Arch Microbiol (1995) 163:373–379

ORIGINAL PAPER

G. V. Mukamolova · N. D. Yanopolskaya · T. V. Votyakova
V. I. Popov · A. S. Kaprelyants · D. B. Kell

Biochemical changes accompanying the long-term starvation of *Micrococcus luteus* cells in spent growth medium

Received: 7 December 1994 / Accepted: 26 January 1995

Abstract Changes in the biochemical properties of *Micrococcus luteus* cells were studied during the transition to a dormant state after incubation in an extended stationary phase. The overall DNA content after 150 days of starvation was similar to its initial level, while the RNA content decreased by 50%. Total lipids and protein, phospholipids and membrane proteins declined rapidly within the first 1-10 days of starvation. After 180 days of starvation, cells contained 43% of the protein and 35% of the lipid initially present. Starvation for 120 days resulted in the loss of phosphatidylglycerol and, to some extent, of phosphatidylinositol, giving a membrane whose phospholipids consisted mainly of cardiolipin. The membrane fluidity declined during starvation, as judged by diphenyl hexatriene fluorescence anisotropy measurements. Oxidase activities declined to zero within the first 20–30 days of starvation, while the dehydrogenases and cytochromes were more stable. The activities of some cytoplasmic enzymes were lost very rapidly, while NADPH-linked isocitrate dehydrogenase had 30% of its initial activity after 120 days of starvation. For all parameters tested there were significant fluctuations during the first 10–20 days of starvation, which may reflect cryptic growth in the culture.

Key words *Micrococcus luteus* · Stationary phase · Anabiosis · Dormancy · Cytochromes · Phospholipids · Macromolecules

G. V. Mukamolova · N. D. Yanopolskaya · T. V. Votyakova
A. S. Kaprelyants
Bakh Institute of Biochemistry, Russian Academy of Sciences, Leninskii Prospekt 33, Moscow 117071, Russia

V. I. Popov
Institute of Cell Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, 142292, Russia

D. B. Kell (✉)
Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed SY23 3DA, UK
Tel. +44-1970-622334; Fax +44-1970-622354
email dbk@aber.ac.uk

Abbreviations *MPN* Most probable number · *DPH* Diphenyl hexatriene

Introduction

In recent work, we have found that cells of the nonsporulating, copiotrophic, gram-positive coccus *Micrococcus luteus* starved for 3–7 months in spent growth medium following growth to stationary phase in batch culture can persist in a dormant state, exhibiting very low viability ($< 10^{-4}$), as estimated by plating on agar plates, while the total count remains close to its initial value (Kaprelyants and Kell 1993; Kaprelyants et al. 1993). Using flow cytometry with appropriate probes and conditions (Kell et al. 1991), we found that at least 50% of *M. luteus* cells in 3-month-old populations could be resuscitated to normal, colony-forming bacteria (Kaprelyants and Kell 1993) under conditions that excluded any significant regrowth of initially viable cells. We confirmed this by using the most-probable-number (MPN) method when we resuscitated cells in media which, statistically, contained no "initially viable" cells. When the medium also contained spent growth medium from a culture in early stationary phase, we found a substantial increase (1,000- to 100,000-fold) in the number of viable bacteria in such starved populations compared to those estimated with the agar-plate method (Kaprelyants et al. 1994). While these and other data (Votyakova et al. 1994) led to the conclusion that, from a physiological point of view, a significant number of cells in such starved *M. luteus* populations were dormant and not dead, little is known about the biochemical properties of such cells.

Broadly similar comments may be made about two other groups of nonsporulating bacteria, which are sometimes considered to be dormant forms in natural water habitats, viz. the so-called viable-but-non-culturable (VBNC) forms (Colwell et al. 1985; Roszak and Colwell 1987) and ultramicrobacteria (Morita 1990). (For reviews see Kaprelyants et al. 1993; Kjelleberg 1993). Siegele and Kolter (1992) collected a limited amount of information

on the changes in some of the biochemical properties of some bacteria when cells enter the stationary phase, and special attention has been paid to the regulation of gene expression and the synthesis of novel proteins and other substances in stationary phase cultures of *Escheria coli* (e.g. Kolter 1992; Matin 1992; Kjelleberg 1993; Huisman and Kolter 1994; Loewen and Hengge-Aronis 1994). More detailed biochemical studies have been performed with respect to the relatively short-term starvation of non-sporulating bacteria in unsupplemented water or in buffers (Kjelleberg et al. 1983; Hood et al. 1986; Smigielski et al. 1989; Lappin-Scott and Costerton 1990; Holmquist and Kjelleberg 1993). It is not known, however, whether the populations of starved bacteria in these studies contain any dormant bacteria, so that the properties observed are plausibly not characteristic of the dormant state. The aim of the present study was therefore to characterize the biochemical properties of bacterial cultures under conditions in which the concentration of dormant cells was known to be substantial.

