FEMS Microbiology Reviews 104 (1993) 271–286

Published by Elsevier

FEMSRE 00275

Dormancy in non-sporulating bacteria

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Received 7 September 1992
Revision received 24 October 1992
Accepted 26 October 1992

Key words: Starvation-survival; Viability; Culturability; Ultramicrobacteria; Cryptobiosis

"All stood amazed, until an old woman, tottering out from among the crowd, put her hand to her brow, and peering under it in his face for a moment exclaimed, 'Sure enough! it is Rip Van Winkle — it is himself. Welcome home again, old neighbour — Why, where have you been these twenty long years?'"

Irving, W. (1899) Rip Van Winkle, In: The Sketchbook, p. 39, Desmond Publishing, Boston.

"Time flows because metabolism flows."

Welch, G.R. (1992) Progr. Biophys. Mol. Biol. 57, 71–128, An analogical "field" construct in cellular biophysics: history and present status.

"These germs — these bacilli — are transparent bodies. Like glass. Like water. To make them visible you must stain them. Well, my dear Paddy, do what you will, some of them won't stain; they won't take cochineal, they won't take any methylene blue, they won't take gentian violet, they won't take any colouring matter. Consequently, though we know as scientific men that they exist, we cannot see them."

Sir Ralph Bloomfield-Bonington. The Doctor's Dilemma. George Bernard Shaw.

1. INTRODUCTION

The environment(s) of bacteria in nature are generally very different from those of the laboratory, and oligotrophic conditions are more the rule than the exception. To survive such nutritionally unfavourable conditions, bacteria must adapt their metabolism to a less profligate way of life than that adopted in conditions of nutrient excess [1]; this would suggest that in the case of complete exhaustion of exogenous nutrients they can go into an anabiotic (dormant) state, which helps the cells to survive for a long time without growth and multiplication. By 'dormancy' we mean a reversible state of low metabolic activity, in which cells can persist for extended periods without division; we shall see that this often cor-

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responds to a state in which cells are not 'alive' in the sense of being able to form a colony when plated on a suitable solid medium, but one in which they are not 'dead' in that when conditions are more favourable they can revert to a state of 'aliveness' as so defined.

Until recently, dormancy has mainly been connected with bacterial forms which are obviously morphologically specialized, viz. spores and cysts ('constitutive dormancy'; see [2]), structures which can be formed by only a limited number of bacterial species. However, there has recently been much discussion with regard to the possible existence of dormant states of vegetative, non-sporulating bacteria. Parenthetically, we may draw attention to the very well-documented existence of cognate phenomena of dormancy as studied by plant physiologists [3,4]; indeed, Lang et al. [5] have listed no less than 54 terms that have been used to describe dormancy, the most common of which were 'rest' and 'quiescence'. Hibernation in animals [6] may also be considered to be a form of dormancy, whilst Bell [7] discusses in detail the problem of whether protozoans 'die', and what that means. Watson [8] reviews the many difficulties that have been experienced in deciding whether even humans are 'alive' or 'dead'. Notwithstanding, and whilst much of what we have to say in general is likely to be true of a variety of other microorganisms, including yeast, algae and fungi, the chief aims of this short review are (i) critically to consider the main evidence which suggests the prevalence of such 'exogenous dormancy' [2] amongst vegetative, non-sporulating bacteria, (ii) to review some recent progress in analysing such dormant states, and (iii) to point up areas of present ignorance, and the significance of dormancy in applied microbiology and biotechnology.

2. DORMANCY IN NATURAL AND ARTIFICIAL ENVIRONMENTS

The basic argument that is often used in favour of the widespread existence of dormant forms of vegetative, non-sporulating bacteria is that "in most natural microbial environments only a very small fraction of the microbes present can be enumerated using agar plate techniques" [9]. This discrepancy (between 'viable' and total counts) has been found for bacteria in soil [10,11] and water [11,12] environments, and may be obtained in laboratory conditions during progressive starvation of Gram-negative bacteria [13–15]. Bacteria in such conditions often form ultramicrobacteria (in Morita's terminology [13]), which have also been found in natural oligotrophic habitats [10,11,16]. It was suggested that these ultramicrobacteria (or dwarf cells) represented dormant forms of non-sporulating bacteria, which formed as a result of the adaptation of the culture to the low substrate concentrations, and which were not able to produce colonies on the agar surface [11,17].

Another example of 'vegetative dormancy' is the formation of 'viable but non culturable' (VBNC) forms of some Gram-negative bacteria [18]. These forms are not revealed by simple agar plating and can be monitored in a starved bacterial population by their metabolic activity as demonstrated via the so-called direct viable count technique [19] of Kogure et al. [20].

The biochemical characteristics of dormant cells, and the mechanism(s) of the transition of vegetative cells from active into dormant states, are as yet little understood. However, there is evidence that the transition is itself an active metabolic process which needs protein synthesis [21,22] (the formation of 'starvation proteins' [23,24] and see later), and (for aerobes) an active respiratory chain and energised membrane [25]. Further information on these forms is available elsewhere [11–13,18,26].

