seventeen revertants were isolated having exactly the same characteristics as the wild-type Klebsiella, so it seems that only one mutation is responsible for the different disturbances of the mutant. The possibility of membrane damage on which the morphology and division disturbances may depend is discussed.

INTRODUCTION

The interdependence between bacterial functions and structures such as the cell membrane is becoming evident (Hirota, Mordoh & Jacob, 1970; Rogers, 1970). The physical integrity and replication of DNA, for example, may depend on the state of the cytoplasmic membrane (Schachtele, Anderson & Rogers, 1968; Shapiro, Siccardi, Hirota & Jacob, 1970; Siccardi, Shapiro, Hirota & Jacob, 1971). Inhibition of DNA replication can cause changes in membrane protein composition (Inouye & Pardee, 1970; Walker & Pardee, 1968) and inhibition of septum formation (Walker & Pardee, 1968). The timing of action of some hydrolases located in the membrane has been reported to be correlated with genome replication (Schwarz, Asmus & Frank, 1969). The inhibition of septation can be caused by partial inhibition of the synthesis of mucopeptide (Rogers, 1970). Slight or gross alteration of the wall is frequently associated with an aberrant invagination of the septum (Boylan & Mendelson, 1969; Cole, Popkin, Boylan & Mendelson, 1970; Grula & King, 1970; Rogers, MacConnell & Burdett, 1970).

A possible hypothesis is that in such relationships a key role is played by a limited area in the membrane which, according to various models (Jacob, Brenner & Cuzin, 1963; Donachie & Begg, 1970), might be the site at which the genome replicates, growth of the cell wall and the cytoplasmic membrane take place, and septum invagination occurs. Some experimental data already support this possibility (Jacob et al. 1963; Ryter, 1967; Donachie & Begg, 1970; Ryter, 1971) and further support could be obtained from the isolation of conditional membrane mutants damaged in more than one of these functions.

Meloni & Monti-Bragadin (1962) have isolated in this laboratory a strain of Klebsiella pneumoniae (Mir M7) which shows a rod-shaped morphology at pH 5.5 and a coccoid morphology at pH 7. Recently, we have noticed that this mutant shows disturbances of
the membrane and of cell division dependent on conditions of incubation (Satta, Schito & Meloni, 1969). This paper records further observations on these phenomena.

METHODS

Bacterial strains. Mir M7, a morphological mutant of Klebsiella pneumoniae, has been isolated and described by Meloni & Monti-Bragadin (1962). Mir A12 is a revertant with the physiological and morphological characteristics of the wild-type Klebsiella pneumoniae (G. Satta & R. Fontana, unpublished).

Media and pH. PL medium contained (g/l): peptone, 10; lactose, 10; K₂HPO₄, 0·3; KH₂PO₄, 0·1; Na₂SO₄, 0·2. The pH varied between 5·3 and 5·7 and was brought to the desired value by adding NaOH or HCl. In the experiment at constant pH, the pH was measured every 30 min, and when necessary adjusted by a calculated amount of HCl or NaOH.

Preparation of round and rod-shaped cells. When Mir M7 was grown or maintained on a slant of PL agar at pH 7 and 37 °C the cells were round. To yield round cells for harvesting, growth was on PL at pH 7 and 37 °C, to an optical density (O.D.) of 0·5 as measured by a Beckman DU spectrophotometer at 650 nm. Rod-shaped cells were obtained by transferring a loopful of Mir M7 from the slant into PL at pH 5·5, and incubating at 30 °C for 36 h. A 2 ml portion of this culture was transferred to PL at pH 5·5 and incubated at 30 °C to an O.D. of 0·5. Cells of either shape were harvested by centrifugation at 4000 g for 5 min.

Revertants. Three techniques were used to isolate revertants. One exploited the relative fragility of mutant Mir M7 compared with the wild-type Klebsiella when subjected to repeated freezing and thawing. The second utilized lysis of the round cells by DL-methionine in PL at pH 5·5. The third technique employed the inhibition of division at 42 °C.

