

Cell Division, Macromolecular Synthesis and Morphology Dependent on the State of the Envelope in a Mutant of *Klebsiella pneumoniae*

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SUMMARY

Mutant Mir M7 of *Klebsiella pneumoniae* exhibits a pH-dependent morphological interconversion; growing cells are rod-shaped at pH 5.5 but round at pH 7. Round cells cannot divide or make DNA, RNA and proteins at pH 5.5. At this pH, cell division and DNA synthesis are resumed either when substances such as Mg²⁺, Na⁺, sucrose or spermine are added to the growth medium or immediately after the appearance of a normal rod morphology. Round cells, but not rods or the wild type, are lysed by low concentrations of detergents and are killed by DL-amino acids. Their growth rate is not improved by vigorous aeration and they make spheroplasts at an incubation temperature of 8 °C. This behaviour is the opposite of that of rods and the original strain. The transition from rods to round cells at pH 7 does not occur when cell division is inhibited by penicillin. Round cells must divide several times in an acid medium to give regular and uniform rods.

The possibility of a different state of the membrane in round and rod-shaped cells and a dependence of cell division, DNA synthesis, and morphology on the state of the membrane are discussed.

INTRODUCTION

The Mir M7 strain of *Klebsiella pneumoniae* shows pH-dependent damage to the mechanism governing morphogenesis and temperature-dependent damage of the division mechanism (Satta, Schito & Meloni, 1968; Satta & Fontana, 1973). These characteristics make the Mir M7 strain suitable for studying the nature of any interrelation between the membrane and replication of the genome, the conservation of morphology, and regular cell division.

This paper studies division capacity, macromolecular syntheses, and morphology in relation to the function of the membrane of round and rod-shaped cells. The role played by cell division in the morphological transition is also described. The results obtained suggest that the two shapes of mutant Mir M7 are associated with two different states of the cytoplasmic membrane. Cell division is indispensable both for transition to the round shape and for the formation of regular-shaped rods.

METHODS

Bacterial strains and preparation of round and rod-shaped cells. The organisms and techniques employed in the preparation of round and rod cells were as previously described (Satta & Fontana, 1973).

Media, buffers and pH. PL medium prepared as described (Satta & Fontana, 1974) was

employed at pH 5.5 and 7. In some cases 0.4 M-sucrose, 0.05 M-MgCl₂ or 0.1 M-NaCl were added to the PL pH 5.5 medium.

Solid media PL agar 1% (PLA) pH 5.5 and pH 7 were also employed. PLA pH 7 was buffered with 0.025 M-phosphate buffer.

Analysis of the morphological transition. Round and rod-shaped cells were transferred respectively into PL pH 5.5 or PL pH 7 at 37 °C. At regular time intervals samples were taken for counting colony forming units (c.f.u.), for observing morphology and for measurement of the dry weight. For the latter, 200 ml of the culture were centrifuged, the cells washed twice in distilled water and heated at 90 °C until the weight remained constant (Koch, 1970).

In order to evaluate the morphological change of single bacteria, round or rod-shaped cells were diluted to a concentration of approximately three cells/ml. Samples (0.1 ml) of these dilutions were then transferred into 1200 tubes each containing 1 ml of PL pH 5, PL Mg²⁺ pH 5 (round cells) or PL pH 7 (rod-shaped cells). After incubation at 37 °C for 48 h, the number of tubes which had become turbid was counted and the morphology of the cells checked. The cells from 40 tubes of PL pH 5.5 and 40 of PL pH 7 were then transferred to PL pH 7 and PL pH 5.5 respectively, so as to evaluate the morphological reversibility of the cells in such clones. In another experiment, round or rod-shaped cells were spread on PLA pH 5.5 and PLA pH 7 plates respectively, to give 20 to 50 cells per microscopic field, and the fate of single cells was studied using a phase contrast microscope and photographs taken at intervals.

