Changes in Permeability and Ultrastructure of Starved *Streptococcus lactis* in Relation to Survival

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SUMMARY

When *Streptococcus lactis* was starved in phosphate buffer the intracellular amino acid pool was rapidly released into the external medium from the onset of starvation whereas lactic dehydrogenase and DNA appeared in the suspending buffer only as organisms began to lose viability. Addition of spermine enhanced survival and suppressed the release of ultraviolet-absorbing material.

Thin sections of *Streptococcus lactis* were examined by electron microscopy at intervals during starvation in a number of environments. Ribosome particles rapidly disappeared from organisms starved in the absence of Mg^{2+} and non-viable organisms remained structurally intact; in the presence of Mg^{2+} ribosomes were retained but, after prolonged starvation, some organisms autolysed soon after the death rate increased. Addition of a suitable energy source maintained cell structures intact for a much longer period but again lysis occurred as the death rate increased. Starvation led to unfolding or extrusion of the mesosomes and dislocation of the nuclear material. The death rate of starved *S. lactis* organisms in phosphate buffer partly depended on the presence of Mg^{2+} , which probably acted by promoting polymer stability, particularly RNA. In this environment, a suitable exogenous energy source further enhanced survival which may ultimately be a function of cell wall and/or membrane stability.

INTRODUCTION

Vegetative bacteria starved in buffer at their optimum growth temperature may survive for several days but death ultimately occurs. The reasons for bacterial death in starvation environments may be impossible to define but some authors have claimed that certain possibilities can be excluded. For instance, starved *Aerobacter aerogenes* were considered to maintain functional membranes after death (Postgate & Hunter, 1962). An earlier investigation (Thomas & Batt, 1969*a*) showed that a substantial amount of phospholipid breakdown occurred on prolonged starvation of *Streptococcus lactis*. This process would be expected to impair permeability barriers since phospholipids are important membrane components (Salton, 1967). The present paper describes a study of the changes in permeability and ultrastructure of starved *S. lactis* in relation to survival.

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METHODS

General procedures. The organism (*Streptococcus lactis* ML₃), the conditions of growth and starvation and the methods used for determining bacterial mass and viability were described in preceding papers (Thomas & Batt, 1968, 1969a).

Thin-layer chromatography. Amino acids were separated by thin-layer chromatography (TLC) on mixed cellulose-silica gel layers and detected with ninhydrin (Turner & Redgwell, 1966). Plates were developed with phenol-water (80:20, w/v) in the first direction (5 hr) and dried overnight at 40 to 50°. Butanol:acetic acid:water (5:1:4, v/v/v, upper phase) was used in the second direction (4 hr) and the spots were identified by comparison with standard amino acids. Supernatant buffer samples were desalted (Dreze, Moore & Bigwood, 1954) before analysis.

Enzyme assays. Lactic dehydrogenase was assayed using the method described by Kornberg (1955). Supernatant samples (2.8 ml.) were adjusted to pH 7.4, 10 mM-Na pyruvate (0.1 ml.) and 4 mM-nicotinamide-adenine-dinucleotide (NADH, 0.1 ml.) were added and the rate of oxidation of NADH at 30° was determined using a Zeiss PMQII recording spectrophotometer at 340 m μ . The unit of activity was taken as the amount of enzyme which caused an initial rate of oxidation of 1 μ mole NADH per minute. β -Galactosidase activity of supernatant samples was assayed by the *o*-nitrophenyl- β -D-galactopyranoside hydrolysis method as described by Citti, Sandine & Elliker (1965).

Deoxyribonucleic acid. Cell-free DNA was estimated by the method of Burton (1956).

Electron microscopy. The organisms were examined in the electron microscope as thin sections, prepared by the following methods.

Fixation. Method 1. The organisms were fixed with osmium tetroxide and post-fixed in uranyl acetate according to the method of Kellenberger, Ryter & Sechaud (1958). This method is subsequently referred to as R-K fixation.

