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Viability and activity in readily culturable bacteria: a review and discussion of the practical issues

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Abstract

In microbiology the terms 'viability' and 'culturability' are often equated. However, in recent years the apparently self-contradictory expression 'viable-but-nonculturable' ('VBNC') has been applied to cells with various and often poorly defined physiological attributes but which, nonetheless, could not be cultured by methods normally appropriate to the organism concerned. These attributes include apparent cell integrity, the possession of some form of measurable cellular activity and the apparent capacity to regain culturability. We review the evidence relating to putative VBNC cells and stress our view that most of the reports claiming a return to culturability have failed to exclude the regrowth of a limited number of cells which had never lost culturability. We argue that failure to differentiate clearly between use of the terms 'viability' and 'culturability' in an *operational* versus a conceptual sense is fuelling the current debate, and conclude with a number of proposals that are designed to help clarify the major issues involved. In particular, we suggest an alternative *operational* terminology that replaces 'VBNC' with expressions that are internally consistent.

Introduction and background: 'viable' and 'nonviable' cells

At the simplest level, bacteria may be classified into two physiological groups: those that can and those that cannot readily be grown to detectable levels *in vitro*. Leaving aside organisms such as *Mycobacterium leprae* that can only be seen to reproduce in a foreign host, and demonstrably syntrophic organisms (McInerney et al., 1981), the well-established view is that *culturability* reflects *viability*.

In this review we will be concerned only with bacteria that are normally readily culturable by standard methods. In particular, we will discuss the significance of apparently intact cells which, at the time of sampling, are not able to grow on media appropriate for the organisms concerned. We will also discuss how the lack of a widely accepted and consistently applied terminology has led to a debate that has centred as much on semantics as the underlying scientific issues. In an attempt to disentangle these issues, we have focussed on the *operational* (practical) domain, aiming to avoid the philosophical problems which arise when such terms are used *conceptually* (i.e. plausibly, but without the possibility of direct experimental analysis in many cases). Moreover, by excluding from the discussion organisms which have yet to be grown axenically (*in vitro*), we have been able to concentrate on the area of current controversy relating to the culturability of species which are claimed to maintain viability in spite of failing to grow on media which normally support their growth.

The notion that, for readily culturable organisms, culturability and viability are synonymous, is supported by many reviews and texts e.g. (Hattori, 1988; Postgate, 1969)). From this point of view, one rôle of the

microbial physiologist has been to establish appropriate conditions to maximise the growth potential of individual cells (propagules). The critical issue here is the detection of growth to a level appropriate to the organism concerned. This inevitably involves an increase in biomass although it should be realised that the degree to which this reflects cell division will depend on specific growth patterns. Thus, for filamentous organisms or those in which fission is not complete, detection of growth does not necessarily require that extensive fission should have occurred.

The term '*viable count*' usually refers to the number of individual organisms in a sample that can be grown to a detectable level, for example by forming colonies on an agar-based medium. Under these conditions the number of viable cells approximates to the number of colony-forming units, and similar concepts apply to the related technique of slide culture (Postgate, 1969). Another method for quantitating viability *via* growth is the Most Probable Number (MPN) technique (e.g. Koch, 1994; Meynell & Meynell, 1970; Postgate, 1969; Russek & Colwell, 1983). Other methods for enumerating 'viable cells' which, nevertheless, are not necessarily predicated on direct observation of their replication, fall within the purview of the field of 'rapid microbiology', and have been widely reviewed (e.g. Fry, 1990; Harris & Kell, 1985; Herbert, 1990; Hobson et al., 1996; Jarvis & Easter, 1987; Kell et al., 1990; Sonnleitner et al., 1992). In this review we consider only some of the optically-based rapid methods.

In a simple, two-valued logic system, the usual convention would maintain that, if a bacterial cell is *not* viable, it is *nonviable* or *dead*. Even with higher organisms (Watson, 1987), such definitions are strictly operational, and have been established by convention and law to serve practical purposes (e.g. organ transplantation programmes). It is recognised that amongst sexually reproducing organisms, fertility, the ability to multiply at the level of the organism, is not synonymous with being alive. In contrast, the consensus within the microbial sphere assumes that only cells which can multiply should be considered 'viable'.