Materials and methods

Organism and media

Micrococcus luteus NCIMB 13267 (previously described as "Fleming strain 2665") was grown oxically at 30° C in shake flasks in lactate minimal medium containing L-lactate described previously (Kaprelyants and Kell 1992). When the culture reached stationary phase, agitation was continued at 30° C for up to 3 months. Cultures were then held oxically at room temperature without agitation for a period of up to 3 more months.

Preparation of membrane and cytoplasmic fractions

Harvested cells were washed with 50 mM potassium phosphate buffer, pH 7.4, by centrifugation. The pellet was suspended in the same buffer supplemented with 5 mM $MgSO_4$ and incubated with lysozyme (1 mg/g wet cells) for 30 min at 30° C with a few crystals of DNase. Membranes were sedimented by centrifugation at 22,000 × *g* for 30 min, and the membrane pellet was suspended in phosphate-Mg buffer.

Polarographic measurments

The oxygen consumption rates of cells and membranes were measured by a Clark oxygen electrode at 30° C in the above phosphate-Mg buffer.

Determination of cytochrome concentration

Reduced-*minus*-oxidized difference spectra were recorded at ambient temperature with a Hitachi-557 spectrophotometer. Cytochromes were oxidized with potassium ferricyanide (5 mM) and reduced with a few crystals of sodium dithionite. The cytochrome content was calculated using the following molar absorption coefficients (cm^{-1} mM^{-1}): for cytochrome *c* 21, for cytochrome *b* 20, and for cytochrome *a* 13 (Artzatbanov et al. 1991).

Determination of dehydrogenase activity

Malate, lactate and NADH dehydrogenase activities in membrane fractions were measured spectrophotometrically using 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor (Ostrovsky et al. 1976). Malate-, lactate-, and ethanol-dependent NADH production and isocitrate-dependent NADPH production catalyzed by the cytoplasmic fraction were measured fluorimetrically at room temperature using a Hitachi MPF-4 fluorescence spectophotometer (excitation 350 nm, emission > 450 nm) in 50 mM phosphate buffer, pH 7.5, containing 1 mM NAD(P). Substrate concentrations were: malate (10 mM), lactate (20 mM), ethanol (50 mM), and isocitrate (2 mM).

Membrane fluidity

M. luteus cells were stained with diphenyl hexatriene (DPH) by incubating cells (1.5–2 mg dry weight ml^{-1}) with 10 mM DPH in 0.2 M phosphate buffer containing 0.5 mM $MgSO_4$ at 30° C for 15 min. The stained cell suspensions were diluted tenfold with phosphate buffer before measurement. DPH fluorescence polarization anisotropy was measured at ambient temperature using a Hitachi MPF-4 spectrofluorimeter equipped with the Perkin Elmer 063–0568 polarization accessories. The fluorescence anisotropy *r* was calculated from the relationship: $r = (I_{par} - I_{per})/(I_{par} + 2I_{per})$, where I_{par} and I_{per} are the fluorescence intensities for the parallel and perpendicular positions of analyzers (430 nm) with respect to the exciting photons (348 nm).

DNA and RNA

DNA was determined in cells with diphenylamine as described by Burton (1956). For total RNA determination, bacterial cells were lysed with lysozyme, followed by membrane centrifugation (22,000 × *g*, 30 min) and supernatant dialysis. RNA was determined in both membrane and cytoplasm fractions with orcinol (Kerr and Seraidarian 1945).

Lipids

Lipids were extracted from cells with chloroform/methanol according to Folch, as described by Ansell and Hawthorne (1964). The total lipid concentration in extracts was determined by heating with H_2SO_4 (Gribanov and Sergeev 1975). Two-dimensional TLC of standard phospholipids and lipid extracts was performed with the first solvent, chloroform/methanol/water (65/35/5, v/v), and the second solvent, chloroform/methanol/water (65/25/4 v/v). Phospholipid spots were visualized by treatment with molybdate blue (Dittmer and Lester 1964). Phospholipid content was determined with Victoria blue R dye in lipid extracts (Eryomin and Poznyakov 1989) and individual phospholipids after scraping silica gel from the approrpiate areas of TLC plates by heating with H_2SO_4 (Gribanov and Sergeev 1975).

Protein

Protein in cell extracts and membranes was determined according to the modification of Lowry et al. (1951).