Method 2. The organisms underwent a primary fixation in a glutaraldehyde preparation followed by a secondary fixation in osmium tetroxide according to the following schedules: (a) 2 ml. fixative (1 % glutaraldehyde in 0.1 M-phosphate buffer, pH 6.1, containing 6 mM-MgCl₂) was added to 20 ml. of culture. After mixing, the culture was centrifuged (1000 g for 5 min.) and the organisms resuspended in fixative (2 ml.) and left for 3 hr at 4°. After further centrifugation, the organisms were washed twice in 0.1 M-phosphate buffer (pH 6.1) containing 0.2 M-sucrose. The pellet was then resuspended in 0.1 M-phosphate buffer (pH 6.1) containing 1 % osmium tetroxide and left for 2.5 hr at 4°. After two further washes in phosphate buffer the organisms were set in 2 % agar (aq.) at 45°; the agar blocks were cut into small pieces and immersed in uranyl acetate (0.5 %, aq.) for 2 hr at room temperature (Kellenberger *et al.* 1958). (b) 2 ml. fixative (1 % glutaraldehyde in 0.05 M-cacodylate buffer, pH 6.3, containing 0.01 M-MgCl₂) was added to 20 ml. of culture. After mixing, the culture was centrifuged (1000 g for 5 min.) and the organisms were resuspended in fixative (2 ml.) and left for 3 hr at 4°. After further centrifugation, the organisms were washed twice in 0.05 M-cacodylate buffer, pH 6.3, containing 0.5 % NaCl. The pellet was resuspended in 0.05 M-cacodylate buffer (pH 6.3) containing 1 % osmium tetroxide and 0.5 % NaCl, and left for 2.5 hr at 4°. The suspensions were diluted with cacodylate buffer and centrifuged. The organisms were then set in agar and small pieces of the agar blocks were immersed in uranyl acetate according to the procedure of Kellenberger *et al.*

(1958). (c) Using the fixative (1 % glutaraldehyde in 0.05 M-cacodylate buffer (pH 6.3) containing 0.5 % NaCl) the same procedure as (b) was followed.

These double fixation methods are subsequently referred to as G-Os (a) (b) and (c).

Dehydration and embedding. The agar blocks obtained from each fixation method were dehydrated in graded aqueous ethanol solutions according to the following schedule: 25 %—15 min., 50 %—15 min., 75 %—overnight at 4°, 95 %—30 min., 100 %—30 min. (twice). The blocks were then washed with propylene oxide and embedded in Araldite resin using the method of Luft (1961).

Sections were cut with either a glass or diamond knife, using an L.K.B. ultramicrotome and picked up on copper grids having either a collodion-carbon supporting film or no supporting film. Sections were stained by floating the grids on lead salt solutions (Millonig, 1961; Reynolds, 1963) for 1 min. followed by washing with distilled water and they were subsequently examined in a Philips EM200 electron microscope.

RESULTS

Measurement of bacterial lysis. Chromatographic analysis indicated a progressive loss of intracellular amino acids with a corresponding increase in the concentrations of most of the supernatant amino acids, suggesting that leakage of the intracellular pool occurred on starvation.

The release of an intracellular enzyme, such as β -galactosidase, into the external medium has been used as an index of cell lysis (Pollock, 1961). In the present study however, no detectable β -galactosidase activity was found in cell-free samples of *Streptococcus lactis* suspensions starved at a bacterial density of 4.8 mg. dry wt/ml.

Table 1. *Release of lactic dehydrogenase (LDH) and deoxyribonucleic acid (DNA) from starved Streptococcus lactis*

Washed organisms were resuspended at 4.6 mg. dry wt/ml. in phosphate buffer containing 10 μ M-EDTA + 1 mM-MgSO₄. Samples were removed at the times indicated, centrifuged and the supernatant buffers assayed as described in Methods.

Starvation period (hr)	Supernatant		Viability (%)
	LDH (Units/ml.)	DNA (μ g./ml.)	
1	0.000	0.0	—
3	0.002	0.0	99
5	0.006	0.2	—
8	0.011	0.2	99
10	0.069	0.7	—
12	0.138	1.3	94
21	0.319	4.0	32
24	0.505	6.8	17
29	0.547	11.2*	3

* Equivalent to 6.6 % total cell DNA.

Citti *et al.* (1965) reported that the β -galactosidase released from five out of six strains of *S. lactis* by shock treatment was very unstable, so that if this enzyme had been released in the present study its activity may have been rapidly lost. Assays for lactic dehydrogenase (LDH) indicated that this enzyme was released slowly from viable organisms (Table 1) but release of LDH appeared to increase markedly at the onset

of cell death. Control experiments showed that the LDH in cell-free samples was unstable under the incubation conditions used. Assays of 24 hr samples (Table 1), after 5 hr and 22 hr incubation, indicated a loss of 11 % and 53 % of the original activity. Therefore, the results for LDH release in Table 1 are only approximate.

