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On resuscitation from the dormant state of *Micrococcus luteus*

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Abstract

It has been found previously that a significant number of *Micrococcus luteus* cells starved in a prolonged stationary phase (up to 2 months) and then held on the bench at room temperature without agitation for periods of up to a further 2–7 months can be resuscitated in liquid media which contained (statistically) no initially-viable (colony-forming) cells but which were fortified with sterile supernatant from the late logarithmic phase of batch growth. Here it was found that such resuscitation can be done only within a defined time period after taking the first sample from such cultures, necessarily involving agitation of the cells. The duration of this period depends on the age of the starved culture: cells kept on the bench for 3 months possess a 2 month period of resuscitability while cells starved for 6 months can be resuscitated only within 10 days after the beginning of sampling. It is suggested that the input of oxygen to the starved cultures while they are agitated may exert a negative influence on the cells, since cultures stored in anaerobic conditions (under nitrogen) had a more prolonged 'survival' time. The cells which experienced between 10 and 60 days of starvation on the bench could be resuscitated, although the number of resuscitable cells depended strongly on the concentration of yeast extract in the resuscitation medium. This concentration for cells stored on the bench for more than 2 months was 0.05% while '1-month-old' cells displayed a maximum resuscitability in the presence of 0.01% of yeast extract. Application of the fluorescent probe propidium iodide revealed the formation of cells with a damaged permeability barrier if resuscitation was performed by using concentrations of yeast extract of 0.1% and above. Thus the successful resuscitation of bacterial cultures under laboratory conditions may need rather strictly defined parameters if it is to be successfully performed for the majority of cells in a population.

Introduction

The persistence of nonsporulating bacterial cells in 'non-culturable' or dormant forms is the subject of intensive discussions (Barer, 1997; Kell et al., 1998). Dormant cells are defined as metabolically inactive cells which often are not 'alive' in the sense of being able to form a colony when plated on a suitable solid medium but capable of making a transition to a culturable state by a procedure referred to as resuscitation (Kaprelyants et al., 1993). For many cases it was found that the starvation of bacterial (especially Gram-negative) cells led to cultures with low 'culturability' (for review see (Barer et al., 1993; Oliver 1993; Kell et al., 1998) although the evidence for any *reversibility* of this state is hard to come by (and we consider that demonstrating the possibility of a 'backward' transition from 'non-culturable' to culturable cells is a key issue if 'non-culturable' cells are to be considered as having a significant importance for microbiology). Indeed, many attempts to resuscitate potentially dormant bacteria have been undertaken, some claiming positive results (Allen-Austin et al., 1984; Nilsson et al., 1991; Oliver & Bockian, 1995; Wai et al., 1996; Bovill & Mackey, 1997; Kell et al., 1998), while further experiments disproved this conclusion when the

problem of the presence of a small number of 'initially viable' cells in the population before resuscitation had been taken into consideration by using heavily diluted cell cultures (Morgan et al., 1991; Rose et al., 1990; Weichart & Kjelleberg, 1996). The failure of resuscitation in these experiments might be due to inappropriate conditions chosen for the resuscitation of putative dormant bacteria (MacDonnell & Hood, 1982).

In our own studies we have found that cells of the nonsporulating, copiotrophic, Gram-positive bacterium *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 in cultures which overall exhibited a very low viability ($< 10^{-4}$, given as a ratio of cfu number: total count) (Kaprelyants & Kell, 1993; Kaprelyants et al., 1994; Mukamolova et al., 1995). Using flow cytometry with appropriate probes and conditions (Davey & Kell, 1996) we found that a high percentage of *M. luteus* cells in 3-month-old populations could be resuscitated to normal, colony-forming bacteria (Kaprelyants & Kell, 1993), under conditions which excluded any significant regrowth of initially viable, colony-forming cells. We confirmed this by exploiting the Most Probable Number (MPN) approach, and resuscitating cells in media which contained (statistically) no initially-viable cells but which were fortified with sterile supernatant from the late logarithmic phase of batch growth (Kaprelyants et al., 1994). From this, operationally, dormant cells are cfu-negative but (in the presence of spent medium) MPN-positive cells. It was concluded that viable and active cells of *M. luteus* can secrete a pheromone-like substance, which is apparently necessary (though not sufficient) for the resuscitation of starved, dormant cells of the same organism (Kaprelyants & Kell, 1996).