In fact, the evidence that ultramicrobacteria and VBNC cells are dormant is presently rather ambiguous, since dormant bacteria are in states that are intermediate between what we normally consider 'alive' and 'dead' (see above, and [27,28]). By definition, the dormant state must be reversible, i.e. dormant cells are potentially 'alive'. Discrimination between viable and dead microorganisms is of course a (the?) fundamental problem of microbiology. It seems that the most traditional, commonly used and biologically reasonable approach is to follow the ability of bacterial

cells to multiply, using appropriate liquid or solid media [27]. This criterion seems to be the only true 'benchmark' for assessing the living microbial load in a sample, in spite of the many indirect (but faster) approaches proposed [12,29,30]. Thus, to consider a bacterium as being in the dormant state one has to prove that the cell of interest cannot grow under the present conditions of incubation but is able to produce daughter cells under appropriate conditions. Let us consider the problem of dormancy from this point of view, using the available (but very limited) experimental data.

2.1. Ultramicrobacteria

According to Bakken and Olsen [10] only a limited fraction of the cells in a given population of ultramicrobacteria isolated from soil are capable of forming colonies on agar. However, the resuscitation of some of these bacterial forms on a liquid medium or diluted nutrient agar enables their transition to normal bacteria [13,24,31]. MacDonell and Hood [32] described a recovery method for ultramicrobacteria from estuarine waters, which included the use of a dilute nutrient broth (where it may be noted that the concentration range of nutrients which allowed cells to grow was rather narrow). Cell recovery and adaptation to nutrient-rich media were accompanied by a pronounced increase in cell size (from 0.5 to 2–2.5 μm) [32]. However, this study did not clarify what percentage of the initial forms were able to recover during resuscitation in liquid media to produce colonies on the agar surface. It may well be that only a small proportion of the ultramicrobacteria (with some specific properties) were responsible for the growth observed. In this regard, it has been shown that a population of ultramicrobacteria from sea water consisted of two cell types, which could be converted either to normal vegetative cells giving macrocolonies on plates or to ultramicrocolonies which were not able to multiply further [33].

A rather better-designed recovery experiment was carried out with starved marine bacteria (*Vibrio* sp.) in glucose-containing liquid media [34]. In this study, the total cell number, average cell volume, and respiratory and biosynthetic activities were monitored during the recovery process. An increase in average cell volume from 0.5 μm^3 to 3–4 μm^3 was demonstrated under conditions of constant cell number (and see also [35]), which effectively excluded the possibility of multiplication of normal (viable) bacteria in the starved population. However, the starved cells in this study had 100% viability even after 200 h starvation (notwithstanding the decreased cell volume). It was stressed that these cells could not be classified as dormant because of the preservation of pronounced metabolic activity [34].

Morita concluded that most ultramicrobacteria are not able to multiply, but possibly because the proper conditions for their resuscitation had not been found [11]. However, according to an opposite view, ultramicrobacteria (or at least most of them) are not small forms of the more 'usual' cells but are cells of novel genera and species which happen to be very small (and thus they can not be reverted to 'normal' cells even in principle) [10]. In other words, the question is whether ultramicrobacteria are small forms of normal bacteria or normal forms of small bacteria (or of course both). Very recently, there has been some significant progress in the analysis of this question.

Schut et al. [36] noted that bacteria represent a substantial biomass component in oligotrophic environments [37], and that many methods have been used to estimate the activity of marine bacteria in situ. These methods have included assays of amino acid uptake [38], protein synthesis [39], RNA synthesis [40], electron transport activity [38,41] and direct viable counts [42]. Whilst the existence of these activities is not obviously consistent with the idea that such bacteria are dormant, the organisms responsible for these activities have yet to be identified. To this end, Schut et al. [36] studied seawater taken from Resurrection Bay (near Seward, Alaska) and from the North Sea off the Dutch coast, and used 0.2-μm-filtered samples of this seawater that were subsequently autoclaved to enrich dominant heterotrophic marine bacteria by diluting the inocula to extinction. The purpose of this protocol was to avoid the possibility that an originally non-dominant but eutrophic organism could outcompete

the putatively dominant but oligotrophic organism(s) of interest. Using this protocol, Schut et al. [36] established that the typical organism which developed from the highest dilutions under these conditions (in over 50% of the cases) was a small, straight rod with a cell volume of some 0.06 μm^3, and a DNA content of approx. 1.5 fg/cell (some 38% that of the *E. coli* genome), i.e. an ultramicrobacterium. It could be persuaded to form smooth, translucent, yellow-pigmented colonies (diameter approx. 0.5 mm) after incubation on ZoBell agar for 6 weeks, and was obligately (rather than facultatively) oligotrophic upon first cultivation. Its viability in the original samples appeared to be as much as two orders of magnitude greater when judged from the dilution-to-extinction method than when judged by agar plating techniques. Schut and colleagues concluded, in contrast to earlier results of Moyer and Morita [43], that bacteria with such low cell volumes and DNA contents were not necessarily exhibiting a starvation–survival response but may be actively growing bacteria. On this basis, such bacteria are therefore not to be considered dormant.