Release of diphenylamine reactive material (expressed as DNA—Table 1) from starved organisms increased markedly as the death rate accelerated.

Fig. 1. Effect of spermine on starved *Streptococcus lactis*. Organisms were washed and re-suspended at 20 μ g. dry wt./ml. in 0.075 M-phosphate buffer (pH 7.0, 10 μ M-EDTA). Viabilities of suspensions containing: no addition, $\circ$; spermine (1 mM), $\bullet$; MgSO_4 (1 mM), $\square$; MgSO_4 (1 mM)+spermine (1 mM), $\blacksquare$. Release of u.v.-absorbing material is indicated by dashed lines; symbols as above.

Effect of spermine. Certain aliphatic diamines, especially spermine, have a marked stabilizing effect on osmotically sensitive bacteria and protoplasts (Mager, 1959; Tabor, 1962), suppressing the leakage of ultraviolet (u.v.)-absorbing material and cell lysis. Incubation of starved *Streptococcus lactis* organisms with spermine reduced the death rate and the release of u.v.-absorbing material (Fig. 1). Spermine did not however greatly enhance the normal stabilizing effect of Mg^{2+} .

General ultrastructural features of Streptococcus lactis. The morphology of organisms from the growth phase (Plate 1) reflects various stages in the division cycle. This sequence illustrates separation of the nuclear material followed by septum formation.

Prominent mesosomes (Fitz-James, 1960) are associated with the ingrowing cell wall and membrane and also with the nuclear material. The cytoplasmic membrane is 70 to 80 Å thick and consists of two prominent electron-dense layers separated by a light layer (Plate 1). The cell wall occurs as a diffuse band approximately 200 to 250 Å thick. Cole (1965) investigated cell wall growth in *Streptococcus pyogenes* using an immunofluorescence technique and considered that cell wall replication was initiated along an equatorial ring. Transverse septa grew centripetally with the peripheral wall replicating simultaneously so that the new hemispheres of the daughter cocci were initiated back-to-back. New equatorial sites of wall and transverse septa formation were initiated before completion of the previous transverse septum. In contrast, Chung, Hawirko & Isaac (1964) found that *Streptococcus faecalis* had only one site of cell wall synthesis per coccus. This latter mode of cell wall replication appears to operate in *Streptococcus lactis* since only one site of transverse septum formation per coccus was seen.

The fixation method of Kellenberger *et al.* (1958) (Method 1) preserved the nuclear material with a typical fibrillar structure (Plate 1). Glutaraldehyde-osmium tetroxide fixation (Method 2) procedures clearly demonstrated electron-dense, granular particles 100 to 200 Å in diameter (Pl. 2, fig. 4). Most investigators consider that these particles are ribosomes (see Kellenberger & Ryter, 1964).

Ultrastructural changes in starved cells. Although the appearance of ribosomes in thin sections from any system showed some variation, examination of a large number of sections prepared by different techniques suggested a rapid depletion of ribosomes when organisms were starved in buffer without Mg^{2+} (Pl. 2, fig. 5). In only a few of these organisms were ribosomes seen. Magnesium starvation of *Escherichia coli* (Morgan, Rosenkranz, Chan & Rose, 1966) and *Aerobacter aerogenes* (Kennell & Kotoulas, 1967) resulted in similar ribosome depletion. Starvation of *Streptococcus lactis* in the presence of Mg^{2+} and amino acids substantially reduced the depletion (Pl. 2, fig. 6).

The death of *Streptococcus lactis* in buffer without Mg^{2+} occurred while peripheral cell structures appeared to remain intact (Pl. 2, fig. 5). However, R-K fixation procedures showed disappearance of mesosomes and a pronounced dislocation of nuclear material. After 17 hr starvation intrusions of the membrane and cell wall were occasionally observed and the membrane appeared to be in direct contact with the nuclear material (Pl. 3, fig. 7). The appearance of these organisms is similar to that of organisms which have extruded their mesosomes (Ryter & Landman, 1964; Ryter, 1968). In this connexion it has been reported that mesosome extrusion may occur without loss of viability (Ryter, 1968).