At the same time, the application of supernatants for resuscitation resulted in a rather broad variation of the final number of resuscitated cells of cultures with different 'age' (Kaprelyants et al., 1994), which might be taken to indicate the necessity of optimisation of the conditions for resuscitation of dormant cells in any particular culture.

The main goal of present study was thus to characterise in more detail the conditions for the successful production of dormant cells and their resuscitation. Consistent with the sometimes controversial nature of this phenomenon, we found that such conditions are rather strict for the particular organisms used. This finding might be of significance regarding the high number of uncultured organisms now widely recognised (McVeigh et al., 1996).

Materials and methods

Organism and media

Micrococcus luteus NCIMB 13267 (previously described as 'Fleming strain 2665') was grown aerobically at 30 °C in shake flasks (200 ml of culture in a 900ml flask) in lactate minimal medium (LMM) containing L-lactate as described previously (Kaprelyants & Kell, 1993). When the culture had reached stationary phase agitation was continued at 30 °C for up to 2 months. Cultures were then held under nominally aerobic conditions at room temperature without agitation for periods of up to a further 2–7 months. The taking of the first sample from particular cultures for cell resuscitation, necessarily involving agitation, was taken as the beginning of the experiment.

In some experiments, 2 ml aliquots of culture after their period of agitation at 30 °C were distributed into different test tubes (10ml) and kept under identical conditions on the bench. For sampling at one particular time of storage one tube was used. When necessary, cells were maintained in anaerobic conditions during storage after agitation at 30 °C by bubbling nitrogen through 3ml of culture for 10 min, and storing on the bench in 15 ml bottles sealed with a rubber seal. The sampling in this case was performed using a syringe. To obtain cultures with different cell densities stationary phase cells were centrifuged and resuspended in their spent medium to the desired concentration.

Each version of the above experiments was repeated 2–5 times; those shown in Results represent typical data.

Spent medium preparation

Supernatant was obtained after the centrifugation of late logarithmic phase *M. luteus* cultures grown in lactate minimal medium. The inoculum consisted of 2% (by volume) of a cell suspension grown in rich medium (Broth E, Lab M, Amersham) after washing them in lactate minimal medium lacking lactate. The supernatants were passed through a 0.22 μm filter (Whatman) before use.

Estimation of viable cell number by plating

Plates consisting of 1.3% Nutrient Broth E (Lab M) or lactate minimal medium plus 1.5% of agar (LabM Agar N1, Amersham) were used. Cell dilutions were made in quadruplicate with centrifuged and autoclaved

spent medium taken from the starved culture. 0.1 ml of an appropriate dilution was spread using a spatula. Plates were incubated at 30 °C for 3–5 d.

Cell resuscitation and estimation of viable cell number by MPN assay

The MPN assay was performed in a Bioscreen C optical growth analyser (Labsystems, Finland) using lactate minimal medium supplemented by 0.5% lactate and 0–0.1% of yeast extract as a resuscitation medium (Kaprelyants et al., 1996). Dilutions of starved cells were made as described (Kaprelyants et al., 1994). 5–10 μl of each dilution (5–10 replicates) were added to a well containing 200 μl of either lactate minimal medium supplemented by 0.5% lactate and 0.05% of yeast extract with supernatant (100 μl). A 600 nm filter was used to monitor the OD. Plates were incubated at 30 °C with intensive continuous shaking. The calculation of the MPN was based on published Tables (Meynell & Meynell 1965).

Total cell counts

Unstained cells were counted with a phase-contrast microscope and an improved Neubauer counting chamber (Kaprelyants & Kell 1992).

Propidium iodide staining

Unfixed cells were stained by propidium iodide with final concentration of 3.8μM for 2–5 min. For permeabilization cells before staining were treated with octanol (50μl/ml of a solution diluted in ethanol (1:10)) for 1–2 min. A Leica epifluorescent microscope (LEITZ DM RB) with an N2 filter block (excitation 530-560 nm, band stop 580, emission $>$ 580 nm) was used to monitor the fluorescence.

Chemicals

Nutrient Broth E, yeast extract and agar were obtained from Lab M, whilst L-lactate (Li salt) was obtained from Sigma. Propidium iodide was obtained from Molecular Probes. Other chemicals were of analytical grade and were obtained from Sigma or BDH.