2.2. Viable but non-culturable cells (VBNC cells)

Colwell et al. [18] introduced the term "viable but non-culturable cells" to describe particular cells which appeared in populations of some Gram-negative bacteria (*E. coli, Salmonella* and *Vibrio* spp.) starved in aquatic environments [18]. As described above for ultramicrobacteria, VBNC cells are usually smaller than the cells from which they are produced [19], and of course it has long been known that cell volume is approximately linear with growth rate in chemostat cultures ([44], and see later). However, in the case of *E. coli*, sometimes the cells did not change size, and appeared intact [15,45]. These cells could not form colonies on solid media but displayed metabolic activity [15] and the ability to elongate [12,18] after the administration of nutrients. It should be stressed that the number of VBNC cells in starved population was *very* much lower (several orders of magnitude) than the total cell count estimated with dyes such as Acridine orange (e.g. [18,46]). (Recently the same behavior was reported for some Gram-positive bacteria [47].

A number of studies have indicated that the VBNC cells can start to multiply on solid media after the application of a resuscitation procedure. Colwell and colleagues used the passage of VBNC of Gram-negative cells through an animal ileal loop for resuscitation [18]. However, in contrast to previous data and conclusions of Allen-Austin et al. [48] from experiments with *A. salmonicida*, and as pointed out by Morgan et al. [49] and Rose et al. [50] on the basis of cognate experiments, it is possible that a limited number of intact cells in the population could have been responsible for the growth that occurred in the ileal loop experiment. Nonetheless, the failure to observe true resuscitation of dormant *A. salmonicida* [49,50] may be due to the inability of these authors to find the appropriate nutritional conditions. To resuscitate starved *S. enteritidis*, the cells were incubated in liquid nutrient media prior to plating out. In the course of the resuscitation of cells that had been starved for 4 days, a stepwise increase in the number of colonies that formed on agar was demonstrated [46]; this could have been interpreted as VBNC cell recovery if data on the total count had been presented as well. In this experiment such a recovery could not be observed if cells were held in a non-culturable state (starved) for a longer time (21 days), despite the fact that they still possessed metabolic activity (the capability to elongate in the presence of the antibiotic nalidixic acid) [19]. The recovery of such cells could nevertheless be effected using incubation in the ileal loop [19].

In perhaps the most persuasive and detailed paper on the resuscitation of starved *Vibrio vulnificus*, recently published by Nilsson et al. [51], a temperature shift was applied to effect cell recovery. These bacteria were starved at 5°C, and exhibited a time-dependent loss in viability on agar plates, under conditions of a constant total number of cells. The viability could be completely restored by incubation of the culture at room temperature in salt media without any nutrition. Transitions from rods (normal cells) to small cocci (the end of starvation) and vice versa (during resuscitation) were observed. Careful monitoring

of the total cell counts, viable counts (measured on agar plates) and microscopic images in the course of the resuscitation demonstrated definitively that the cells were really in a dormant state [51]. It is of interest to note that the resuscitation was shown to be an active metabolic process, which needed protein synthesis [51] ('resuscitation' proteins?). However, at least six other *Vibrio* spp. did not display such behaviour [52].

Parenthetically, it is worth pointing out that the phenomenon of dormancy can be of great significance in medical microbiology, since VBNC cells are potential reservoirs of infection, even following a course of antibiotic treatment.

3. DORMANCY IN LABORATORY CULTURES

3.1. Dormancy in chemostats

If one accepts that non-sporulating bacteria in oligotrophic conditions in nature adopt dormant forms, it is reasonable to envisage the same in chemostat cultures of such bacteria at low dilution rates. It is well established that the steady-state viability of bacteria is lowered at decreased dilution rates, under both carbon and nitrogen limitation, as judged by direct count of viable bacteria on agar plates [53–55]. A stepwise drop of the viability of *C. johnsonae* was reported after prolonged cultivation in chemostats at both high dilution rate (after 120 generations) and low dilution rate (after 10 generations). It could be calculated that if 90% of the cells in the chemostat were dead, the dividing population would have to grow with a doubling time faster than 1 h in a chemostat with low overall dilution rate. Since this is very unlikely, the author proposed that 90% of the cells in his chemostat had lost the ability to grow on agar plates "without losing [their] viability or metabolic activity in the continuous culture" [55]. The term viability as used here is not correct (since some of the cells obviously *had* lost the ability to form colonies). However, it is plausible that the loss of the viability that does occur under conditions of prolonged cultivation in chemostats can be related to the shock of sudden nutrient abundance in the plating medium [56,57]. Such a phenomenon, known as substrate-accelerated death, can occur when the substrate that limited the growth of a population accelerated its death when added to starved cells [57].

It is necessary to stress that the adaptation of cell metabolism under continuous cultivation at low dilution rates was shown to be an active process, which results in both biochemical and morphological changes [53,54,56]. At dilution rates of less than 0.05 h^{-1} the mean size of *Aerobacter aerogenes* cells was decreased and the culture became morphologically heterogeneous (it contained both long cells and small cocci) [53]. A decrease in diameter with decreasing D is evidently common to many bacteria, and has been reviewed in extenso by Gottschal [31], although the differentiation of the culture into two (or more) forms is consistent with the view that the larger cells are growing (if not dividing) more rapidly than the smaller ones, and if unbalanced growth occurs might even become bigger than those in cultures whose overall (macroscopic) dilution rate may be higher. *S. cerevisiae* cells grown under glucose limitation in chemostat culture at low growth rates adopted two forms (phase-bright and phase-dark), both with sizes that were small relative to those observed at high growth rates. The phase-bright cells were unable to multiply whilst the phase-dark ones were apparently capable of multiplying normally. It was proposed that the cell population was differentiated under these conditions, and formed cells which had stopped in the G1 phase of the cell cycle, similar to the behaviour of cells in the stationary phase grown in batch culture [58]. It is possible that such cells were dormant, but the reversibility of the process was not examined. Of course the induction of bacterial sporulation at low growth rates is also well known [59].