Chemicals

These were of analytical grade wherever possible, and were obtained from Sigma (Poole, Dorset, UK) or from Reachim (Moscow, Rusia). Water was singly distilled in an all-glass apparatus.

Electron microscopy

Cell deposits were fixed with 2.5% glutaraldehyde solution prepared in 0.1 M Na-cacodylate buffer (pH 7.6–7.8) for about 2–12 h at room temperature. All specimens were postfixed with 0.8–1%

osmium tetroxide containing 0.1% potassium dichromate. Osmium fixation lasted not less than 12 h at room temperature. Dehydration was perfomed in ethanol (50, 70, 80, 100% for 15–20 min each) and in 100% acetone (4 changes, 15–20 min each). The dehydrated material was kept overnight in the following mixture: 1 vol. unit of 100% acetone plus 1 vol. unit of Epon 812/Araldite M (Serva, Heidelberg, Germany). Specimens were embedded overnight in epoxy resin at 37–40° C. Thin sections (50–80 nm thick) were cut with an LKB Ultratome using glass knives. Sections were stained in 70% ethanol solution saturated with uranyl acetate for 5–10 min. Thin sections were studied with a JEM 100B electron microscopes. Magnifications were calibrated for each experiment by using cross-grating replicas.

Results

Cell composition

Earlier we found that prolonged incubation of *M. luteus* in spent growth medium resulted in a rapid decrease in the number of colony-forming units after the first 10 days of starvation, while the total count of bacteria changed only slightly during 6 months of starvation (Kaprelyants and Kell 1993). Also, cells gradually lost protein in contrast to their DNA content, which was stable as judged by flow cytometric measurements (Kaprelyants and Kell 1992). Figure 1 shows that the decrease in protein content is due mainly to the degradation of cytoplasmic proteins, while the membrane protein content was more stable. As with 3-month-old cells (Kaprelyants and Kell 1993), 5–6 months of starvation did not result in DNA degradation as judged by the conventional diphenylamine method (2.9 ± 0.4 fg DNA cell^{-1} at the onset of starvation and 2.8 ± 0.5 fg cell^{-1} after 5 months of starvation). However, the RNA content decreased from 15 ± 1 fg RNA cell^{-1} at the onset of starvation to 8 ± 1 fg cell^{-1} after 5 months of starvation.

The losses of lipid material by cells were more pronounced during the first 10–20 days of starvation (Fig. 2), and the final content of both total lipids and phospholipids was approx. 50% of that at the onset of starvation. The protein:lipid ratio in membrane fractions was estimated as 2.8:1 at the onset of starvation, 2.7:1 at 65 days of starvation, and 3.8:1 after 6 months of starvation. After 6 months of starvation, the cells had almost completely lost their main carotenoid, lutein (data not shown).

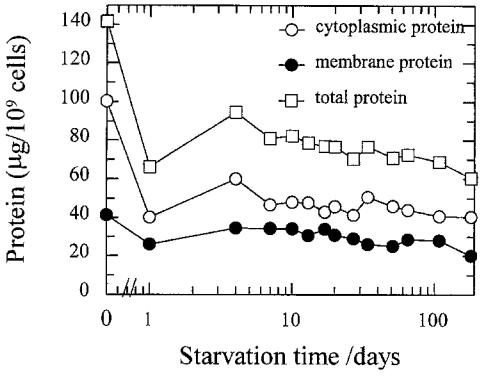

Fig. 1 Changes in the total, membrane and cytoplasmic protein contents of *Micrococcus luteus* starved in a prolonged stationary phase. Starvation was performed and proteins were assayed as described in Materials and methods

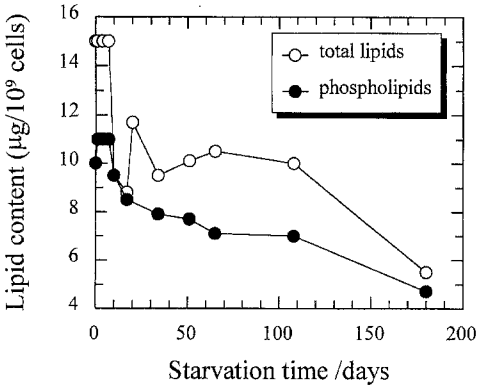

Fig. 2 Changes in the total lipids and phospholipid content of *Micrococcus luteus* starved in a prolonged stationary phase. Starvation, lipid extraction and total lipid and phospholipid determinations were as described in Materials and methods