Freezing and thawing. A log phase culture of Mir M7 in PL pH 7 was harvested and resuspended in 0·84 m-NaCl at 2 × 10⁸ cells/ml. The solution was rapidly and repeatedly frozen and thawed till the O.D. of the suspension had fallen to 10 % of the original value. A 10 ml portion of this suspension was centrifuged for 5 min at 4000 g. The pellet was resuspended and subdivided into 100 tubes of PL pH 7 which were incubated at 37 °C until the O.D. reached 0·5. The tubes were frozen and thawed till the bacterial suspension was clarified, after which the suspension was again incubated at 37 °C for 4 h. All the tubes in which growth had occurred were examined and those cultures containing rod-shaped cells were plated on PL agar pH 7 plates. From each isolation a colony of rod-shaped cells was chosen and transferred to a slant for subsequent experiments.

Growth in PL at 42 °C. About 4 × 10⁷ cells were spread on each of 30 PL agar pH 5·5 plates and incubated at 42 °C for 72 to 96 h. An average of five colonies from each plate was transferred and incubated again for 24 h. All the colonies that survived this last transfer were grown in PL pH 5·5 and incubated at 42 °C for 36 h. Cultures whose cells were rod-shaped were diluted to about four cells/ml and 0·1 ml samples were transferred to PL and incubated at 42 °C for 36 h. A turbid tube containing rod-shaped cells from each diluted sample was transferred to a slant for subsequent experiments.

Growth in PL pH 5·5 containing DL-methionine. Round cells prepared and harvested as indicated were transferred to 200 tubes containing 5 ml of PL pH 5·5 plus DL-methionine (0·3 mg/ml) to give a final O.D. of 0·1. The tubes were incubated at 37 °C and after 16 h, tubes in which the O.D. had dropped to below 0·020 were selected and incubated again for 12 h at the same temperature. Turbid tubes were diluted to final concentration of about
Mutant with altered morphology and division

Fig. 1. Effect of various incubation pH's on the growth of (a) Klebsiella pneumoniae Mir M7 and (b) Mir A12 in PL medium. Equal quantities of round cells of Mir M7 or of Mir A12 in log phase, were transferred to each of the media and incubated at 37 °C without shaking. At regular time intervals the O.D. was measured. The pH was kept constant as described in Methods.

four cells/ml and 0.1 ml samples were inoculated into each of 15 tubes. After 12 h incubation at 37 °C a turbid tube for each series of 15 tubes was taken at random; the cellular morphology was examined and a loopfull was transferred to a slant for subsequent experiments.

RESULTS

Growth and morphology at different pH values

Fig. 1 and 2 and Table 1 show the growth curves and the morphology of round cells of the mutant Mir M7 and of the strain Mir A12 (a revertant with the physiological and morphological characteristics of the wild-type Klebsiella pneumoniae) in PL medium at various pH values. At present we have no explanation for the non-linearity of these growth curves. Preliminary results suggest that the reduction in growth rate between the second and fourth hour at pH 6, 7 and 7.5 might be due to the induction of a bacteriophage.
Fig. 2. Polymorphism and atypical divisions in cells of mutant Mir M7. Cells of Mir M7 grown in PL pH 7 to log phase, were transferred to a PL pH 7 medium and incubated at 37 °C without shaking. The pH was kept constant as described in Methods. Scale markers represent 8 μm.

However, the growth curves of the strain Mir A12 and of the mutant Mir M7 between pH 6 and 8, are similar.

Strain Mir M7 can grow at pH values between 5 and 8 with an optimum between 6.5 and 7 (Fig. 1). It is very sensitive to alkaline pH values; it grows badly at pH 7.5, the optimum for Mir A12 strain, and is inhibited more by pH values around 8 than is the Mir A12 strain (Fig. 1). At pH values between 6 and 8 the mutant and Mir A12 strain differ only in growth rate; but at pH 5.5 the mutant characteristically lags for about 2 h, during which time the turbidity drops 10 to 20%.