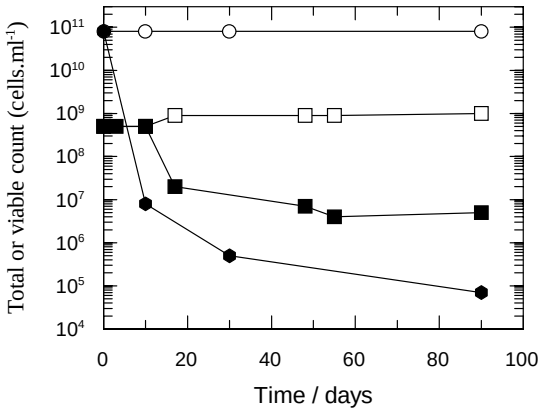

Figure 1. Changes in the content of colony-forming cells and in the total count during prolonged incubation under aeration of stationary-phase *M. luteus* having different initial cell concentrations. Stationary phase cells were concentrated by centrifugation and diluted in spent medium with final concentrations of 9.10^{10} cells ml^{-1}(circles) or 5.10^{8}cells ml^{-1}(squares). Open symbols- total count, closed symbols- cfu count. A typical experiment of two performed is shown.

Results and discussion

It was reported previously that prolonged incubation of *M. luteus* in spent growth medium resulted in a rapid decrease in the number of viable cells as judged by plating on agar plates while the total count was stable over several months of storage (Kaprelyants & Kell 1993). Here we found that the behaviour of the starving culture during a prolonged stationary phase depended on the initial cell concentration, an increase in which resulted in a more rapid and pronounced fall of cell viability (as judged by cfu) while a decrease had the opposite effect (Figure 1). Nevertheless, the total cell count remained almost unchanged during the period tested. Note that in these experiments diluted and concentrated cultures were subjected to starvation in the same spent medium obtained from the original culture (see Materials and methods).

Earlier we also found that supernatant (SN) taken from actively growing *M. luteus* in LMM promotes the resuscitation of starved bacteria in liquid medium as detected by an MPN assay (Kaprelyants et al., 1994; Votyakova et al., 1994). In a similar vein, Figure 2 shows that there are significant differences between the number of viable cells calculated on the basis of cfu (plates) and by MPN assay in the presence of supernatant (the MPN count performed in the absence of SN was very close to the cfu number). The difference between these two decreased with time after the start of sampling since the MPN count decreased while the cfu

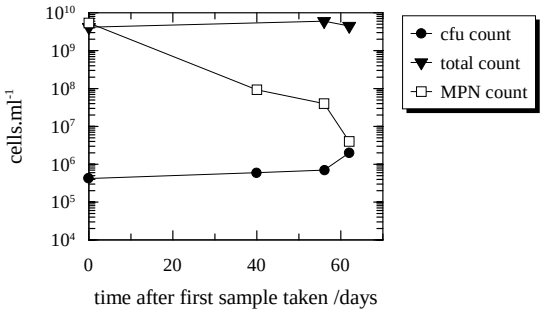

Figure 2. Changes in the total counts and the viable counts as judged by agar plates or by a Most Probable Number (MPN) method in a culture of *M. luteus* after 5 months of starvation in spent medium (2 months in an agitated flask and 3 months kept on the bench). For the MPN assay supernatant taken from a batch culture of the organism grown in LMM (plus 0.5% lactate) to an OD of 2, slightly before the beginning of stationary phase, was added (0.1ml of supernatant per well containing 0.1ml resuscitation medium and starved cells - see Materials and Methods). A typical experiment of 4 performed is shown.

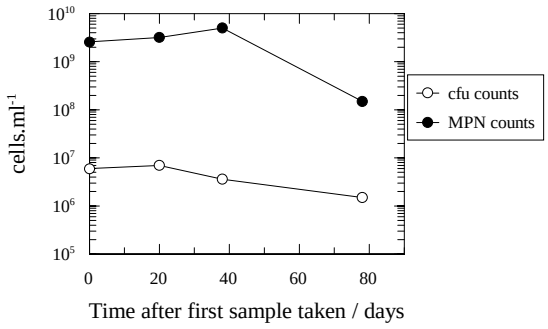

Figure 3. Changes in the total counts and the viable counts as judged by agar plates or by a Most Probable Number (MPN) method in a culture of *M. luteus* after 4 months of starvation in spent medium (2 months in agitated flask and 2 months kept on the bench). Before the period in which the culture was kept on the bench nitrogen was passed through the culture. The total count (3. 10^9cells.ml^{-1}) of the culture through the experiment was almost constant. For other details see the legend to Figure 2 and 'Materials and methods'. A typical experiment of 4 performed is shown.