After 30 hr starvation in buffer containing Mg^{2+} , viability had fallen to 42 % and examination of micrographs indicated that at least 12 % of the population had lysed. Intact organisms (G-Os fixation [a]) retained a dense ribosome pattern and organisms fixed by the R-K method showed fragmentary mesosomes. By 40 hr most of the organisms had lysed and the viability was < 1 % (Pl. 3, fig. 8–11). Lysis appeared to result from rupture of both the membrane and cell wall, and after lysis membranes tended to remain relatively intact while cell walls fragmented (Pl. 3, fig. 11). It is not clear whether the extrusion of cytoplasmic material occurred at specific sites but the ruptures did not appear to be randomly distributed over the cell surface. The micrographs of lysing cocci (Pl. 3, fig. 8–11) appear similar to those of lipase-treated bacteria

(Ghosh & Murray, 1967) where breakdown of the cytoplasmic membrane and cell wall eventually resulted in the liberation of cytoplasmic material.

It seems that autolytic enzymes are present in most bacteria, and appear to be activated whenever normal growth of the organism is disrupted (Stolp & Starr, 1965; Shockman, 1965). For example, Shockman (1965) has observed that cell wall preparations from *Streptococcus faecalis* in the growth phase were slowly autolysed when incubated in phosphate buffer. It is generally considered that the bacterial cell wall has a predominantly mechanical role, conferring cell shape, rigidity and resistance to the high osmotic pressure created by the selectivity of the cytoplasmic membrane. As *Streptococcus lactis* lysed, the cytoplasmic membrane pulled away from the rigid wall and eventually collapsed (Pl. 3, fig. 8–11), presumably due to the decreasing osmotic pressure. The membranes of Gram-positive bacteria do not appear to be bound to the cell walls (Salton, 1967).

In this study, exogenous amino acids have been shown to maintain organisms intact and viable for a longer period (Pl. 3, fig. 12). A dense ribosome pattern was still visible after prolonged starvation (Pl. 2, fig. 6) and lysed organisms appeared as the death rate began to increase (Pl. 3, fig. 12). Sections prepared by the R-K fixation procedure showed some organisms with normal mesosome structures consisting of concentric whorls of membrane but most mesosomes appeared to have unfolded (Pl. 4, fig. 13).

Maximum survival of starved *Streptococcus lactis* was achieved by the continuous feeding of low concentrations of glucose (Thomas & Batt, 1969*b*). After 120 hr starvation the population was 40 % viable and thin sections of these organisms showed marked changes in the mesosomes and nuclear material (Pl. 4, fig. 14, 15; Pl. 5, fig. 16, 17). Unfolding of mesosomes often resulted in what appeared to be a multiple cytoplasmic membrane system (Pl. 4, fig. 14, 15). In some Gram-positive bacteria a close morphological and functional relationship has been shown to exist between the mesosomes and the nuclear material (Ryter & Landman, 1968). When mesosome extrusion or unfolding occurred in the present study, the nuclear material become dislocated from its central position contiguous with the mesosome and often appeared in direct contact with the cytoplasmic membrane (Pl. 5, fig. 17). Changes similar to these seem to occur in all bacteria which have lost their mesosomes, e.g. protoplasts (Ryter & Landman, 1968). Although some of the changes observed in starved *Streptococcus lactis* are typical of Gram-positive bacteria undergoing plasmolysis, extruded mesosomes were only rarely observed in pockets between the cell wall and cytoplasmic membrane as described by Fitz-James (1964). This finding, together with the fact that the cell walls of lysed organisms were broken down into small fragments, may indicate that cell wall autolysis is responsible for the loss of viability of *S. lactis* under these conditions. Lysed organisms appeared in viable cultures containing amino acids (Pl. 3, fig. 12) and also in glucose-fed populations (Pl. 5). In the latter system, some organisms appeared to have undergone considerable growth without division; ribosomes were still present (Pl. 5, fig. 16) and surviving organisms had short division lags similar to those at the onset of starvation (Thomas & Batt, 1968, 1969*a*).

DISCUSSION

The steady leakage of intracellular amino acids from starved *Streptococcus lactis* into the external medium is consistent with the absence of an endogenous energy source (Thomas & Batt, 1969*a*) since bacteria having such energy sources appear to retain their free amino acid pools when starved (Dawes & Holms, 1958; Postgate & Hunter, 1962; Dawes & Ribbons, 1965). Conditions promoting release of components from metabolic pools may initiate polymer breakdown and increase the death rate and this would be consistent with the observation that spermine enhanced survival and suppressed the release of u.v.-absorbing material from starved *S. lactis*. As well as promoting ribosome stability, added Mg^{2+} could have an important function in maintaining permeability barriers. The finding that high EDTA concentrations were lethal (Thomas & Batt, 1968) supports this view, since EDTA appears to remove cell wall and membrane Mg^{2+} by chelation. After approximately 12 hr starvation of *S. lactis* in phosphate buffer containing Mg^{2+} , the death rate began to increase and was accompanied by the release of lactic dehydrogenase and DNA. The release of intracellular macromolecules, before lysis was detectable by electron microscopy, may have resulted from a partial rupture of the cell wall and membrane.