Evaluation of the synthesis of macromolecules during the shape transition. Round and rod-shaped cells from exponentially growing cultures were transferred to PL pH 5.5 and PL pH 7 media, respectively, containing (per ml): 1 μC of [³H]thymidine and 20 μg of unlabelled uridine, or 1 μC of [³H]uridine and 20 μg of unlabelled thymidine, or 1 μC of a mixture of five different ³H-labelled amino acids. At regular intervals samples of 0.2 ml were taken to measure the radioactivity incorporated as described by Inouye (1969).

RESULTS

Dynamics of the shape transition

To study the mechanism by which the transition between the two shapes takes place, the evolution of morphology compared with mass and c.f.u. were analysed (Fig. 1 and Table 1). In cultures of round cells at pH 5.5 some of the cells tend to lengthen progressively with time, so that after 110 to 130 min the first definite rods can be seen and after about 180 min of incubation they represent the prevailing cell type, although 15 to 30% of round elements still persist. During all this time the rod-shaped cells are rather short, fat, and polymorphous or monstrous. As incubation proceeds the rods become more and more uniform and polymorphous and atypical cells decrease in number, until after about 10 to 14 h incubation the culture is composed almost entirely of regular rods which cannot easily be distinguished from the original strain. During this change, turbidity and dry weight remain constant for 2 h and then dry weight increases faster than turbidity. The viable count instead decreases by 30 to 50% during the first 40 to 50 min, then remains constant for 50 to 60 min and, after 120 min, resumes increasing at a slightly slower rate than that of the dry weight. It seems that the time at which cells begin to divide, coincides with that at which rod-shaped cells begin to appear.

In cultures of the rod-shaped cells in PL pH 7, shorter cells appear, most of which become round after 150 min. After 3 h all cells are round, and large polymorphous cells appear

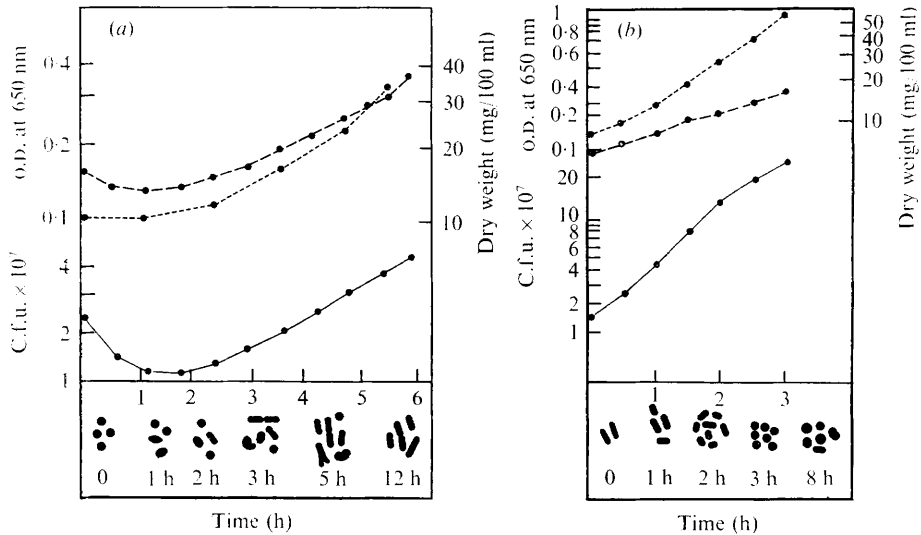


Fig. 1. Growth characteristics of round cells in PL pH 5.5 (a) and rods in PL pH 7 (b) of the mutant Mir M7. Round and rod-shaped cells prepared as indicated in the text were transferred respectively to PL pH 5.5 and PL pH 7 and incubated at 37 °C without aeration. At different time intervals their morphology was observed and their O.D. (●—●), viable count (●—●) and dry weight (●---●) were evaluated.

later. The viable count increases quickly, doubling after about 50 min and 10 to 12-fold after 150 min. The dry weight does not increase significantly immediately and after 150 min increases only four to sixfold.