At low pH values the coccal-shaped cells become rod-shaped after 3 to 6 h incubation (Table 1). The highest pH value for the transition to the rod morphology is pH 6.5. At lower pH values the transition is more rapid and longer rods are formed. At the pH optimum for growth, cells remain roundish and most of the divisions are atypical (Fig. 2). At alkaline pH values the cells become uniformly round, and the higher the pH, the bigger they ultimately become, until at pH 8, they look like spheroplasts.
Table 1. Influence of pH on the morphology of Mir M7 and Mir A12 strains growing in PL medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (h)</th>
<th>5</th>
<th>5.5</th>
<th>6</th>
<th>6.5</th>
<th>7</th>
<th>7.5</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mir M7</td>
<td>3</td>
<td>Round cells</td>
<td>Short polymorphous rods and round cells</td>
<td>Very short polymorphous rods; atypical divisions</td>
<td>Fat polymorphous cells; atypical divisions</td>
<td>Polymorphous cells; atypical divisions</td>
<td>Uniform round cells</td>
<td>Big, uniform round cells</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Very short rods</td>
<td>Polymorphous rods</td>
<td>Short polymorphous rods; atypical divisions</td>
<td>Very short polymorphous rods; atypical divisions</td>
<td>Round polymorphous cells; atypical divisions</td>
<td>Big, uniform round cells</td>
<td>Spheroplast-like cells and big uniform round cells</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>Rods</td>
<td>Very long rods</td>
<td>Long rods</td>
<td>Fat rods; atypical divisions</td>
<td>Round polymorphous cells; atypical divisions</td>
<td>Big round cells; a few spheroplast-like cells</td>
<td>Spheroplast-like cells</td>
</tr>
<tr>
<td>Mir A12</td>
<td>9</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Short rods</td>
<td>Short rods</td>
</tr>
</tbody>
</table>
Fig. 3. Effect of incubation temperature on rod-shaped cells. Rods of mutant Mir M7 were transferred into PL pH 5.5 medium. The culture was subdivided into 3 parts which were incubated respectively at 30 °C (□—□), 37 °C (○—○) and 41 °C (●—●) without shaking. At regular time intervals (a) the colony forming units and (b) O.D. were determined.

**The effect of temperature on growth and morphology in PL at pH 5.5**

The effect of temperature on growth and morphology of round and rod-shaped cells growing at pH 5.5 is illustrated in Fig. 3 and 4, and Table 2. At 30 °C both round and rod-shaped cells grow and divide normally, and after growth for 48 h they cannot be distinguished from the original strain (Satta & Fontana, 1974). At 37 °C round and rod-shaped cells grow and divide regularly for 10 to 14 h. Subsequently the viable count remains constant and the O.D. continues to increase. Both the cocci and rods form filaments. At 41 °C growth is always less, and round and rod-shaped cells differ in behaviour. Within the first four hours, the former drop slightly in O.D. and 70 to 80% in viable count. From then on, the O.D. slowly increases for 12 h, whereas the viable count remains constant.
A large percentage of cells stay rod-shaped for the whole experiment (probably cells which are dead), whereas others lengthen, forming filaments after about 10 h. The filaments sometimes become very long, and are always fatter than normal rods and polymorphous (Fig. 4). Cells incubated as rods at 41 °C grow from the beginning of incubation. The O.D. increases during the whole period of incubation; the viable count maintains its original value for 24 h, before decreasing slightly (Fig. 4). With continued incubation the rods grow longer and longer, forming long uniform filaments which have the same diameter as normal rods.

**Growth and morphology at 41 °C in PL at pH 7**

Fig. 5 and Table 2 show the morphological evolution of round cells in PL pH 7 at 41 °C. Even at this pH about 50 to 70% of the round cells die during the first few hours and the viable count does not subsequently increase. The O.D. drops during the first few hours after which it shows a slow increase. Fifty to 80% of the population are round cells which remain unaltered all through the experiment and are most probably dead cells.
Table 2. Effect of temperature on the morphological evolution of the round and rod-shaped cells of Mir M7 incubated at pH values 5.5 and 7

<table>
<thead>
<tr>
<th>Original morphology</th>
<th>Incubation time (h)</th>
<th>Morphology at different incubation temperatures</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30 °C</td>
</tr>
<tr>
<td><strong>Rods at pH 5.5</strong></td>
<td>8</td>
<td>Uniform rods</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Uniform rods</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Uniform rods</td>
</tr>
<tr>
<td><strong>Round cells at pH 5.5</strong></td>
<td>8</td>
<td>Polymorphous rods</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Uniform rods</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>Uniform rods</td>
</tr>
<tr>
<td><strong>Round cells at pH 7</strong></td>
<td>8</td>
<td>Round polymorphous cells</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Round polymorphous cells</td>
</tr>
</tbody>
</table>

Fig. 5. Giant and monstrous cells originating from round cells incubated in PL pH 7 medium at 41 °C. The pH was maintained constant as described in Methods. Scale markers represent 15 μm.