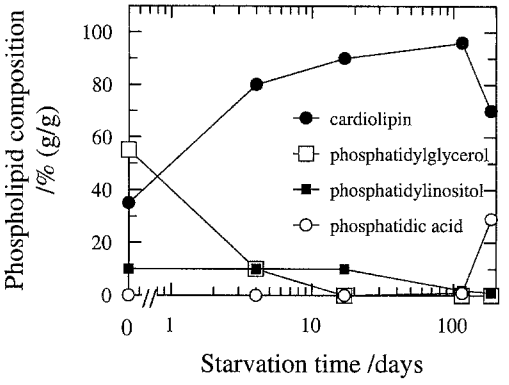

Fig. 3 Changes in the phospholipid composition of *Micrococcus luteus* subject to starvation in a prolonged stationary phase. Starvation, lipid extraction and TLC analyses were as described in Materials and methods

More detailed studies of the phospholipids revealed dramatic changes in their composition during starvation (Fig. 3). Phosphatidylglycerol disappeared almost completely, leaving cells possessed of membranes that contained cardiolipin as the dominant phospholid after 10–100 days of starvation. Subsequently, there was a noticeable accumulation of phosphatidic acid at the expense of cardiolipin and phosphatidylinositol (Fig. 3). After 10 days lysophospholipids (mainly lysocardiolipin) appeared in the lipid extracts, amounting to 5–10% of the total phopholipids, a figure which did not change significantly during further cell incubation (data not shown).

Membrane and cytoplasmic enzyme activities

We tested the activities of some of the enzymes responsible for the energy metabolism of these cells during starvation. Figure 4A shows that the oxidase activities for malate, lactate and NADH in isolated membranes had de-

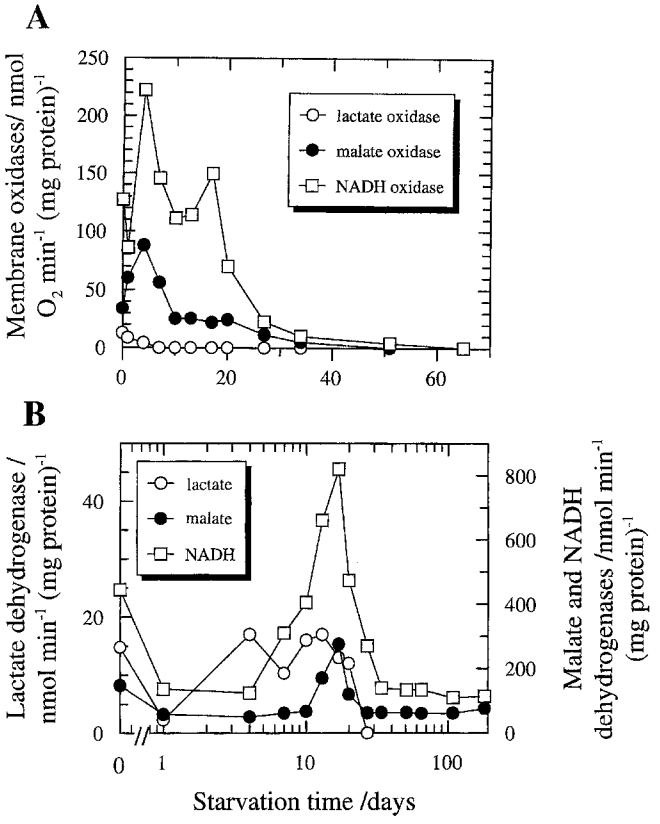

Fig. 4 Changes in oxidase (**A**) and dehydrogenase (**B**) activities of membranes isolated from *Micrococcus luteus* cells starved in a prolonged stationary phase. The starvation regime, the preparation of cytoplasmic membranes and the enzyme assays were performed as described in Materials and methods

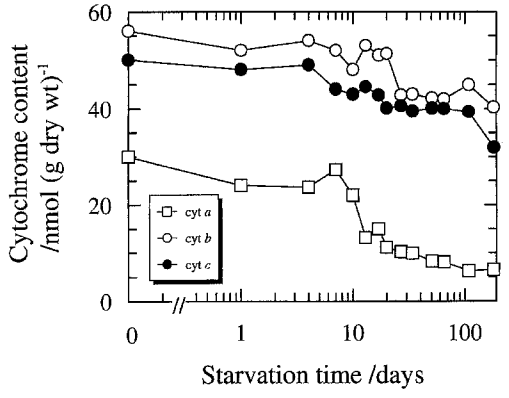

Fig. 5 Changes in the cytochrome content of membranes isolated from *Micrococcus luteus* cells starved in an extended stationary phase. Measurements were carried out exactly as described in Materials and methods

creased to unmeasurable levels after 2 months of starvation, while Fig. 4B indicates that malate and NADH-dehydrogenases retained some activity (about 30 and 25 % of their initial activities, respectively) for up to 6 months of starvation. Similarly, the cytochrome contents decreased during the first 20 days of starvation, while further incubation of cells in spent medium resulted in only a minor further loss of cytochromes. After a total of 6 months' starvation their content was 22% for cytochrome *a*, 71% for cytochrome *b* and 64% for cytochrome *c* relative to those obtained initially (Fig. 5).