The experiments described suggest that round cells become rods by extending in only one direction without dividing, until one diameter has become several times greater than the other, and that the rod-shaped cells become round by repeatedly dividing before doubling in mass, thus leading to progressively shorter cells until they appear round. A confirmation of this suggestion has been obtained by analysing the morphological evolution of single round and rod-shaped cells respectively in acid or neutral liquid and solid media. Table 2 shows that 43% of 420 round cells transferred to tubes of PL pH 5.5 die, and the rest produce clones of rod-shaped cells. All rod-shaped cells transferred under similar conditions to neutral medium survive to form clones of round cells. Single round cells spread on PLA pH 5.5 grow along one of the diameters, lengthening gradually and acquiring more regular borders and a rod shape, after which they divide producing microcolonies of rods in 10 h. The rods spread on PLA pH 7 divide early, before significant growth in length. The cells of each fresh generation divide while they are still smaller than the mother cell at birth, thus producing microcolonies of round cells in 8 h.

Macromolecular synthesis during the two morphological transitions

To see if any other changes in cell physiology are associated with the shape changes, macromolecular syntheses of round cells in PL pH 5.5 and rods in PL pH 7 were evaluated. Fig. 2 and 3 show the results obtained. No peculiarities were seen in the macromolecular synthesis of rods changing into round shapes.

During the formation of rods, the synthesis of DNA, RNA and protein is markedly reduced for about 110 min, starts again very slowly between 110 and 120 min, and then

Table 1. *Morphological evolution of rods incubated in PL pH 7 and round cells incubated in PL pH 5.5*

Original morphology	Media	Morphology at different incubation times							
		60 min	90 min	120 min	150 min	180 min	6 h	9 h	14 h
Rods	PL pH 7	Rods and short rods	Short rods and very short rods	Very short rods and round cells	Round cells and a few very short rods	Round cells	Round cells	Round polymorphous cells	Round polymorphous cells
Round	PL pH 5.5	Round cells	Round cells and a few fat, very short rods	Round cells and fat, very short rods	Very short, fat, polymorphous rods and round cells	Round cells	Uniform rods, fat polymorphous rods	Uniform rods, a few polymorphous rods	Uniform rods

Table 2. *Survival and clone morphology of single round cells in PL pH 5.5 and rods in PL pH 7*

Morphology of the cells inoculated	Media	No. of cells inoculated	No. of cells which gave a clone	Clone morphology	Morphology reversibility of the clones (%)
Round	PL pH 5.5	About 420	240	All 240 rods	100
	PL Mg ²⁺ pH 5.5	About 390	380	All 380 rods	100
Rod	PL pH 7	About 410	395	All 395 round cells	100

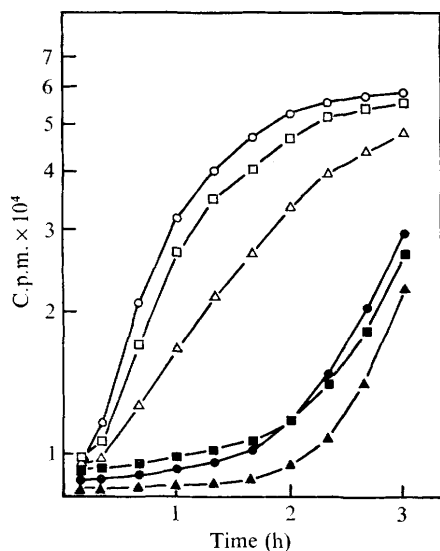


Fig. 2

Fig. 2. Synthesis of DNA (▲, △), RNA (●, ○) and protein (■, □) in round cells of the Mir M7 mutant in PL pH 5.5 (solid symbols) and PL pH 7 (open symbols). For details see Methods.