The remaining cells show a progressive increase in diameter, forming giant and amorphous cells which can reach an average diameter 4 to 6 times greater than the original (Fig. 5).

Effect of sucrose, MgCl₂ and NaCl on the growth in acid medium at 37 °C and 41 °C

High osmolarity and some ions can protect fragile cells (MacQuillen, 1960). A study has been made of the growth of round and rod-shaped cells in PL pH 5.5 containing different concentrations of sucrose, MgCl₂ or NaCl, at 37 and 41 °C. At 37 °C (Fig. 6)
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Fig. 6. Effect of the various sucrose (●—●), NaCl (○—○) and MgCl₂ (□—□) concentrations on the growth of round cells of Mir M7 incubated in PL pH 5.5 medium. Round cells of Mir M7 in log phase were transferred into PL pH 5.5 medium containing each of these three substances at various concentrations between 1 M and 1 × 10⁻² M. The original O.D. was the same in all samples. After about 6 h of incubation without shaking, the O.D. was determined.

all the substances tested abolished the typical 2 h lag before growth, led to an increase in the growth rate, and prevented the formation of filaments which otherwise appear after 10 to 14 h. Mg²⁺ ions were found to have their maximum efficiency at a molarity between 5 × 10⁻² and 1 × 10⁻¹, while sucrose has its maximum effect on growth rate at 4 × 10⁻¹ M, having a negligible effect at 5 × 10⁻² to 1 × 10⁻¹ M (Fig. 6). At 41 °C, sucrose, Mg²⁺ and Na⁺ ions are effective at the same respective concentrations. They are able to prevent the drop in O.D. and viable count, and the 4 h lag before growth, all of which are typical of round cells in the acid medium. They also overcome the inhibition of division of rod-shaped and round cells. Fig. 7 shows that in the presence of 0.4 M-sucrose, 0.1 M-Na⁺ or 0.05 M-Mg²⁺, both the O.D. and the viable count of cultures inoculated with rods in PL pH 5.5 increase immediately and in parallel.

Effect of deoxycholate and spermine

The behaviour of the mutant Mir M7 was studied in the presence of deoxycholate, which can lyse cells with damaged membranes (Nagel de Zwaig & Luria, 1967; Hirota et al. 1969; Hirota et al. 1970; Rolfe & Onodera, 1971), and of spermine, which protects membranes (Cohen & Lichtenstein, 1960; Mager, 1959a, b; Puck, 1960). The concentration of deoxycholate inhibiting growth of the mutant is one fifth of the minimum concentration that has any significant inhibitory effect on the growth of the wild type (Fig. 8). Spermine (5 × 10⁻³ M)
Fig. 7. Effect of sucrose, Mg$^{2+}$ and Na$^+$ on the growth of rods. Cells of mutant Mir M7 in log phase were transferred to a PL pH 5.5 medium (■—■), and PL pH 5.5 medium added with 0.4 M-sucrose (○—○), 0.05 M-MgCl$_2$ (□—□) or 0.1 M-NaCl (○—○), and incubated at 41 °C without shaking. At regular time intervals the viable count (a) and the O.D. (b) were determined.

allowed the round cells incubated in PL pH 5.5 to grow immediately and with a growth curve similar to that obtained in the same medium supplemented with NaCl, MgCl$_2$ or sucrose. In addition, spermine prevents the formation of filaments after extended incubation (12 h at 37 °C) and allows the formation of uniform and regular rods after 4 to 6 h.
Mutant with altered morphology and division

Fig. 8. Effect of deoxycholate at various concentrations on the growth of Mir A12 (a) and of mutant Mir M7 (b). Cells of the two strains were incubated in PL pH 7 medium to log phase and transferred to PL pH 7 (□—□) and to PL pH 7 containing sodium deoxycholate at 0.1% (○—○) and 0.5% (●—●) and incubated at 37°C without shaking. At regular time intervals the O.D. of the culture was determined.