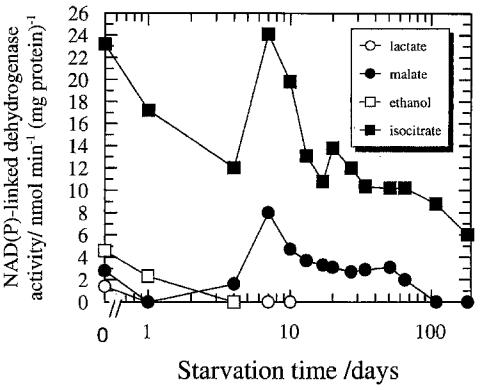

Fig. 6 Changes in the activities of various cytoplasmic enzymes of *Micrococcus luteus* cells starved in an extended stationary phase. Measurements were carried out exactly as described in Materials and methods

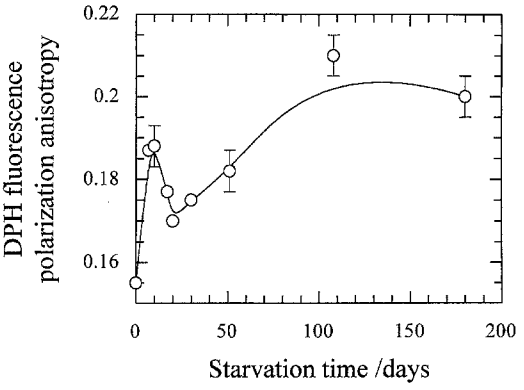

Fig. 7 Changes in the fluorescence polarization anisotropy of DPH in *Micrococcus luteus* cells starved in an extended stationary phase. Measurements were carried out and the fluorescence polarization anisotropy calculated as described in Materials and methods

In contrast to the membrane enzymes tested, cytoplasmic dehydrogenases were much less stable during starvation except for NADP-dependent isocitrate dehydrogenase, which retained 26% of its initial activity after 6 months of starvation (Fig. 6). It is worth stressing that all the activities tested demonstrated significant fluctuations during the first 10–20 days of starvation, revealing a transient increase of activities a few days after the stationary phase.

Membrane fluidity

Study of isolated membranes with the fluorescent probe DPH revealed a general tendency: the fluorescence anisotropy of DPH increased during starvation, a finding which indicates a decrease in the fluidity of the bulk lipid phase (Fig. 7). However, there were also fluctuations in the DPH anisotropy during the first 20 days of starvation.