Study of the behaviour of bacterial cells growing at very low dilution rates (let us say lower than 0.01 h^{-1} for typical laboratory strains) in steady-state chemostat culture is beset with technical difficulties (since five volume changes at such a dilution rate require 25 days). Using a recycling fermentor, which allows one to study very slowly growing cells, it was found that *E. coli*

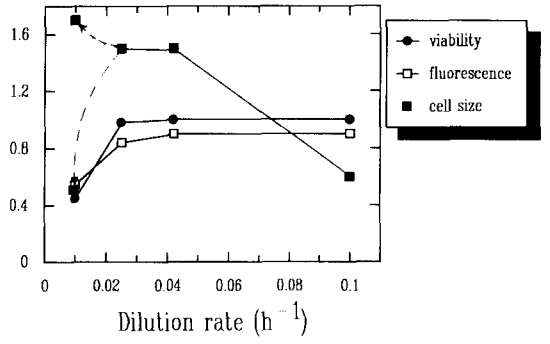

Fig. 1. Effect of dilution rate on the viability (by plating), mean size (by flow cytometry) and fraction of M. luteus cells that accumulated rhodamine 123 beyond channel 85 in a flow cytometric experiment. The experiment was performed, with a rhodamine 123 concentration of 0.3 μM, exactly as described elsewhere [35]. The ordinate represents the fractional viability, the fraction of cells whose fluorescence was in a channel number above 85, or the equivalent cell diameter in μm. For further discussion, see the text.

and *Paracoccus denitrificans* cells with doubling time corresponding to a dilution rate lower 0.014 h^{-1} manifested the transition associated with the stringent response (accumulation of ppGpp) [60]. Evidently this is somehow related to the initial phase of starvation mentioned below. Unfortunately, the lack of viability data in these experiments does not allow one to establish whether cells in a dormant state are accumulated in the recycling fermenter under these conditions. Since the effective starvation time in the recycling fermentor is rather small, it is at least plausible that the well-known loss in viability (as judged by plate counts [53,61]) that occurs in true chemostats at low dilution rates will not be mirrored in the recycling fermentor.

In order to describe more adequately the behaviour of chemostat cultures at very low dilution rates, Pirt assumed that a certain fraction of the cells were in a dormant state [62]. However, Mason et al. [63] describe chemostat cultures in terms of the existence of alive, dead and non-viable but metabolically active bacteria.

Using flow cytometric light scattering, and rhodamine 123 as a probe sensitive to membrane energization [35,64], we studied the dependence of the size of *M. luteus* cells on their dilution rate in lactate-limited chemostat culture. As the dilution rate was decreased from 0.1 h^{-1} to 0.025 h^{-1} the culture consisted of a single population with a viability of approx. 90%, but the mean cell size actually increased (Fig. 1). A further decrease of D (to 0.01 h^{-1}) resulted in a greatly increased heterogeneity of the culture such that it consisted of two populations representing very big and very small cells (Fig. 1). The steady-state viability of this culture was now only some 40%, virtually all of the viable cells being represented by the larger cells, which were also the population which was the most effective in accumulating rhodamine 123. In fact this approach allowed us to discriminate viable, dead and dormant cells. The latter population was revealed via resuscitation of a fraction of the small cells, accompanied by an increase of membrane energization, an increase

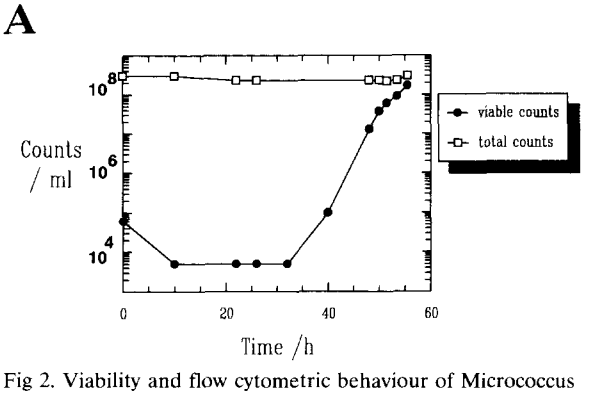

Fig 2. Viability and flow cytometric behaviour of Micrococcus luteus during resuscitation after starvation following batch culture. M. luteus was grown to stationary phase in a lactate minimal medium [35], then starved (without washing) for 2 months. By this stage, the apparent viability as judged by plate counts was only approximately 10^{-5} (although the total counts of bacteria in the starved population were not changed significantly). The cells were then washed, inoculated into fresh growth medium ('time zero') and incubated for 10 h in the presence of penicillin, G. The cells were washed in growth medium lacking lactate, and further incubated in growth lactate minimal medium containing 0.05% yeast extract, in the absence of penicillin. Flow cytometric measurements were performed, with a rhodamine 123 concentration of 0.3 μM, as described previously [35,64,85]. **A.** Viable (by plating) and total (microscopic) counts. **B.** Flow cytometric behaviour of cells at the beginning of the resuscitation period (after penicillin treatment). Most cells are in one population of small cells with a low fluorescence (rhodamine uptake). **C.** Flow cytometry of cells after 56 h of resuscitation. It is evident that there is now a population of larger cells with higher fluorescence.