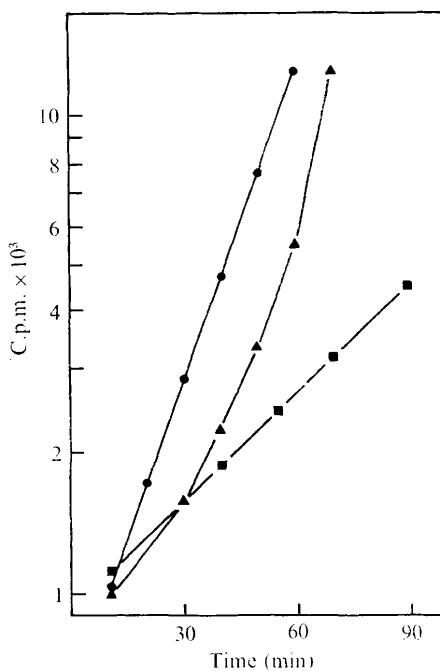


Fig. 3

Fig. 3. Synthesis of DNA (▲), RNA (●) and protein (■) in the Mir A12 strain in PL pH 5.5 medium. For details see Methods.

grows at a high rate (see also Fig. 1). After the same shift from PL pH 7 to PL pH 5.5, the Mir A12 strain (a revertant with the physiological and morphological characteristics of the wild-type *Klebsiella pneumoniae*) shows a macromolecular synthesis which is normal and proportional to the growth rate from the beginning of incubation (Fig. 3). DNA synthesis seems to be inhibited for longer, and more completely than protein or RNA synthesis. This was particularly evident in pulse-labelling experiments (Fig. 4). It is to be stressed that macromolecular synthesis (especially that of the DNA) resumes at the same time as that at which rods appear in the culture and begin to divide.

It has been shown that high osmotic pressure and substances such as divalent cations which have a protecting action on the membrane (Mayer, 1959; MacQuillen, 1960; Ray & Brock, 1971) facilitate the growth of round cells in PL pH 5.5 media (Satta & Fontana, 1973). Therefore the pattern of DNA synthesis, viable count and O.D. of round cells in PL pH 5.5 medium containing 0.4 M-sucrose or 0.05 M-MgCl₂ was studied. Fig. 5 shows that in the presence of these substances cells divide regularly and show a normal increase in turbidity, as well as regular synthesis of DNA which continues without lag after the transfer to PL pH 5.5 while morphologically the culture still consists of round cells.

With regard to the relationship between growth and morphology, the cells in 0.4 M-sucrose remain very short throughout the experiment, whereas those in MgCl₂ take on a round shape after a 3 h incubation period.

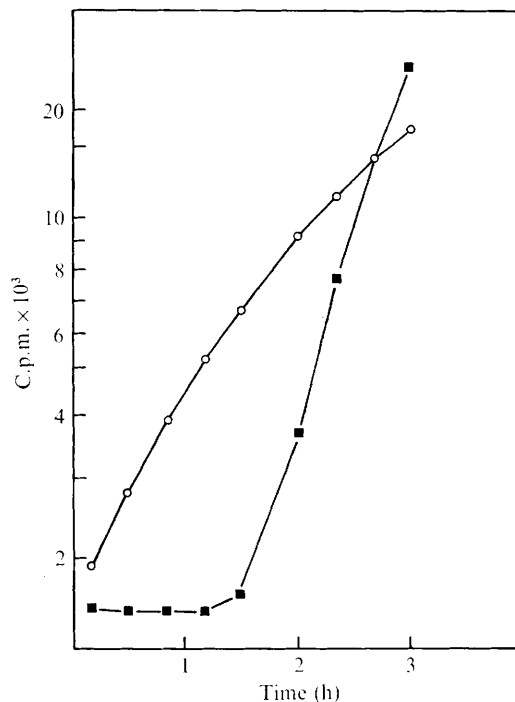


Fig. 4. Pulse labelling of the DNA of Mir M7. Mutant round cells growing in PL pH 5.5 were transferred to PL pH 5.5 (■) and PL pH 7 (○) and incubated at 37 °C without shaking. At regular time intervals 1 ml samples were taken and transferred to tubes containing 5 μ C of [³H]thymidine and 100 μ g of unlabelled uridine. After a 3 min incubation period at 37 °C each sample was mixed with 4 ml of 6 % cold TCA, kept 30 min at 4 °C and filtered on Whatman GF filters and the filters counted.