number increased (Figure 2). At the beginning of sampling of this particular culture that had been kept on the bench there were almost 100% dormant (resuscitable) cells, whilst at 40 days the population contained only 2% of resuscitable cells. Figure 2 represents one particular *M. luteus* culture, although such behaviour was typical for the majority of many cultures starved for 3–9 months. To check if the decrease in the number of resuscitable cells depended on a possible oxygen supply after agitation of the flask we used cultures stored under nitrogen. Under these conditions an MPN assay revealed resuscitation even after 80 days of cell storage (Figure 3).

It was also found that in conditions similar to those of Figure 2 the time gap after the first sample was taken when the MPN assay could reveal that resuscitation had been successful (for 10–100% of cells in the population) depended on the duration over which the culture had previously been stored: cells kept on the bench for 3 months possess a 2 month period of resuscitability while cells starved for 6 months can be resuscitated only within 10 days after the beginning of sampling.

While the first sample taken from cultures with a storage time between 2–6 months demonstrated an almost constant and significant resuscitability, shorter periods of storage revealed a pronounced decrease in the number of resuscitable cells. To characterise in more detail the behaviour of cells between 0–2 months of storage experiments with nonagitated cultures were conducted (to this end aliquots of culture were distributed between different test tubes and kept in identical conditions on the bench until sampling) (Figure 4). Under these conditions only ca 1% of resuscitable cells were found at the onset of storage in the standard conditions used previously while after 45 days the MPN count was close to the total count (50–100% resuscitability). The viable count of the cells judged by plates was almost constant during the whole period (Figure 4), as was the total count (not shown). However 'young' cells (between 10–30 days) could be also resuscitated when a lower concentration of yeast extract (0.01%) was used in resuscitation medium while for more 'mature' cells (more than 40 days storage) this concentration was not appropriate.

In separate experiments 0.05% was found to be the optimal concentration of yeast extract for the successful resuscitation of dormant cells stored on the bench for more than 1.5 months (4×10^6, 2.6×10^9, 2.8×10^8resuscitable cells /ml by using 0.01%, 0.05% and 0.1% yeast extract respectively).

To study the behaviour of the cells during resuscitation in the presence of different concentrations of yeast extract the state of the permeability barrier of the cells was monitored by staining with propidium iodide (a DNA staining probe). We first established that this does not penetrate through the cytoplasmic membrane of intact or starved *M. luteus* and that octanol administration to the cell suspension (0.5%) resulted in 100% stained cells (see also (Humphreys et al., 1994; Lloyd & Hayes, 1995)).

Figure 5 clearly shows that there is a significant increase in the proportion of cells with a damaged per-

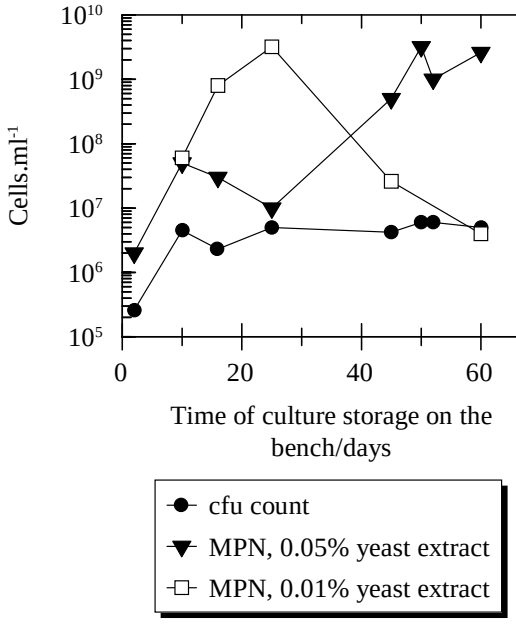

Figure 4. Effect of yeast extract concentration in the resuscitation medium on the resuscitation of starved *M. luteus* of different 'age'. Starved cultures were prepared as shown in Figure 4 while before starvation on the bench aliquots of culture were distributed through different test tubes and kept in identical conditions on the bench until sampling. Periodically the culture was checked for viable count /by plates and MPN assay (as described in the legend to Figure 2). Each point on the graph represents one test tube with nonagitated culture. A typical experiment of 5 performed is shown.