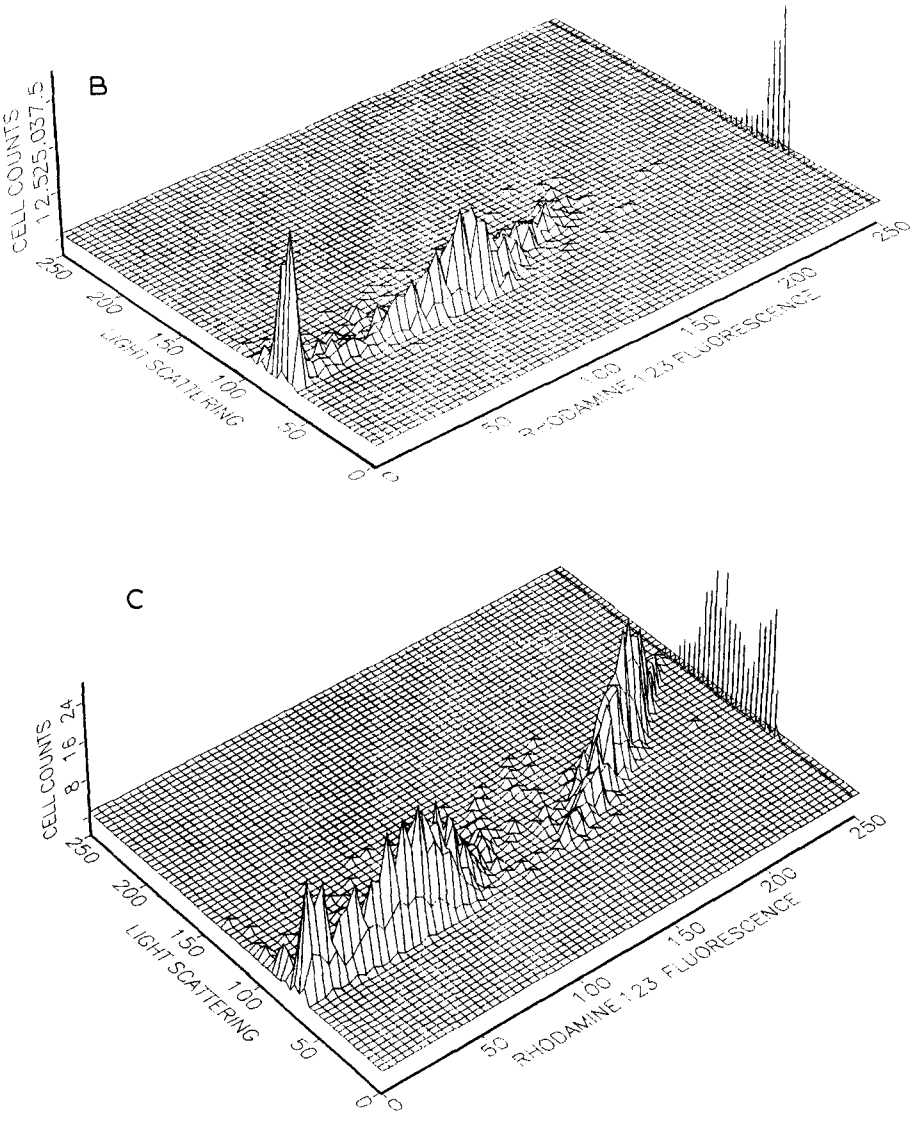

Fig. 2. (continued).

of the mean cell size, and a comparable increase in viability, under conditions of a constant total cell count during the resuscitation process. In this case, the viability of *M. luteus* cells became 80–90% at the end of resuscitation [35].

3.2. Dormancy in 'old' bacterial cultures ('death phase'?)

The transition of cells from the log phase of a batch culture to the stationary phase is usually due, or attributed, to the exhaustion of growth substrate(s). On this basis, it is very likely that cells that have spent a long time in stationary phase, when this is induced by the exhaustion of appropriate nutrients, just as those subjected to 'true' starvation (when cells are washed and re-suspended in nutrient-free buffer or are grown in chemostat culture with known limiting factor(s)), would adapt their metabolism to this perhaps more familiar type of starvation. Indeed, Postgate

[64a] showed that the dependence on growth rate of the viability of chemostat cultures of *Klebsiella aerogenes* was profoundly influenced by the nature of the growth-limiting nutrient, decreasing at significantly greater growth rates for Mg^{2+}- or glycerol-limited cultures than for those limited by ammonium or phosphate. The textbook view of events would have it that in batch culture a relatively brief stationary phase is followed by a 'death' phase, in which the number of viable cells decreases with time. The question arises, however, as to whether bacteria are really dying in the 'death' phase. When viability is measured by plating, it may be rapidly decreased to a few percent from an initially high level, a number which is then typically maintained for many days more [57]. Notwithstanding, it is often unclear as to whether this viability is calculated on the basis of the total counts *at the beginning* of the death phase or for each measurement point *during* the death phase. It has been known for many years that some morphological changes occur in old bacterial cultures [57]; just as during the production of VBNC, cells in stationary phase become much smaller and almost spherical [65].