Morphological transition and protein synthesis

Protein synthesis was inhibited by transferring round and rod-shaped cells to buffer, to medium without leucine, or to chloramphenicol (CAP)-containing medium, at pH 5.5 and pH 7 respectively. The cell morphology was observed after 6 h. The results obtained suggest that new protein synthesis is required for the transition. When protein synthesis is inhibited, change of shape is also inhibited and this happens if protein synthesis is stopped at any time during these transitions.

A comparison between the states of the membranes in round and rod-shaped cells

Detergents. A comparison of the action of Brij, SDS and sarkosyl on the growth of round and rod-shaped cells shows that the cocci are more sensitive. They either fail to grow or lyse at concentrations of the detergents which have no effect on the rods (Table 3).

Aeration. Respiratory enzymes are located in the membrane (Salton, 1967; Rogers & Perkins, 1968). Therefore the effect of good aeration on the growth of round and rod-shaped cells has been studied. Fig. 6 shows that with aeration round cells in PL pH 7 grow only slightly faster, whereas rod-shaped cells in PL pH 5.5 grow very much faster, than in the unaerated culture. Aeration, therefore, stimulates growth of rod-shaped cells in acid media, but only slightly affects the round cells growing in neutral media. Aeration only increases the growth rate of round cells at PL pH 5.5 after 2 h, which corresponds to the time at