Revertants

Three groups of revertants were isolated according to three different criteria: (i) The greater resistance of the rod-shaped cells to the action of rapid freezing and thawing was utilized; bacteria having a normal morphology were selected. (ii) Revertants were isolated by their ability to grow at 42°C. (iii) Bacteria were selected which were resistant to the effect of a mixture of DL-amino acids, which caused lysis only of the round cells, without effect on rod-shaped cells or on the wild type. Such behaviour has been correlated with the function of specific permeases (Cohen & Monod, 1957) in the membrane. Therefore, in a population of round-shaped cells incubated in the presence of DL-methionine, only revertants with a normal membrane may grow.

By these methods all revertants isolated were found to possess a stable rod-shaped morphology, the ability to divide regularly at 42°C, and an insensitivity to DL-methionine or deoxycholate. The simultaneous recovery of membrane function, morphology and cell division, is consistent with the existence of a single pleiotropic mutation.

DISCUSSION

The present paper confirms and extends an earlier communication from this laboratory which showed that mutant Mir M7 exhibits disturbed morphology (Meloni & Monti-Bragadin, 1962; Satta et al. 1969), forming round cells when grown at a pH above 7 but normal rod shapes at pH values below 7.

Several observations suggest that the round morphology is associated with a weakening and/or a disorganization of the rigid layer of the cell wall. In fact Mir M7 round cells are much more fragile than the rod-shaped cells of both the mutant and the wild type. They are easily lysed by freezing and thawing, and by mild sonication (R. Fontana & G. Satta, unpublished). Growing under conditions in which division is inhibited, for example at 41°C or in the presence of penicillin or nalidixic acid (R. Fontana & G. Satta, unpublished), they form giant polymorphous cells at pH 7 and long filaments at acid pH. Increasing the pH above 7
yields larger and larger round cells; at pH 8 spheroplast-like cells are formed. Also at pH values of 6 and 7.5, the mutant grows respectively as rods and as polymorphous round cells although the growth rate is higher at the acid pH.

The Mir M7 strain also shows disturbances in cell division, which probably depend upon a damaged envelope. Evidence which supports this possibility includes the effects of sucrose, Mg\(^{2+}\) ions, Na\(^+\) ions and spermine, which prevent the inhibition of the division of round and rod-shaped cells (or Mir M7 strain) at 41 °C and eliminate the 2 h lag and the drop in viable count which characterizes the growth curve of round cells at 37 and 41 °C (Satta & Fontana, 1974). It is already known that sucrose and Mg\(^{2+}\) ions can support the cytoplasmic membrane of fragile cells, spheroplasts and protoplasts (MacQuillen, 1960), spermine has been recognized to have specific protective action on membranes (Cohen & Lichtenstein, 1960; Mager, 1959a, b; Puck, 1960), and Hirota, Ryter & Jacob (1968) have demonstrated an envelope damage in a temperature-sensitive cell division mutant in which the blockage of cell division can be relieved by the addition of NaCl (Shapiro et al. 1970; Siccardi et al. 1971). Nagel de Zwaig & Luria (1967), Holland & Holland (1970), Samson & Holland (1970), and Rolfe & Onodera (1971) described enhanced sensitivity to deoxycholate in colicin-tolerant mutants of *E. coli*, the last two named finding an alteration in the protein composition of the membrane, while Hirota *et al.* (1969, 1970) described a cell division mutant which again showed greater deoxycholate sensitivity and a change in membrane protein composition. We similarly find that round Mir M7 cells are prone to lysis in the presence of deoxycholate at a concentration which has no effect on the Mir A12 strain.

It is known that the membrane is involved in cell wall synthesis (Rogers, 1970) and the observations described above for the effects of Mg\(^{2+}\), Na\(^+\), spermine and deoxycholate suggest that the damage of the division is the result of an envelope defect. Damaged envelope would then be responsible for the pH-dependent alteration of the morphology and for the temperature-dependent inhibition of division. The reversion experiments suggest that in Mir M7 strain a single mutation is responsible for the damage to the envelope, alteration of the morphology and disturbance of cell division.

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