Kolter and co-workers [66] recently reported some interesting results from the study of old *E. coli* cultures. Special (*sur*) genes were discovered, the expression of which in stationary phase resulted in prolonged preservation of the ability of bacteria to survive in both stationary and *post*-stationary phases. Radiolabelling of old *E. coli* cells demonstrated the incorporation of label into specific proteins. One of these proteins (20 kDa) was cloned and was shown in vitro to form heat-stable complexes with DNA, in which the DNA was protected from nuclease-mediated hydrolysis. Whilst population effects (i.e. *favourable* cell–cell interactions) on survival during starvation have been known for a long time [57], it was argued in this case that cells that had been in stationary phase for a long time possessed a 'killer phenotype', dramatically decreasing the survival of young cells [66]. This phenomenon presumably reflects the synthesis and excretion of some specific compounds by old cells which are able to decrease the *apparent* viability of young cells; as in many other studies in which viability was estimated simply by plate counts, it was not excluded that such compounds actually served to induce dormancy in the 'young' population.

We too were interested in the behaviour of cells that were left in stationary phase for an extended period. Figure 2 shows the results of an experiment (Kaprelyants and Kell, unpublished) in which *M. luteus* was grown to stationary phase, then starved (without washing) for 2 months. By this stage, the apparent viability as judged by plate counts was only approximately 10^{-5} (although the total count of bacteria in the starved population was not changed significantly). It may be mentioned that the mean diameter of the cells after this starvation became rather homogeneous (in contrast to chemostat starvation) as judged by flow cytometric light-scattering measurements, and was less than 0.5 μm, within the range of ultramicrobacteria (see above). The cells were then washed, inoculated into fresh growth medium and incubated for 10 h in the presence of penicillin G to remove all 'viable' cells, since penicillin kills only actively dividing cells (a method exploited in the well-known penicillin enrichment method [67]). After such treatment, viability decreased by a further order of magnitude. Washing, and further incubation of cells in growth medium in the absence of penicillin, permitted the resuscitation of cells to give a viability of some 70–90% (Fig. 2A), as judged both by standard plate counts and by flow cytometry of the fluorescence of rhodamine 123. Resuscitation was accompanied by the appearence of a cell population with both a larger size (with diameters exceeding 1 μm) and greater accumulation of rhodamine (Fig. 2B, C). The percentage of cells that have changed their rhodamine uptake during the resuscitation period was well correlated with the viability on plates (cf. Fig. 2A, C). It is worth noting that during resuscitation under conditions of a constant total cell count the percentage of small cells in the population was decreased by some 15–20%, with an equivalent increase in the percentage of larger cells; this indicates the conversion of small, dormant cells into metabolically active cells, and argues further against a possible multiplication of cells during this period. It may be concluded that the majority of a cell popula-

tion may become dormant not only in chemostat culture but also in batch culture following a lengthy stationary phase.

4. BIOCHEMICAL AND GENETIC BASIS OF DORMANCY

At present there is only limited information concerning the biochemical mechanisms underlying the phenomenon of dormancy. Most data have been obtained with bacteria under conditions of starvation, the observed changes including changes in cell morphology, an increase in surface hydrophobicity, a decrease in lipid and RNA content (in contrast to the more stable DNA), changes in fatty acid composition, the utilization of reserves such as poly-β-hydroxybutyrate, and protein degradation (for review see [14,16,24,65,68]).

Some time after the onset of starvation (the time depending on the bacterial species), the rate of endogenous respiration sharply decreased [16,21], possibly as a result of the loss of potential respiratory chain activity. The ATP content of starved bacteria steadily decreased as well [9,13,16,69] (however, see [13]). At the same time, it was shown that the initial period of starvation of marine bacteria is characterized by an activation of cell metabolism (increase in endogenous respiration, etc.) [24]. In this period, cells were much more sensitive to inhibitors of oxidative phosphorylation than at later times [24,70]. Whilst the synthesis of some new proteins takes place as well in later phases of starvation [24], the inhibition of protein synthesis by chloramphenicol had the greatest effect on the survival of *E. coli* when added in the early phase of starvation [45].

The starvation-dependence of the extent of membrane energization is rather uncertain: a very fast and significant drop in the apparent protonmotive force following the initiation of starvation was shown for *Thiobacillus acidophilus* [71], *Streptococcus cremoris* [72] and *Staphylococcus epidermidis* [73], whilst the marine bacterium *Vibrio fluvialis* maintained its apparent protonmotive force at a constant level during 14 days of starvation [73].

However, it is not clear what particular processes (formation of dormant forms, cell lysis or both [9]) were occurring in many of the experiments of the type described above, since the data displayed usually consisted of graphs of a time-dependent decrease in viability but with no information on the total number of bacteria during the starvation regime [72,73]. For *Micrococcus luteus* grown slowly in chemostat culture we suggested that the dormant state could be characterized (or at least was accompanied) by a level of membrane energization (as judged by the uncoupler-sensitive extent of rhodamine 123 uptake) lower than the values typical of normal bacteria. Decrease of this membrane energization to even lower values is likely to result in a very low probability of cell recovery [35].