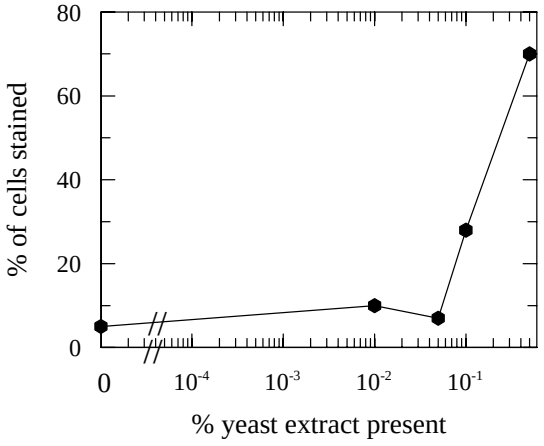

Figure 5. Effect of the yeast extract concentration in the resuscitation medium on the percentage of *M. luteus* cells stainable by propidium iodide after 7 hours incubation in fresh medium. The 2 month-starved cells were diluted 20-fold and incubated in fresh lactate minimal medium with different concentration of yeast extract. Cells were stained by propidium iodide and studied under the fluorescence microscope. A typical experiment of 3 performed is shown.

meability barrier after 7 hours of incubation of dormant cells in lactate medium supplemented by yeast extract when the latter is at a concentration above 0.05%. After 10–15 hours of incubation of these cells in media with any concentration of yeast extract the initially viable cells in the culture started to multiply, which did not allow us further to monitor dormant cells under these conditions. 90% of cells in media without any yeast extract became stainable cells after 25 hours of incubation.

The decrease in cfu count of *M. luteus* during a prolonged stationary phase under conditions in which the total count was almost constant (Figure 1) was found to reflect a *transition* of viable and active bacteria to cells which were dormant and apparently non-culturable (until resuscitation) (Kaprelyants & Kell, 1993; Kaprelyants et al., 1994). In contrast to the well known phenomenon in which the rate of decrease of the cfu count of copiotrophic bacteria under starvation conditions is lower in more dense populations (Harrison, 1960; Postgate & Hunter, 1963), which could be explained in part by cryptic growth (Mason et al., 1986; Postgate, 1967), in this study varying the cell concentration in the culture at the beginning of starvation showed that the rate and the extent of the decrease in cfu counts are maximal in more dense cultures (Figure 1). It may be assumed that a loss of culturability of the remaining cells in the population during starvation in a stationary phase could be promoted by the secretion of some substances from the same cells, or by the more rapid creation of anaerobic (or microaerophilic) conditions in the denser populations (see later).

In this study it was found that the conditions in which flasks with dormant bacteria are stored is a very important factor for the maintenance of their dormancy. The decrease in the number of resuscitable cells and the increase of the number of dead cells after the flask was agitated (Figure 2) may simultaneously reflect the beginning of attempted cell resuscitation (due to presence of viable and active cells which could stimulate resuscitation (Votyakova et al., 1994)), and cell lysis and cryptic growth (see Mason et al., 1986). This activation of the cell population in the flask could be due to oxygen input to the bacteria during sampling because anaerobic cells stored under nitrogen revealed a longer maintenance of resuscitability (Figure 3) since the absence of oxygen concentration will have suppressed any metabolic processes in the strictly respiratory *M. luteus.* It is of course also possible that oxygen *per se* can be inimical to dormant cells