Without exogenous Mg^{2+} ribosome particles rapidly disappeared from the cytoplasm in agreement with earlier chemical evidence (Thomas & Batt, 1969*a*) and loss of viability occurred while organisms retained intact cell wall and cytoplasmic membrane structures. In the presence of exogenous Mg^{2+} some lysis was evident as the death rate increased while intact organisms still retained dense ribosome patterns. When organisms were starved with Mg^{2+} plus a suitable energy source, cell structures and ribosomes were maintained for a longer period. However, lysis eventually occurred when the death rate increased and this could have led to some high viability estimates, since lysed organisms may not always be counted on slide-cultures.

Conclusions. The existence of minimum growth rates for bacteria (Tempest, Herbert & Phipps, 1967) implies a state of minimum subsistence which must be maintained for survival. In this state, essential polymers are present at the lowest level capable of initiating regrowth and any breakdown of these components results in loss of viability. It is now well established for some species of bacteria that organisms produced at maximum growth rates may contain material in excess of these minimum levels so that considerable degradation may take place before death occurs. The products of polymer hydrolysis in most bacteria are metabolized producing energy but with *Streptococcus lactis* these products generally appear to be released into the external medium in an undegraded form.

Although it has been suggested that maintenance of bacteria in a viable state requires energy, the amount of energy is ill-defined. *Streptococcus lactis* maintained complete viability for approximately 15 hr when starved in buffer containing Mg^{2+} , yet no absolute requirement for an energy source could be determined. At the onset of starvation an ATP pool was probably present and small amounts of energy may have been produced by endogenous metabolism, especially if *S. lactis* contains constitutive kinases for nucleotide metabolism (see Gronlund & Campbell, 1965). However, leakage of the intracellular free amino acid pool occurred as soon as the organisms were starved, there was no appreciable protein or RNA synthesis and, in addition, starved organisms were extremely pH sensitive. This suggested that an

appreciable endogenous energy source was not involved in maintaining the viability of organisms starved in phosphate buffer. It would appear that these organisms remained viable as long as the degradation of polymers, perhaps RNA, had not proceeded beyond some irreversible point and the present evidence suggests that the death rate was dependent on the presence of compounds promoting polymer stability. When Mg^{2+} was added to buffered suspensions, a suitable exogenous energy source further enhanced survival. This energy source appeared to suppress the release of the free amino acid pool and allow limited polymer synthesis and pH control. In this environment survival may then be a function of cell wall and/or membrane stability. *S. lactis* does not appear to have any survival mechanism based on an endogenous metabolism. The ability to withstand low pH values may be the most important factor for the survival of this organism in milk.