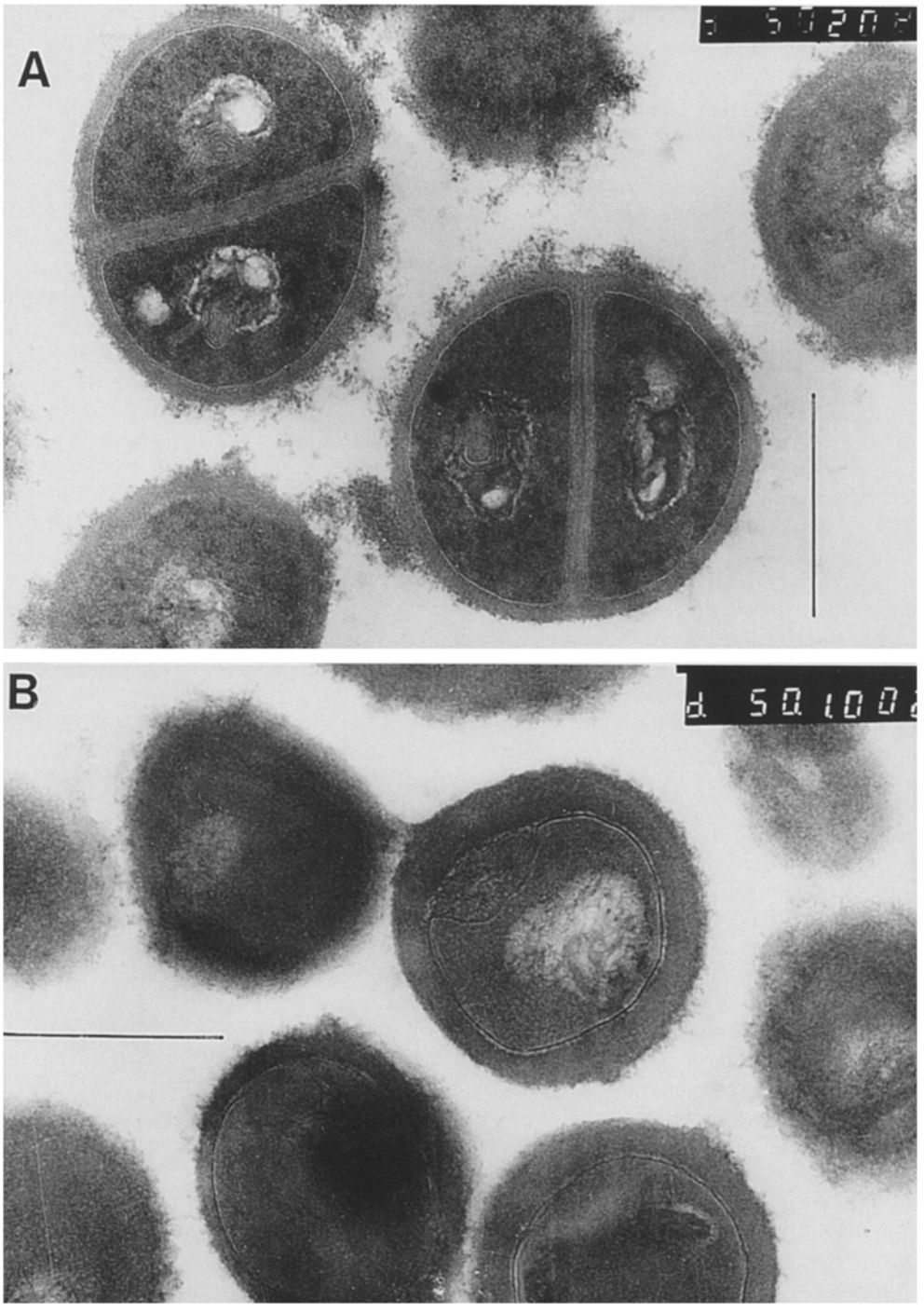

Fig. 8 A, B Transmission electron micrographs of thin sections of *Micrococcus luteus*. **A**. Nonstarved cells harvested from an exponential phase. **B**. Cells that had been starved for 4 months under the conditions described in Materials and methods. (*Bar* 0.5 µm)

Cell morphology

Although their cytoplasmic membranes were permeable to a normally membrane-impermeant nucleic acid stain (Votyakova et al. 1994), electron microscopy revealed that most cells in a population that had been starved for 6 months were grossly morphologically intact, with a thicker cell wall and a denser cytoplasm in comparison with growing bacteria. There were no signs of cell lysis or of dividing bacteria. However, the structure of the nucleoid region in starved cells differed somewhat from that in fresh cells (Fig. 8). A small fraction of the population was represented, however, by partially plasmolysed cells (data not shown).

Discussion

In some respects, the behavior of *M. luteus* cells held in a prolonged stationary phase resembles that of bacteria held under starvation conditions in water or buffers. Thus, a rapid and significant decline in RNA, lipid and protein

content has been reported during the starvation of *Escherichia coli* (Lappin-Scott and Costerton 1990, Oliver 1993), *Selenomonas ruminantium* (Mink et al. 1982) and *Vibrio cholerae* (Hood et al. 1986). Generally, DNA appeared to be more stable even during prolonged starvation (Lappin-Scott and Costerton 1990, Siegele and Kolter 1992), although in some cases the DNA content per cell apparently declined gradually over a period of time (Mink et al. 1982; Hood et al. 1986; Moyer and Morita 1989; Galdiereo et al. 1994). In the present work we found that the DNA content of *M. luteus* cells starved in spent medium for a period of 6 months was not changed significantly. In previous experiments using flow cytometry, *M. luteus* held for 3 months in similar conditions showed a DNA distribution between cells that was even more homogeneous relative to that observed at the onset of starvation (Kaprelyants and Kell 1993). Especially because under these conditions a significant proportion of these old, starved cells could be resuscitated to normal bacteria (Kaprelyants and Kell 1993; Votyakova et al. 1994), it seems reasonable that the maintenance of DNA integrity is a prerequisite for cell resuscitation from a dormant state.

