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

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(Received 19 February 1973)

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, Siccaldi, Hirota & Jacob, 1970; Siccaldi, 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; K_2HPO_4 , 0.3; KH_2PO_4 , 0.1; Na_2SO_4 , 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 loopfull 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 2×10^8 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 4×10^7 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

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 μ m.

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*

Strain	Time (h)	Morphology at different pH values						
		5	5.5	6	6.5	7	7.5	8
Mir M7	3	Round cells	Short polymor- phous rods and round cells	Very short poly- morphous rods; atypical divisions	Fat polymorphous cells; atypical divisions	Polymorphous cells; atypical divisions	Uniform round cells	Big, uniform round cells
	6	Very short rods	Polymorphous rods	Short polymor- phous rods; atypical divisions	Very short poly- morphous rods; atypical divisions	Round polymor- phous cells; atypical divisions	Big, uniform round cells	Spheroplast-like cells and big uni- form round cells
	9	Rods	Very long rods	Long rods	Fat rods; atypical divisions	Round polymor- phous cells; atypical divisions	Big round cells; a few sphero- plast-like cells	Spheroplast-like cells
Mir A12	9	Rods	Rods	Rods	Rods	Rods	Short rods	Short rods

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.

Fig. 4. Filaments originating from round cells of Mir M7 incubated in PL pH 5.5 medium at 41 °C. Round cells prepared as described in Methods were transferred to PL pH 5.5 and incubated at 41 °C without shaking. The photos were taken after 8 h incubation. Scale markers represent 12 μ m.

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*

Original morphology	Incubation time (h)	Morphology at different incubation temperatures		
		30 °C	37 °C	41 °C
Rods at pH 5.5	8	Uniform rods	Uniform rods	Uniform filaments
	14	Uniform rods	Filaments and uniform long rods	Uniform long filaments
	48	Uniform rods	Uniform very long filaments	Long filaments
Round cells at pH 5.5	8	Polymorphous rods	Long polymorphous rods	Fat and polymorphous short filaments and single round cells
	14	Uniform rods	Polymorphous filaments and long rods	Fat polymorphous and diaphanous filaments
	48	Uniform rods	Very long filaments	
Round cells at pH 7	8	Round polymorphous cells	Round polymorphous cells	Big amorphous cells and round cells
	14	Round polymorphous cells	Round polymorphous cells	Giant amorphous cells and round cells

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 μ m.

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)

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, 1959*a, 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.

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, 1959*a, 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.

We are most grateful to Dr H. J. Rogers for very kindly reading the manuscript and for his useful criticism, advice and discussions. Very useful to us also were stimulating discussions with Dr H. Pooley and the suggestions he made. The kind help that Carla Olivari gave us in planning and doing experiments is also gratefully acknowledged.

REFERENCES

- BOYLAN, R. J. & MENDELSON, N. H. (1969). Initial characterization of a temperature-sensitive *rod*⁻ mutant of *Bacillus subtilis*. *Journal of Bacteriology* **100**, 1316-1321.
- COHEN, G. N. & MONOD, J. (1957). Bacterial permeases. *Bacteriological Reviews* **21**, 169-194.
- COHEN, S. S. & LICHTENSTEIN, J. (1960). Polyamines and ribosome structure. *Journal of Biological Chemistry* **235**, 776-782.
- COLE, R. M., POPKIN, T. J., BOYLAN, R. J. & MENDELSON, N. H. (1970). Ultrastructure of a temperature-sensitive *rod*⁻ mutant of *Bacillus subtilis*. *Journal of Bacteriology* **103**, 793-810.
- DONACHIE, W. D. & BEGG, K. J. (1970). Growth of the bacterial cell. *Nature, London* **227**, 1220-1224.
- GRULA, E. A. & KING, R. D. (1970). Inhibition of cell division in *Micrococcus lysodeikticus* dis. II. *Canadian Journal of Microbiology* **16**, 317-324.
- HIROTA, Y., MORDOH, J. & JACOB, F. (1970). On the process of cellular division in *Escherichia coli*. III. Thermosensitive mutants of *Escherichia coli* altered in the process of DNA initiation. *Journal of Molecular Biology* **53**, 369-387.