of an organism which is normally resistant to oxygen. In particular *M. luteus* cells have a significant amount of catalase (Dhaese, 1996) and the recently discovered cyclopyrophosphate (Ostrovsky, 1995) which is supposed to serve as an oxygen stress defence system and which could be damaged during prolonged starvation. The effect of the 'age' of starved cultures of *M. luteus* on the period of successful resuscitation of the cells after the first sample was taken from the flask may also reflect a decrease of cell resistance to oxygen with their 'starvation age'. At all events, microaerophilic conditions which presumably are set up in unagitated dense culture (when some cells in the starved population remain active) must be important for the maintenance of the dormant state of the cells in the population. Indeed, it is worth noting that Wayne found that microaerophilic conditions are particularly appropriate for the formation of dormant forms of *M. tuberculosis* under laboratory conditions (Wayne, 1994). Recently Wayne and Hayes reported on the necessity of keeping *M. tuberculosis* in microaerophilic conditions for a sufficient time before they would effect a transition to a nonreplicating (and purportedly dormant) stage, in which they could further survive anaerobiosis (Wayne & Hayes, 1996). The evident similarity between the protocol adopted for obtaining such *M. tuberculosis* and our 2-stage procedure for *M. luteus* starvation in a prolonged stationary phase allows us to suggest that it may be that oxygen tension may play an additional role in determining the transition of *M. luteus* to a dormant state.

The other important condition for successful resuscitation of dormant cells is the composition of the resuscitation medium. As did McDonell and Hood (recovery of *Vibrio, Aeromonas, Pseudomonas* and *Alcaligenes* spp. (MacDonell & Hood, 1982)), we found a very narrow concentration range of yeast extract which would permit maximum resuscitation. Moreover, the optimal concentration of yeast extract varied with the age of the culture (Figure 4). Study of the cell permeability barrier using propidium iodide (Figure 5) showed that elevated concentrations of yeast extract induced a permeabilization of the cell membrane of 2-months stored cells during their resuscitation. Earlier it was reported that the usage of rich medium for the resuscitation of *M. luteus* resulted in cell lysis and prevented the restoration of the permeability barrier judged from flow cytometry and the DNA-staining probe PO-PRO-3 (Kaprelyants et al. 1996). (In contrast to propidium iodide PO-PRO-3 demonstrated an injured barrier in almost all cells in the starved population before resuscitation (Kaprelyants et al., 1996), similar to that found for NADH (Mukamolova et al., 1995), although it is not possible to exclude the contamination of PO-PRO-3 by a membrane-permeant contaminant.) Taken together these findings demonstrate that the term 'membrane permeability barrier', like 'viability' (Davey & Kell, 1996; Kell et al., 1998), is rather arbitrary, and depends on the degree of injury: a membrane could be intact if tested by one particular probe but injured and capable (or incapable) of repair if tested by another.

We would suggest that a successful exit from dormancy (and thus by definition its demonstration) needs rather strict conditions, since cells in the initial phase of resuscitation possess an unbalanced metabolism and are very sensitive to an excess of substrates ('nutrient shock' (Jensen et al., 1996)). Figure 4 also shows that the formation of dormant cells continues for a long time of storage of unagitated cell cultures at room temperature on the bench, the different optimum of yeast extract concentration with different culture age presumably reflecting different depths of dormancy. Thus these results, in turn, clearly show that the application of a single set of conditions is not enough for the correct monitoring of the number of potentially culturable cells in an environmental sample.

Finally, we would like to stress that both entry into the dormant state and exit from it (resuscitation) for bacterial cultures under laboratory conditions need strictly defined parameters if they are to be effected successfully for the majority of cells in the population. Indeed, it is likely that such conditions are different for each microorganism.

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References

Allen-Austin D, Austin B & Colwell RR (1984) Survival of *Aeromonas salmonicid*a in river water. FEMS Microbiol. Lett. 21: 143–146

Barer MR (1997) Viable but non-culturable and dormant bacteria: time to resolve an oxymoron and a misnomer? J. Med. Microbiol. 46: 629–631

Barer MR, Gribbon LT, Harwood CR & Nwoguh CE (1993) The viable but non-culturable hypothesis and medical microbiology. Rev. Med. Microbiol. 4: 183–191

Bovill RA & Mackey BM (1997) Resuscitation of 'non-culturable' cells from aged cultures of *Campylobacter jejuni*. Microbiology 143: 1575–1581

Davey HM & Kell DB (1996) Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single cell analysis. Microbiol. Rev. 60: 641–696

Dhaese P (1996) Catalase - an enzyme with growing industrial potential. Chimica Oggi-Chemistry Today. 14: 19–21

Harrison AP (1960) The response of *Bacterium lactis aerogenes* when held at growth temperatures in the absence of nutrient: an analysis of survival curves. Proc. Roy. Soc., B. 152: 418–428