The relative stability of membrane protein can account for the fact that the protein:lipid ratio increases in membranes when cells are starved, which can in turn account for the decrease in membrane fluidity (Fig. 7). As with the starvation of *Vibrio fluvialis* in buffered medium (Smigielski et al. 1989), oxidase activities in isolated membranes of *M. luteus* decreased during starvation. The inhibition of cell respiration during the transition to the so-called "viable-but-non-culturable" state appears to be a general phenomenon (Oliver 1993). While after 1 month of starvation the overall oxidase activity of the respiratory chain was practically inactive, a number of dehydrogenase activities revealed significant potential activity (especially the membranous malate and NADH dehydrogenases) even after 4 months of starvation. In view of the maintenance of a high level of cytochromes, it would appear that the loss of respiratory chain activity during starvation is due either to degradation of menaquinone or (quantitatively less likely) to changes in the extent of interaction between the respiratory chain components as a result of the modification of lipid composition and membrane fluidity observed (see also Kaprelyants and Ostrovsky 1984). By contrast, the pronounced decrease in the endogenous respiration rate of starved *M.luteus* cultures within the first 10 days of starvation (Kaprelyants and Kell 1993) evidently reflects a decrease in the concentration of endogenous substrates rather than an inhibition or degradation of the respiratory chain per se. It is reasonable that the maintenance of key respiratory chain enzymes in a potentially active (latent) state after long-term starvation allows dormant cells to respond rapidly when nutrients become available.

The observed decrease in membrane fluidity evidently reflects the changes in lipid composition. A similar effect of starvation to produce an increase in the content of cardiolipin at the cost of phosphatidylglycerol in the early stationary phase has been noted in *Staphylococcus aureus* and *E. coli* (Wanner and Egli 1990); an increase in the saturated fatty acids in the membrane during starvation has also been noted (Wanner and Egli 1990).

An interesting peculiarity of the behavior of starved *M. luteus* cells is the significant fluctuation in almost all the parameters tested during the period of ca. 3–20 days following the initiation of starvation. These fluctuations coincided with those in optical density and the total cell count found earlier under the same conditions (Kaprelyants and Kell 1993). Similar fluctuations were also found during starvation of a psychrophilic marine bacterium (Moyer and Morita 1989). These fluctuations presumably reflect cryptic growth in the culture (see Postgate 1976), followed by culture stabilisation and the maintenance of relatively constant enzyme activities and cell composition.

In conclusion, the prolonged starvation of *M.luteus* cells in spent growth medium results in the appearance of cells that possess a significantly changed composition, activities of both cytoplasmic and membrane enzymes, and membrane fluidity. At the same time, most of the cells in the population preserve their morphological integrity even after 4 months of starvation, an evident requirement for the resuscitation observed in these cultures (Kaprelyants and Kell 1993; Kaprelyants et al. 1994; Votykova et al. 1994; Mukamolova et al. 1995). Finally, however, the question of which of the biochemical changes observed are beneficial adaptations for surviving starvation and for permitting a rapid response to improved nutritional fortune (Kell et al. 1995), and which of them are simply a consequence of starvation, remains to be elucidated.

Acknowledgements We are indebted to the Chemicals and Pharmaceuticals Directorate of the Biotechnology and Biological Sciences Research Council, U.K., and the Royal Society, under the terms of the Royal Society/Russian Academy of Sciences Joint Project scheme, for financial support of this work.

References

Ansell GB, Hawthorne JN (1964) Phospholipids. Chemistry, metabolism and function (BBA Library vol 3). Elsevier, Amsterdam London New York, pp 40–44

Artzatbanov V, Sheiko T, Lukoyanova M, Kaprelyants AS(1991) The increase of KCN -sensitive electron flow in the branched respiratory chain of *Micrococcus luteus* grown slowly in carbon-limited culture. J Gen Microbiol 137:1485–1490

Burton K (1956) Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem J 62:315–323

Colwell RR, Brayton BR, Grimes DJ, Roszak DB, Huq SA, Palmer LM (1985) Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Biotechnology 3:817–820

Dittmer JC, Lester RJ (1964) A simple specific spray for the detection of phospholipids on thin-layer chraomatograms J Lipid Res 5:126–127

Eryomin VA, Poznyakov SP (1989) Quantitative determination of phospholipids using the dyes Victoria Blue R and B. Anal Biochem 180:186–191

Galdiereo E, Donnarumma G, Demartino L, Marcatili A, Delero GC, Merone A (1994) Effect of low-nutrient seawater on morphology, chemical composition, and virulence of *Salmonella typhimurium*. Arch Microbiol 162:41–47

Gribanov GA, Sergeev CA (1975) Rapid analysis of total lipids in blood serum. Vopr Med Khim 6:654–657

Holmquist L, Kjelleberg S (1993) Changes in viability, respiratory activity and morphology of the marine *Vibrio* strain S14 during starvation of individual nutrients and subsequent recovery. FEMS Microbiol Ecol 12:215–224