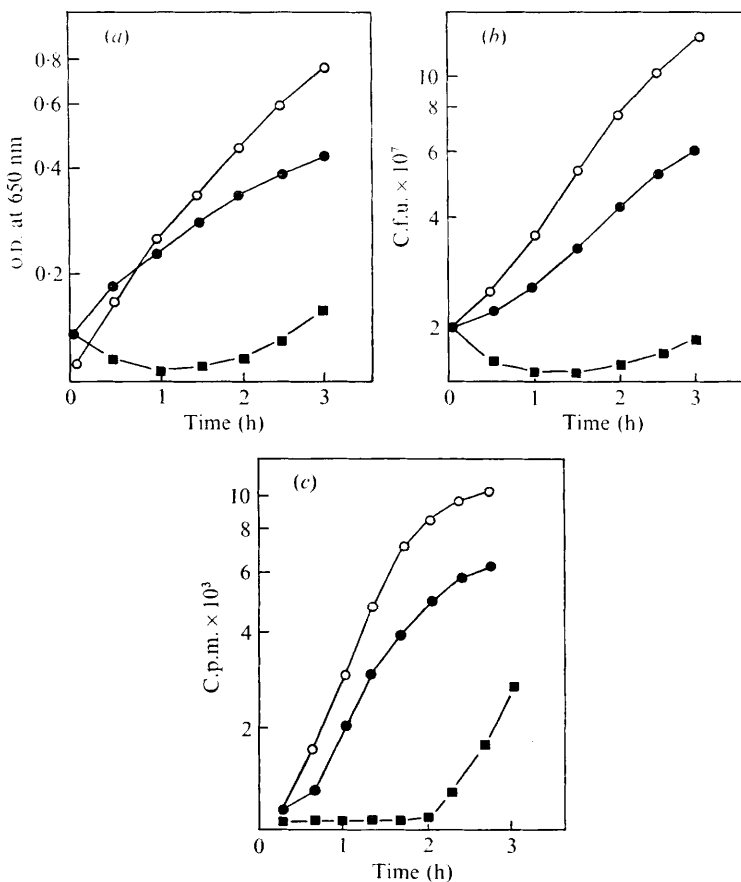


Fig. 5. Growth of round cells in PL pH 5.5 (■—■), PL Mg²⁺ pH 5.5 (●—●) and PL sucrose pH 5.5 (○—○). Round cells in log phase were transferred to PL pH 5.5 + 0.05 M-MgCl₂ and PL pH 5.5 + 0.4 M-sucrose; 1 μC of [³H]thymidine and 20 μg of unlabelled uridine were added to all cultures, which were then incubated at 37 °C without aeration. At various time intervals the O.D. (a), the c.f.u. (b) and the TCA-precipitable radioactivity (c) were evaluated as described in Methods.

which the cell population becomes rod-shaped; aeration only for the first 150 min does not affect the growth rate.

DL-Amino acids. D-Amino acids can kill cells permeable to them, so that an indication of the functioning of the membrane can be obtained by analysing the effect of the DL-amino acids on the two morphological forms.

Table 4 shows that the DL-amino acids have a lethal effect on the round cells, whereas on the rod-shaped cells or on the original strain A12, their effect is indistinguishable from that of the corresponding L-isomers.

The formation of spheroplast-like cells at a low temperature 8 °C incubation. It has been found that the round cells become spheroplast-like cells at low temperature (8 °C). The formation of cell wall, important in shape determination, involves the membrane (Rogers, 1970); therefore the different effects of temperature on the morphology suggest a difference in the membranes. The round cells grown in PL pH 7 at 8 °C produce spheroplast-like cells after a 70 to 100 h incubation period (Fig. 7), whereas rod cells grown in PL pH 5.5 at

Table 3. *Effect of detergents on the growth of round cells in PL pH 7 and rods in PL pH 5.5*

Cells	Time (h)	Optical density		
		1 % SDS	1 % Sarkosyl	1 % Brij 35
Rods in PL pH 5.5	0	0.090	0.100	0.095
	4	0.380	0.520	0.420
Round cells in PL pH 7	0	0.150	0.190	0.160
	4	0.060	0.120	0.160

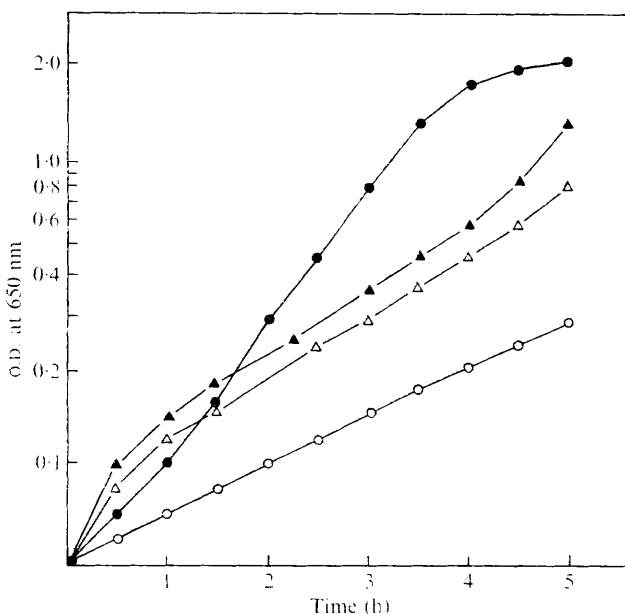


Fig. 6. Effect of aeration on the growth of round and rod-shaped cells of Mir M7. Round and rod-shaped cells in log phase were transferred respectively to PL pH 7 medium and PL pH 5.5 medium. Each of the two samples was divided into two bottles which were then incubated, one with aeration (round cells [▲]; rods [●]) and the other one without aeration (round cells [△]; rods [○]), at 37 °C. At various time intervals the O.D.s of all samples were evaluated.