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REFERENCES

- BURTON, K. (1956). A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**, 315.
- CHUNG, K. L., HAWIRKO, R. Z. & ISAAC, P. K. (1964). Cell wall replication. II. Cell wall growth and cross wall formation of *Escherichia coli* and *Streptococcus faecalis*. *Can. J. Microbiol.* **10**, 473.
- CITTI, J. E., SANDINE, W. E. & ELLIKER, P. R. (1965). β -Galactosidase of *Streptococcus lactis*. *J. Bact.* **89**, 937.
- COLE, R. M. (1965). Symposium on the fine structure and replication of bacteria and their parts. III. Bacterial cell-wall replication followed by immunofluorescence. *Bact. Rev.* **29**, 326.
- DAWES, E. A. & HOLMS, W. H. (1958). Metabolism of *Sarcina lutea*. III. Endogenous metabolism. *Biochim. biophys. Acta* **30**, 278.
- DAWES, E. A. & RIBBONS, D. W. (1965). Studies on the endogenous metabolism of *Escherichia coli*. *Biochem. J.* **95**, 332.
- DREZE, A., MOORE, S. & BIGWOOD, E. J. (1954). Desalting of solutions of amino acids by ion exchange. *Analytica chim. Acta* **11**, 554.
- FITZ-JAMES, P. C. (1960). Participation of the cytoplasmic membrane in the growth and spore formation of bacilli. *J. biophys. biochem. Cytol.* **8**, 507.
- FITZ-JAMES, P. C. (1964). Fate of the mesosomes of *Bacillus megaterium* during protoplasting. *J. Bact.* **87**, 1483.
- GHOSH, B. K. & MURRAY, R. G. E. (1967). The fine structure of *Listeria monocytogenes* in relation to protoplast formation. *J. Bact.* **93**, 411.
- GRONLUND, A. F. & CAMPBELL, J. J. R. (1965). Enzymatic degradation of ribosomes during endogenous respiration of *Pseudomonas aeruginosa*. *J. Bact.* **90**, 1.
- KELLENBERGER, E. & RYTER, A. (1964). In *Modern Developments in Electron Microscopy*. Ed. by B. M. Siegel. p. 335. New York: Academic Press.
- KELLENBERGER, E., RYTER, A. & SECHAUD, J. (1958). Electron microscope study of DNA-containing plasms. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. *J. biophys. biochem. Cytol.* **4**, 671.
- KENNEL, D. & KOTOULOS, A. (1967). Magnesium starvation of *Aerobacter aerogenes*. IV. Cytochemical changes. *J. Bact.* **93**, 367.
- KORNBERG, A. (1955). Lactic dehydrogenase of muscle. *Meth. Enzym.* **1**, 441.
- LUFT, J. H. (1961). Improvements in epoxy resin embedding methods. *J. biophys. biochem. Cytol.* **9**, 409.
- MAGER, J. (1959). The stabilizing effect of spermine and related polyamines on bacterial protoplasts. *Biochim. biophys. Acta* **36**, 529.

- MILLONIG, G. (1961). A modified procedure for lead staining of thin sections. *J. biophys. biochem. Cytol.* **11**, 736.
- MORGAN, C., ROSENKRANZ, H. S., CHAN, B. & ROSE, H. M. (1966). Electron microscopy of magnesium-depleted bacteria. *J. Bact.* **91**, 891.
- POLLOCK, M. R. (1961). The measurement of the liberation of penicillinase from *Bacillus subtilis*. *J. gen. Microbiol.* **26**, 239.
- POSTGATE, J. R. & HUNTER, J. R. (1962). The survival of starved bacteria. *J. gen. Microbiol.* **29**, 233.
- REYNOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208.
- RYTER, A. (1968). Association of the nucleus and the membrane of bacteria: a morphological study. *Bact. Rev.* **32**, 39.
- RYTER, A. & LANDMAN, O. E. (1964). Electron microscope study of the relationship between mesosome loss and the stable L state (or protoplast state) in *Bacillus subtilis*. *J. Bact.* **88**, 457.
- RYTER, A. & LANDMAN, O. E. (1968). Morphological study of the attachment of nucleoid to membrane in bacilli, protoplasts and reverting protoplasts of *Bacillus subtilis*. In *Microbial Protoplasts, Spheroplasts and L-Forms*. Ed. by L. B. Guze. p. 110. Baltimore: Williams & Wilkins.
- SALTON, M. R. J. (1967). Structure and function of bacterial cell membranes. *A. Rev. Microbiol.* **21**, 417.
- SHOCKMAN, G. D. (1965). Symposium on the fine structure and replication of bacteria and their parts. IV. Unbalanced cell wall synthesis: autolysis and cell wall thickening. *Bact. Rev.* **29**, 534.
- STOLP, H. & STARR, M. P. (1965). Bacteriolysis. *A. Rev. Microbiol.* **19**, 79.
- TABOR, C. W. (1962). Stabilization of protoplasts and spheroplasts by spermine and other polyamines. *J. Bact.* **83**, 1101.
- TEMPEST, D. W., HERBERT, D. & PHIPPS, P. J. (1967). Studies on the growth of *Aerobacter aerogenes* at low dilution rates in a chemostat. In *Microbial Physiology and Continuous Culture*, p. 240. London: H.M.S.O.
- THOMAS, T. D. & BATT, R. D. (1968). Survival of *Streptococcus lactis* in starvation conditions. *J. gen. Microbiol.* **50**, 367.
- THOMAS, T. D. & BATT, R. D. (1969*a*). Degradation of cell constituents by starved *Streptococcus lactis* in relation to survival. *J. gen. Microbiol.* **58**, 347.
- THOMAS, T. D. & BATT, R. D. (1969*b*). Metabolism of exogenous arginine and glucose by starved *Streptococcus lactis* in relation to survival. *J. gen. Microbiol.* **58**, 371.
- TURNER, N. A. & REDGWELL, R. J. (1966). A mixed layer for separation of amino acids by thin-layer chromatography. *J. Chromat.* **21**, 129.