- HIROTA, Y., RYTER, A. & JACOB, F. (1968). Thermosensitive mutants of *Escherichia coli* affected in the processes of DNA synthesis and cellular division. *Cold Spring Harbor Symposia on Quantitative Biology* **33**, 677-693.
- HIROTA, Y., WAKIL, S., SHAPIRO, B., RYTER, A., HURWITZ, J. & JACOB, F. (1969). Sur un mutant thermosensible d'*Escherichia coli* présentant des anomalies de la membrane. *Comptes rendus de l'Académie des sciences D* **269**, 1346-1348.
- HOLLAND, E. M. & HOLLAND, I. H. (1970). Induction of DNA breakdown and inhibition of cell division by colicin E2. Nature of some early steps in the process and properties of the E2-specific nuclease system. *Journal of General Microbiology* **64**, 223-239.
- INOUE, M. & PARDEE, A. B. (1970). Changes of membrane proteins and their relation to deoxyribonucleic acid synthesis and cell division of *Escherichia coli*. *Journal of Biological Chemistry* **245**, 5813-5819.
- JACOB, F., BRENNER, S. & CUZIN, F. (1963). On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symposia on Quantitative Biology* **28**, 329-337.
- MACQUILLEN, K. (1960). Bacterial protoplasts. In *The Bacteria*, vol. 1, pp. 249-359. Edited by I. C. Gunsalus & R. Y. Stanier. New York: Academic Press.
- MAGER, J. (1959a). Spermine as a protective agent against osmotic lysis. *Nature, London* **183**, 1827-1828.
- MAGER, J. (1959b). The stabilizing effect of spermine and related polyamines on bacterial protoplasts. *Biochimica et biophysica acta* **36**, 529-531.
- MELONI, G. A. & MONTI-BRAGADIN, C. (1962). Osservazioni sull'isolamento dall'uomo di sferoplasti 'spontanei' derivati da un ceppo di *Klebsiella*. *Annali Scavo* **4**, 143-152.
- NAGEL DE ZWAG, R. & LURIA, S. E. (1967). Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. *Journal of Bacteriology* **94**, 1112-1123.
- PUCK, C. W. (1960). The stabilizing effect of spermine and related amines on mitochondria and protoplasts. *Biochemical and Biophysical Research Communications* **2**, 117-120.
- ROGERS, H. J. (1970). Bacterial growth and the cell envelope. *Bacteriological Reviews* **34**, 194-214.
- ROGERS, H. J., MACCONNELL, R. & BURDETT, J. D. (1970). Isolation and characterization of mutants of *Bacillus subtilis* and *Bacillus licheniformis* with disturbed morphology and division cell. *Journal of General Microbiology* **61**, 155-171.
- ROLFE, B. & ONODERA, K. (1971). Demonstration of missing membrane proteins in colicin-tolerant mutant of *E. coli* K12. *Biochemical and Biophysical Research Communications* **44**, 767-773.
- RYTER, A. (1967). Relationship between synthesis of the cytoplasmic membrane and nuclear segregation in *Bacillus subtilis*. *Acta microbiologica Academiae scientiarum hungaricae* **12**, 284-290.
- RYTER, A. (1971). Étude de la croissance de la membrane chez *Bacillus subtilis* au moyen de la distribution des flagelles. *Annales de l'Institut Pasteur de Lille* **121**, 271-288.
- SAMSON, A. C. R. & HOLLAND, I. B. (1970). Envelope protein changes in mutants of *Escherichia coli* refractory to colicin E2. *FEBS letters* **11**, 33-36.
- SATTA, G. & FONTANA, R. (1974). Cell division, macromolecular synthesis and morphology dependent on the state of the envelope in a mutant of *Klebsiella pneumoniae*. *Journal of General Microbiology* **80**, 65-75.
- SATTA, G., SCHITO, G. C. & MELONI, G. A. (1969). Transizione bastoncinosa in un ceppo di *Klebsiella pneumoniae*. Ultrastruttura delle forme coccoidi tipiche ed abnormi. *Atti del XV° Congresso Nazionale di Microbiologia Torino-Saint Vincent*, vol. 2, pp. 247-253.
- SCHACHTELE, C. F., ANDERSON, D. L. & ROGERS, P. (1968). Mechanism of canavanine death in *Escherichia coli*. II. Membrane-bound canavanine-protein and nuclear disruption. *Journal of Molecular Biology* **33**, 861-872.
- SCHWARZ, U., ASMUS, A. & FRANK, H. (1969). Autolytic enzymes and cell division of *Escherichia coli*. *Journal of Molecular Biology* **41**, 419-429.
- SHAPIRO, B. M., SICCARDI, A. G., HIROTA, Y. & JACOB, F. (1970). On the process of cellular division in *Escherichia coli*. II. Membrane protein alterations associated with mutations affecting the initiation of DNA synthesis. *Journal of Molecular Biology* **52**, 75-89.
- SICCARDI, A. G., SHAPIRO, B. M., HIROTA, Y. & JACOB, F. (1971). On the process of cellular division in *Escherichia coli*. IV. Altered composition and turnover of the membranes of thermosensitive mutants defective in chromosomal replication. *Journal of Molecular Biology* **56**, 475-490.
- WALKER, J. R. & PARDEE, A. B. (1968). Evidence for a relationship between deoxyribonucleic acid metabolism and septum formation in *Escherichia coli*. *Journal of Bacteriology* **95**, 123-131.