This raises certain philosophical issues, in that if the viability of unicellular organisms is equated with the ability to multiply, we can never state that a given cell *is* alive, only that it *was* alive (Postgate, 1976). The point is illustrated by the paradox of Schrödinger's cat:

> *'A cat is locked up in a steel chamber, together with the following infernal machine (which must be protected against the direct grip of the cat). In a Geiger counter there is a minute amount of a radioactive substance, so little that within an hour maybe one of the atoms decays but equally probably none. If an atom decays, the counter triggers and activates a relay so that a little hammer breaks a flask containing prussic acid. If one has left this whole system for one hour, one would say that the cat is still alive if no atom has decayed. The first decay would have poisoned the cat. The* Ψ *function of the whole system would express this in such a way that in it the living and dead cat are mixed or smeared in equal parts.*'
>
> *Certainly everybody would say that either the cat is alive or the cat is dead, but if the chamber is not opened yet, we just do not know..... In this sense, we speak of the paradox of Schrödinger's cat. (Schrödinger, 1935), as translated, recounted and annotated by Primas (Primas, 1981).*

Microbiologists face two problems in addition to that presented by Schrödinger's paradox. These are both concerned with the question of whether the organism could have been expected to multiply over the period of observation. On the one hand, the majority of microorganisms in the biosphere, for which suitable *in vitro* culture conditions have not been defined, cannot be recognised as 'alive or dead' by the culturable/nonculturable dichotomy even by the retrospective criteria outlined above. Indeed, the only definitive statement that can be made about cells of such organisms is that they must be the progeny of cells that were viable. On the other hand, as our understanding of the biology and physiology of readily culturable microorganisms has improved, it has become clear that the simple (and albeit operational) two-valued logic system is inadequate, since there are clear instances (see below) in which cells which could not be cultured at one point in time subsequently became culturable. Moreover, since the time of Leeuwenhoek (see Keilin, 1959), the possibility that culturable microorganisms can adopt 'cryptobiotic' (Keilin, 1959), 'dormant' (Kaprelyants et al., 1993; Stevenson, 1978), 'moribund' (Postgate, 1967) or 'latent' (Wayne, 1994) states in which they exhibit no signs of life as normally defined, yet from which they may indeed be induced to return to a physiologically active state, has been recognised. Such cells have been referred to as 'pseudosenescent' (Postgate, 1976) or 'somnicells' (Barcina et al., 1990; Roszak & Colwell, 1987). The existence of these phenomena alone requires an extension of the number of recognisable physiological states that are

used to describe microbial viability and culturability (Barer et al., 1993; Davey & Kell, 1996; Kaprelyants et al., 1993; von Nebe-Caron & Badley, 1995). In the Schrödinger analogy, these organisms might be viewed as cats that could not be determined as dead or alive even after the box was opened.

We re-emphasise that our discussion will be limited to readily cultured microbial species and have also excluded sporulating bacteria from major consideration; the formation of obviously specialised bacterial forms such as spores and cysts ('constitutive dormancy', Sussman & Halvorson, 1966) is not considered here, except as a model for differentiation. The question of viability has mostly been investigated for laboratory-based studies on cultures whose properties are sufficiently well understood to generate reproducible growth. Under such conditions, culturabilities approaching 100% are expected. However, it must be recognised that in their natural environments, the conditions to which even well-characterised organisms are or have been exposed may influence their behaviour in ways not observed in the laboratory, especially (but not exclusively) under oligotrophic conditions (Poindexter, 1981).

The recognition of discrepancies between culturability and the influence or activity of an organism led to the proposal (Roszak & Colwell, 1985; Roszak and Colwell, 1987; Roszak et al., 1984; Xu et al., 1982) that, under some circumstances, readily culturable bacteria may become nonculturable but retain 'viability' (whose definition would not then be synonymous with culturability). There has been much confusion related to this proposal. Putative 'viable-but-nonculturable' ('VBNC' or 'VNC') bacteria (Oliver, 1993) have been invoked to explain phenomena as diverse as the epidemiology of cholera and the persistence of genetically marked organisms in the environment. The semantics of critical terms such as viability, vitality, active, alive, nonviable and dead have had to be reviewed yet again, and established methodologies called into question. In spite of substantial activity, spanning more than fifteen years, there is little evidence to support the view that a single physiologically-defined 'VBNC' state exists. Moreover, almost all published studies fail to discriminate adequately between resuscitation/recovery and the regrowth of any culturable cells initially present in the population. Here we suggest a framework for understanding the relationships between bacterial culturability, activity and viability.

'Culturable' (and by implication 'nonculturable') can have two rather distinct meanings: (i) immediately culturable (nonculturable) at a specific time using a conventional, single-stage method such as inoculating onto the surface of a suitable agar medium; and (ii) ultimately culturable (or not) under conditions or using protocols that may be different from those routinely used to grow the bacterium. Clearly these are both *operational* definitions, although only nonculturability under the latter circumstances is reasonably equatable to the concept of 'nonviability'. Here, we shall distinguish between the two usages by assigning quotation marks (i.e. 'nonculturable') to the first, immediate, sense and standard text (i.e. nonculturable) to the second, ultimate, sense. Note however, that in these operational definitions the terms are not used to describe any 'innate' properties of a cell but only the results of our measurement of them.