Evidently, the deficiency of nutrients (at least at the beginning of starvation) induces the stringent response in those cells capable of expressing it, as shown in studies of relaxed mutants of *E. coli* [75] and by measuring the ppGpp concentration in starved cells of *Vibrio* sp. [76]. The regulatory role of cAMP during the transition of the cells to starvation conditions has also been demonstrated, at least for *E. coli* [77,78].

What is known about the genes and proteins that respond to starvation conditions? It is now clear that a key role in switching *E. coli* to exhibit a 'starvation' response is played by the product of the *katF* gene, which controls the expression of 32 specific starvation proteins [79]. Cells containing a mutation in *katF* failed to develop resistance to carbon or nitrogen starvation, as well to osmotic, oxidative and heat stresses (see above and ref. [65]).

It is likely that katF is a σ-factor which controls the synthesis of starvation proteins [65]. According to Matin [23,80,81], starvation switches on as many as 40–80 genes in *E. coli* cells (both cAMP-dependent *cst* genes and cAMP-independent *pex* genes) which code for the synthesis of some 35–55 specific polypeptides (the number depending on the type of starvation). (In the case of *Vibrio* sp. starvation induced the time-dependent synthesis of 66 new polypeptides [82].) It would seem that the *cst* genes are concerned with the initial starvation response, whilst the *pex*

genes are connected with the expression of subsequent starvation-resistance [80,81]. Many of the Pex proteins are heat shock proteins, whose role is to prevent the incorrect folding of proteins, and thus possess chaperone activity [80,81]. Other functions of starvation proteins remain unclear.

We wish to stress that these genetic studies deal with starvation processes but not with the transition to dormancy per se (which is evidently one of the possible consequences of starvation). Moreover, in these studies, the decrease of cell viability is judged on the basis of colony counts on agar plates, and changes in viability as an indicator of cell death and thus sensitivity to starvation [23,45]. However, it can not be excluded (and is in our view, on the basis of the material reviewed here, rather likely) that such a decrease actually reflects the formation of dormant cells.

Finally, in this section it is pertinent to ask: which genes and their products are responsible for or induce the dormant state? Do cells synthesize specific 'dormancy' proteins? What factors are responsible for the transition of starved cells to a dormant state or to death, and is the former inevitably on the pathway to the latter? May we expect the existence of specific 'programmed cell death' genes in bacterial cells, similar to the recently discovered gene *ced-9*, which controls the future of certain nematode cells (to remain alive or to suffer apoptotic death [83] and see [84])? All of these questions remain open, and may be expected to be the subject of future work.

5. CONCLUDING REMARKS

It is at least plausible, and certainly can not in general be excluded, that dormant forms of non-sporulating bacteria exist in populations of ultramicrobacteria of marine origin, in starved VBNC cells, in cells grown in chemostats at low dilution rates, and in populations of 'old' stationary-phase cells. It would seem that in spite of the different origins of dormant cells they all generally possess properties such as a decreased cell size and RNA content, a condensed cytoplasm, the accumulation of specific proteins, resistance to environmental insults, and an inability to multiply as judged by traditional plating methods [12,65,81]. However, in most cases strong evidence for dormancy, such as reduced metabolic activity of the cells, their capacity for prolonged survival, and in particular the reversibility of the putatively dormant state, is lacking. The necessity for using all of these criteria is connected with the fact that bacterial populations in both batch and continuous culture are much more heterogeneous than is normally assumed [64,85], and such cultures may consist of several types of subpopulations simultaneously differing in viability, activity and integrity of the cells [9]; comparison of macroscopic (ensemble) and microscopic (individual cellular) measurements can therefore (and will almost inevitably) lead to erroneous conclusions [85,86]. Paradoxically, the metabolic (e.g. respiratory) activity of dormant cells may be much lower than that of 'dead' cells, which whilst unable to divide nevertheless preserve a significant metabolic activity [9]. (This is perfectly reasonable, since, for instance, the addition of a suitable amount of an irreversible inhibitor of the H^+-ATPase, or of an inhibitor of replication, would effectively 'kill' bacteria whilst maintaining respiratory activity for a long time.)

We remain highly ignorant of why cells which cannot grow on solid media may do so after resuscitative procedures, and it is clear that special resuscitation procedures (holding in liquid media before plating) are required for bringing cells out of dormancy [19,35,51]. However, the presence of a small fraction of living (growing) bacteria in the population of interest can significantly influence the results of resuscitation experiments. From this point of view, we would stress the principal difference between the resuscitation of dormant bacteria and the recovery of injured cells (e.g. after sub-lethal stresses). In contrast to dormant cells, injured cells can very often grow on non-selective solid media before recovery (and only the presence of selective additives in agar allows one to detect the cell injuries [87]). Moreover, dormant cells, in contrast to injured cells [17], possess an elevated resistance to salts, etc. [65]. Evidently, this difference (sometimes omitted from consideration [26]) is connected with the character of the processes leading to the injured

(sudden stress) or to the starved or dormant states (slow active adaptation of the metabolism).