Humphreys MJ, Allman R & Lloyd D (1994) Determination of the viability of *Trichomonas vaginalis* using flow cytometry. Cytometry 15: 343–348

Jensen PR, Kauffman CA, & Fenical W (1996) High recovery of culturable bacteria from the surfaces of marine algae. Marine Biology 126: 1–7

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

— (1993) Dormancy in stationary-phase cultures of *Micrococcus luteus* - flow cytometric analysis of starvation and resuscitation. Appl. Environm. Microbiol. 59: 3187–3196

— (1996) Do bacteria need to communicate with each other for growth? Trends in Microbiology 4: 237–242

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

Kaprelyants AS, Mukamolova GV, Davey HM & Kell DB (1996) Quantitative-analysis of the physiological heterogeneity within starved cultures of *Micrococcus luteus* by flow-cytometry and cell sorting. Appl. Environm. Microbiol. 62: 1311–1316

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

Kell DB, Kaprelyants AS, Weichart DH, Harwood CR & Barer MR (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie van Leeuwenhoek, in the press

Lloyd D & Hayes AJ (1995) Vigour, vitality and viability of microorganisms. FEMS Microbiol. Lett. 133: 1–7

MacDonell MT & Hood HM (1982) Isolation and characterization of ultramicrobacteria from a gulf coast estuary. Appl. Environ. Microbiol. 43: 566–571

Mason CA, Hamer G & Bryers JD (1986) The death and lysis of microorganisms in environmental processes. FEMS Microbiol. Rev. 39: 373–401

McVeigh HP, Munro, J & Embley TM (1996) Molecular evidence for the presence of novel actinomycete lineages in a temperate forest soil. J. Ind. Microbiol. 17: 197–204

Meynell GG & Meynell E (1965) Theory and practice in experimental bacteriology. University Press, Cambridge

Morgan JA, Cranwell PA & Pickup R (1991) Survival of *Aeromonas salmonicida* in lake water. Appl. Environ. Microbiol. 57: 1777–1782

Mukamolova GV, Kormer SS, Yanopolskaya ND & Kaprelyants AS (1995) Properties of dormant cells in stationary-phase cultures of *M. luteus* during prolonged incubation. Microbiology (Russian) 64: 284–288

Mukamolova GV, Yanopolskaya ND, Votyakova TV, Popov VI, Kaprelyants AS & Kell DB (1995) Biochemical changes accompanying the long-term starvation of *Micrococcus luteus* cells in spent growth medium. Arch. Microbiol. 163: 373–379

Nilsson L, Oliver JD & Kjelleberg S (1991) Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. J Bacteriol. 173: 5054–5059

Ostrovsky DN (1995) Oxidative stress and organic cyclopyrophosphate in bacteria. Biochemistry (Russian) 60: 9–12

Oliver JD (ed.) (1993) Formation of viable but not culturable cells. Plenum Press, New York, London

Oliver JD & Bockian R (1995). *In vivo* resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Appl. Environ. Microbiol. 61: 2620–2623

Postgate JR (1967) Viability measurements and the survival of microbes under minimum stress, In: Rose AH & Wilkinson JF (Eds) Advances in Microbial Physiology, vol. 1. (pp 1–21.) Academic Press, London

Postgate JR & Hunter JR (1963) The survival of starved bacteria. J. Appl. Bacteriol. 26: 295–306

Rose AS, Ellis AE & Munro A (1990) Evidence against dormancy in the bacterial fish pathogen *Aeromonas– salmonicida* subsp *salmonicida*. FEMS Microbiol. Lett. 68: 105–107

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

Wai SN, Moriya T, Kondo K, Misumi H & Amako K (1996) Resuscitation of *Vibrio cholerae* O1 strain tsi4 from a viable but nonculturable state by heat shock. FEMS Microbiol. Lett. 136: 187–191

Wayne LG (1994) Dormancy of *Mycobacterium tuberculosis* and latency of disease. Eur. J. Clin. Microbiol. & Infec. Dis. 13: 908–914

Wayne LG & Hayes LG (1996) An in *vitro* model for sequential study of shiftdown of *Mycobacterium tuberculosis* through 2 stages of nonreplicating persistence. Infect Immun 64: 2062–2069

Weichart D & Kjelleberg S (1996) Stress resistance and recovery potential of culturable and viable but nonculturable cells of *Vibrio vulnificus*. Microbiology 142: 845–853