Table 4. *Effect of L- and DL-amino acids on the growth of round and rod-shaped Mir M7 and of Mir A12 (wild type)*

Optical density* in PL pH 5.5 plus L- and DL-amino acids† after 16 h incubation

Cells	Serine		Threonine		Alanine		Valine		Phenylalanine		Methionine	
	L	DL	L	DL	L	DL	L	DL	L	DL	L	DL
	Mir M7 (round)	0.180	0.035	0.220	0.065	0.160	0.090	0.180	0.060	0.410	0.065	0.180
Mir M7 (rods)	0.400	0.410	0.240	0.210	0.240	0.220	0.270	0.200	0.260	0.210	0.220	0.200
Mir A12‡	0.600	0.540	0.510	0.490	0.480	0.440	0.610	0.600	0.580	0.570	0.540	0.500

* The O.D. at the beginning of incubation was the same for every sample.

† The amino acid concentration was 1.5 mg/ml for L- and 3 mg/ml for DL-isomers.

‡ Mir A12 was grown in PL pH 7 before transferring to PL pH 5.5 + amino acids.

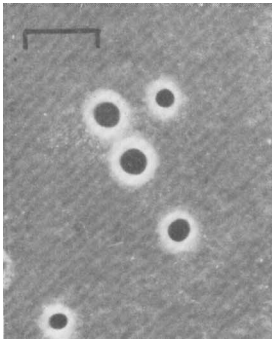


Fig. 7

Fig. 7. Spheroplast-like cells obtained by incubating round cells in PL pH 7 at 8 °C. Scale marker represents 10 μm .



Fig. 8

Fig. 8. Filaments obtained by incubating rods in PL pH 7 + penicillin (8 $\mu\text{g/ml}$). Scale marker represents 12 μm .

the same temperature do not undergo any variation in shape. Rods grown in PL pH 7 produce round cells in 30 to 40 h which in the subsequent 50 to 70 h become spheroplast-like cells. Therefore the effect of low temperature on the change of round shapes to spheroplast-like cells contrasts with the absence of effect on the rod cells in PL pH 5.5 or on their normal change to round cells in PL pH 7 medium.

Morphological transition and cell division

The experiments described above suggest some relation between cell division and change of morphology (round cells become rods by extending in only one direction without dividing, and the rod-shaped cells become round by repeatedly dividing).

In order to verify this, we studied the effect on the rods and round cells of substances which block cell division, such as penicillin (Starka & Moravova, 1967) and nalidixic acid (Goss, Deitz & Cook, 1964). When penicillin is added to a culture of rods growing in neutral medium the rods keep on lengthening, forming very long aseptate filaments in 150 min, during which time round cells would be formed in the absence of the antibiotic (Fig. 8 and 9). Treatment of the round cells under the same conditions causes the formation of giant and polymorphic cells instead of filaments.

Fig. 9 shows the effect of inhibiting the cell division of round cells and of newly formed rods which have divided many times in this medium. In the presence of 0.2 M-NaCl, if cell division is inhibited immediately, fat polymorphous filaments are produced. Uniform filaments with a normal transverse diameter are obtained if cell division is inhibited after a 3 h incubation period. The round cells transferred to PL pH 5.5 formed very fat, very polymorphous filaments in the presence of nalidixic acid added either at the start or after three hours' incubation, whereas they produced filaments which were uniform and with a normal transverse diameter when division was inhibited only after 10 h, when each newly formed rod had divided several times. The same uniform and regular filaments can be obtained by inhibiting DNA synthesis in round cells after a 3 h incubation period in the presence of NaCl, which meanwhile has allowed several divisions per cell.