EXPLANATION OF PLATES

PLATE I

Division sequence of *Streptococcus lactis* ML₃ from the logarithmic growth phase (R-K fixation, Reynolds stain) Scale lines = 0.2 μ

Fig. 1. Initial stage of cell division showing the undivided nuclear material (*N*) and small invaginations where the mesosome (*M*) is continuous with the cytoplasmic membrane (*CM*).

Fig. 2. An intermediate stage showing the divided nuclear material (*N*) in close contact with the mesosome (*M*).

Fig. 3. Transverse wall formation is complete with a clearly defined membrane. The new mesosome (*M*), formed at the next division site, is continuous with the cytoplasmic membrane.

PLATE 2

Ribosome changes

Fig. 4. Organisms from the logarithmic growth phase, showing an even distribution of ribosomes (G-Os fixation (*b*), Reynolds stain).

Fig. 5. Organisms after 17 hr starvation in 0.075 M-phosphate buffer (pH 7.0, 10 μ M-EDTA), viability 16 %, initial bacterial mass 100 μ g. dry wt/ml. Note the absence of ribosomes in most cells (G-Os fixation (*c*), Reynolds stain).

Fig. 6. Organisms after 48 hr starvation in phosphate buffer containing $10\ \mu\text{M}$ -EDTA, $1\ \text{mM}$ - MgSO_4 , $10\ \text{mM}$ -arginine and casamino acids (1% , Difco), viability 92% , initial bacterial mass $100\ \mu\text{g}$ dry wt/ml. Ribosomes are still present and appear to be concentrated near the cytoplasmic membrane (G-Os fixation (*a*), Millonig stain).

PLATE 3

Lysis of *Streptococcus lactis*

Fig. 7. Organisms after 17 hr starvation in phosphate buffer ($10\ \mu\text{M}$ -EDTA), viability 2% . Before any visible lysis, some organisms showed intrusions of the membrane (arrowed) in direct contact with the low density nuclear material (*N*) (G-Os fixation (*a*), Reynolds stain).

Fig. 8–11. Organisms after 40 hr starvation in phosphate buffer containing $1\ \text{mM}$ - MgSO_4 , viability $< 1\%$, $2\ \text{mg}$. dry wt/bacteria/ml. Bacterial lysis has occurred leaving the remains of cytoplasmic membranes (*CM*) and cell walls (*CW*). Membranes tend to remain intact while cell walls fragment. Lysed organisms show the extrusion of cytoplasmic material into the external medium through ruptures (arrowed) in the membrane and cell wall (G-Os fixation (*a*); Fig. 8, 10, Reynolds stain; Fig. 9, 11, Millonig stain).

Fig. 12. Organisms after 48 hr starvation in phosphate buffer containing Mg^{2+} and amino acids (see Pl. 2, fig. 6), viability 92% . Lysed organisms are visible at a high viability (G-Os fixation (*a*), Millonig stain).

PLATE 4

Changes in membrane structures (R-K fixation, Reynolds stain)

Fig. 13. Organisms after 48 hr starvation in phosphate buffer containing Mg^{2+} and amino acids (see Pl. 2, fig. 6), viability 92% . The mesosome (*M*) appears to have unfolded and is still in close contact with the separated nuclear material.

Fig. 14, 15. Organisms after 120 hr starvation in phosphate buffer supplied with a continuous glucose feed ($0.25\ \mu\text{mole}$ glucose/mg. dry wt bacteria/hr—see Thomas & Batt, 1969*b*), viability 40% . In Fig. 14 three membrane layers (*M*) are visible which may result from unfolding of the mesosome (see Fig. 15).

PLATE 5

Changes in membrane structures and nuclear material.

Organisms after 120 hr starvation in phosphate buffer supplied with a continuous glucose feed (see Pl. 4, fig. 14, 15), viability 40% (R-K fixation, Reynolds stain).

Fig. 16, 17. Typical changes in mesosome structures (*M*) and nuclear material (*N*). Ribosomes (*R*) were clearly visible and some organisms had lysed (*L*).

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