Very little is known about the biochemical mechanisms underlying the phenomenon of dormancy. A cell in a dormant state seems to need some level of metabolic activity (and may be capable of undergoing possibly directed mutation [88–92]). This 'dormant' metabolism is likely to be very different from that expressed in cells growing at high growth rates, providing high resistance to environmental insults, and long survival in the hypometabolic state. A search for the specific mechanisms and chemical compounds providing this stability is of particular interest. The unusual bacterial lipids — alkyl resorcinols — initially found in *Azotobacter* cysts [93] and then in vegetative bacteria [94] can play an important role in maintaining cell resistance as membrane stabilizers [95]. Indeed, it has been shown that exogenously administered alkyl resorcinols induced the formation of dormant-like *Bacillus cereus* cells, which were characterized by smaller size, changes in refractive index and buoyant density, decreased metabolic activity, and the ability to resist lysis in nutrient-free buffer [96].

Several classifications of the physiological states of microorganisms have been presented [9,19,60]. We have previously suggested [35] that all the cell types considered could be reduced to three groups, as follows: 'viable' to refer to a cell which can form a colony on an agar plate, 'vital' to refer to one which can only do so after resuscitation and 'non-viable' to refer to a cell which cannot do so under any tested condition According to this terminology, dormant cells are vital (Table 1).

It is presently unknown as to what specific parameters of cell metabolism or cell composition characterize each cell type. For example, both viable and non-viable cells can be metabolically active (e.g. with high respiratory activity) [9,63,97]. At the same time, membrane energization is an apparently good discriminative parameter for Gram-positive cocci [35]. To effect progress, we need approaches (such as flow cytometry [85]) which allow one to evaluate the properties of individual cells. A task for the future is the physical separation of cells comprising the different subpopulations, which would more easily allow their characterization. However, some attempts to do so, based on possible differences in density, proved unsuccessful (J.R. Postgate, personal communication). Other possibilities are to induce in a population as many cells as possible of one type, just as in the case of *M. luteus* starved in stationary phase as described above.

Table 1

Glossary of terms used to describe the three major physiological states defined herein

Physiological state	Phenotype
Viable	Capable of division; will form a colony on an agar plate.
Vital or dormant	Unable to divide or to form a colony on an agar plate without a preceding resuscitation phase.
Non-viable	Incapable of division; will not form a colony on an agar plate under any tested condition.

We use the phrases 'starvation' or 'starving cells' to refer to the environmental conditions under which cells are incubated, rather than to a physiological state. Thus starved cells (or cells that have suffered other stresses) may or may not be dormant. Despite historical usage of these terms, the phrases 'direct viable count' and 'viable-but-non-culturable' are misnomers, since such cells are not viable as defined above.

Finally, it is pertinent to point out that a knowledge of the properties of dormant cells can be of use in applied microbiology and in the biotechnological exploitation of microorganisms. Dormancy, and the development of states that are resistant to antibiotics and other environmental insults, is of obvious signficance in medical and food microbiology. Similarly, one might comment that the maintenance of viability is of importance in all biotechnological processes, since viable microorganisms must constitute the inoculum, may represent the product (e.g. in silage inoculants) or must exhibit an extended 'shelf-life' (e.g. in whole-cell biosensors). However, our interests here are more in the uses of dormant states. Thus, Cusack and co-workers ([98]; see [99]) developed an interesting approach to the recovery of oil from small pores in reservoirs. Ultramicrobacteria produced by the starvation of

Pseudomonas species FC3 were inoculated into model sandcores, and resuscitated in situ. It was found that the ultramicrobacteria had effectively penetrated the sandpack, greatly improving the potential for tertiary oil recovery. The promoters for starvation proteins may also be exploited as growth-rate-dependent signals for mediating the expression of genes of commercial significance. Thus, as outlined by Matin [81], strains of *E. coli* have been constructed in which the gene encoding human growth hormone (HGH) was spliced behind the *cstA* promoter. This encouraged the production of the recombinant protein in dense, non-growing cell cultures. Of course numerous biotransformations catalysed by microorganisms are performed by non-growing cells in nutrient-poor conditions. However, such nutrient-poor conditions often do not permit the expression of enzymes which might serve to degrade or transform the (often xenobiotic) substrate of interest. To overcome this [81], the *tmo* gene encoding toluene monooxygenase was linked to a starvation promoter, resulting in enhanced phenol degradation by non-growing recombinant strains. Thus, the study of apparently recondite aspects of microbial physiology continues to provide useful benefits to those who would exploit such cells for improving the human environment.

ACKNOWLEDGEMENTS

We are indebted to the Science and Engineering Research Council, UK, and the Royal Society, under the terms of the Royal Society/Russian Academy of Sciences exchange agreement, for financial support of this work. We thank Professors J.G. Morris and J.R. Postgate for useful and stimulating discussions. We thank F. Schut and colleagues for allowing us to cite unpublished observations.

NOTE ADDED IN PROOF

Professor J.S. Clegg has kindly drawn our attention to the 1959 Leeuwenhoek lecture by Keilin [100], in which the term 'cryptobiosis' was introduced. This and other pertinent articles are reprinted in the anthology of benchmark papers on anhydriobiosis [101].

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