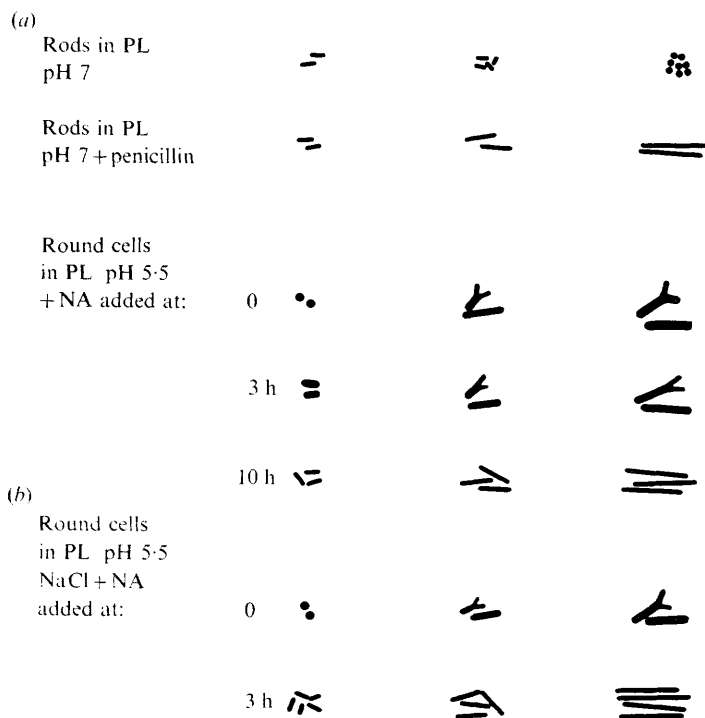


Fig. 9. Relationship between morphology and cell division. (a) With penicillin. Rods in log phase were transferred into PL pH 7, and into PL pH 7 + 8 μ g of penicillin. (b) With nalidixic acid, NA. In one experiment round cells were transferred to three samples of PL pH 5.5 medium to which were added 3 μ g of NA immediately after transfer, and after 3 h and 10 h of incubation at 37 °C. In the second experiment the round cells were transferred to PL pH 5.5 and into PL pH 5.5 containing NaCl (0.2 M) and incubated at 37 °C. Immediately after the transfer and after 3 h, 3 μ g of NA were added to the two samples. The morphology of samples was observed at various time intervals till 3 h after the addition of penicillin and till 6 h after the addition of NA.

DISCUSSION

Round cells of the mutant Mir M7 cannot grow in PL medium pH 5.5. In such an acid medium they slowly grow longer, gradually taking a rod shape; it is only when rods appear that DNA synthesis and cell division resume. The unique behaviour of this strain could be related to a peculiar effect of the acid pH on the damaged envelope. The damage in the envelope may make round cells sensitive to pH 5.5 unless the membrane is supported by substances such as Mg^{2+} and sucrose or by a rigid cell wall. Therefore in PL pH 5.5, 30 to 40 % of the round cells die while the remainder cannot grow, but they are still able to repair the damage to the cell wall rigid layer, becoming rod-shaped and acquiring the membrane support they need to grow in an acid medium. Several findings support this interpretation. It is known that acid pH can cause changes in the properties of bacterial membranes (Den Kamp, Van Iterson & Van Deenen, 1967). Round cells can apparently divide and grow normally in PL pH 5.5 in the presence of substances such as Mg^{2+} and sucrose which are known to protect the membrane (Mayer, 1959; MacQuillen, 1960; Ray & Brock, 1971). Detergents, DL-amino acids, aeration and low temperature have different effects on round and rod cells, possibly indicating that membrane related functions, which are altered in the round cells, become normal after the transition to the rod form.

Filaments, not round cells, are formed when cell division of rods in PL pH 7 is inhibited, and normal rods are obtained when round cells divide many times in PL pH 5.5, suggesting that cell division may be essential to express the envelope lesion at a neutral pH or to permit complete recovery of the envelope damage at acid pH. One possible explanation is that the neutral pH particularly affects the newly formed septa which, when cells become very short or round, account for a large part of the cell surface.

The pleiotropy of the mutation could be explained in terms of the unit cell model of Donachie & Begg (1970), according to which the growth of the bacteria would occur in a discrete area of the membrane, where the growth of the new membrane and wall, the replication of the genome and the formation of the septum take place.

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