Hood MA, Guckert JB, White DC, Fred D (1986) Effect of nutrient deprivation on lipid, carbohydrate, DNA, RNA and protein levels in *Vibrio cholerae*. Appl Environ Microbiol 52:788–793

Huisman GW, Kolter R (1994) Sensing starvation: a homoserine lactone-dependent signaling pathway in *Escherichia coli*. Science 265:537–539

Kaprelyants AS, Kell DB (1992) Rapid assessment of bacterial viability and vitality using rhodamine 123 and flow cytometry. J Appl Bacteriol 72:410–422

Kaprelyants AS, Kell DB (1993) Dormancy in stationary-phase cultures of *Micrococcus luteus*: flow cytometric analysis of starvation and resuscitation. Appl Environ Microbiol 59:3187–3196

Kaprelyants AS, Ostrovsky DN (1984) Mobility and order in the organization of enzymes in the bacterial membrane. In: Skulachev VP (ed) Soviet science reviews (Series.D, vol 5) Harwood, New York, pp 323–356

Kaprelyants AS, Gottschal JC, Kell DB (1993) Dormancy in non-sporulating bacteria. FEMS Microbiol Rev 104:271–286

Kaprelyants AS, Mukamolova GV, Kell DB (1994) Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent culture medium at high dilution. FEMS Microbiol Lett 115:347–352

Kell DB, Ryder HM, Kaprelyants AS, Westerhoff HV (1991) Quantifying heterogeneity: flow cytometry of bacterial cultures. Antonie Van Leeuwenhoek 60:145–158

Kell DB, Kaprelyants AS, Grafen A (1995) On pheromones, social behaviour and the functions of secondary metabolism in bacteria. Trends Ecol Evol 10:126–129

Kerr SE, Seraidarian K (1945) The separation of purine nucleosides from free purines and the determination of the purines and ribose in these fractions. J Biol Chem 159:211–225

Kjelleberg S (ed) (1993) Starvation in bacteria. Plenum Press, New York

Kjelleberg S, Humphrey B, Marshall KC (1983) Initial phase of starvation and activity of bacteria at surfaces. Appl Environ Microbiol 46:978–984

Kolter R (1992) Life and death in stationary phase. ASM News 58:75–79

Lappin-Scott HM, Costerton JW (1990) Starvation and penetration of bacteria in soils and rocks. Experientia 46:807–812

Loewen PC, Hengge-Aronis R (1994) The role of the σ-factor σ^s (katF) in bacterial global regulation. Ann Rev Microbiol 48: 53–80

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275

Matin A (1992) Physiology, molecular biology and applications of the bacterial starvation response. J Appl Bacteriol Symp [Suppl] 73:49S–57S

Mink RW, Patterson JA, Hespell RB.(1982) Changes in viability, cell composition, and enzyme levels during starvation of continuously cultured (ammonia-limited) *Selenomonas ruminantium*. Appl Environ Microbiol 44:913–922

Morita RY (1990) The starvation-survival state of microorganisms in nature and its relationship to bioavailable energy. Experientia 46:813–817

Moyer CL, Morita RY (1989) Effect of growth rate and starvation-survival on the viability and stability of psychrophilic marine bacterium. Appl Environ Microbiol 55:1122–1227

Mukamolova GV, Kaprelyants AS, Kell DB (1995) Secretion of an antibacterial factor during resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase. Antonie Van Leeuwenhoek 67:289–295

Oliver JD (1993) Formation of viable but nonculturable cells. In: Kjelleberg S (ed) Starvation in bacteria. Plenum Press, New York, pp 239–272

Ostrovsky DN, Bulgakova VG, Gukova IG, Kaprelyants AS, Rozancev EG, Simakova IM (1976) Changes in lipid-protein interactions in bacterial membranes induced by gramicidin S. Biochemistry (Russia) 41:175–181

Postgate JR (1976) Death in macrobes and microbes. Symp Soc Gen Microbiol 26:1–18

Roszak DB, Colwell RR (1987) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379

Siegele DA, Kolter R (1992) Life after log. J. Bacteriol. 174:345–348

Smigielski A, Wallace B, Marshall K (1989) Changes in membrane functions during short-term starvation of *Vibrio fluvialis* strain NCTC 11328. Arch Microbiol 151:336–341

Votyakova TV, Kaprelyants AS, Kell DB (1994) Influence of viable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in extended stationary phase. The population effect. Appl Environ Microbiol 60:3284–3291

Wanner U, Egli T (1990) Dynamics of microbial growth and cell composition in batch culture. FEMS Microbiol Rev 75:19–44