Phenomena which have been related to a proposed VBNC state

The central phenomena which led to the proposal of a 'VBNC' state in bacteria are not disputed and require explanation. They remain the starting point for discussions in this area and fall into two broad categories: (i) the detection of some form of activity in cells which could not be induced to replicate and hence do not give rise to turbidity or colonies, and (ii) reports of cells which, while classified as 'nonculturable', apparently regained their capacity for growth as a result of recovery or resuscitation processes.

The activity of bacteria can be assessed in populations (bulk assay) or in single cells (cytological assay). When assigning properties specifically to 'nonculturable' cells, bulk assays require the population to be completely 'nonculturable'; otherwise any attempt to provide a correlation between biochemical activities and culturability is bound to fail (Davey and Kell, 1996; Kell, 1988; Kell et al., 1991). Thus, although some investigations have appeared to use bulk assays successfully (e.g. Rollins & Colwell, 1986), cytological assays in which the outcome is assessed by microscopy (Nwoguh et al., 1995; Zimmermann et al., 1978) or flow cytometry (Davey and Kell, 1996; Kell et al., 1991; Lloyd, 1993; Shapiro, 1995) have been preferred in many studies.

The development of 'nonculturable' cells during laboratory studies on axenic cultures is illustrated in Figure 1. Essentially, in any culture where net growth is arrested at t_0, typically by starvation, discrepancies begin to emerge between the total number of

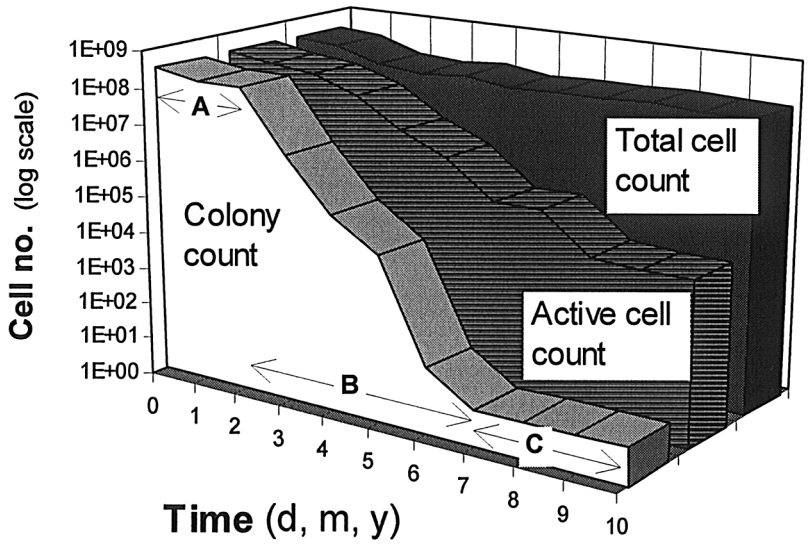

Figure 1. The pattern of changes seen when bacterial cells are inoculated into a microcosm which does not permit net cell growth at time zero. The time scale varies with the organism and the nature of the microcosm. The time periods **A**, **B** and **C** indicate different phases in the relationship between total and colony counts: **A** – indicates the phase of correspondence, **B** – the phase of relative discrepancy, and **C** – the phase of absolute discrepancy onset when colony counts fall below limit of detection. Active cell counts generally continue to fall during phase C if the experiment is extended and total cell counts become increasingly difficult as cell outlines become progressively less distinct. Note that the colony count reaches a detection limit of 10 colonies at some time point, not an absolute count of zero.

cells which can be detected microscopically (total cell count) and the number which can form colonies on agar-based media (colony count). The relationship between the colony and total counts falls into three phases: correspondence, relative discrepancy and absolute discrepancy (i.e. completely nonculturable). During the period of relative discrepancy, 'nonculturable' cells are formed at a rate which is determined by the state of the culture at t_0, the characteristics of the organism itself, the maintenance conditions *and the methods used to determine culturability*. Absolute discrepancy does not occur in all systems and in some cases may be difficult to establish.

It is important to realise that, under conditions of relative discrepancy, it is not possible to establish whether the active and culturable cells are one and the same, or indeed whether culturable cells are always a subset of the active population, as is often confidently assumed. Irrespective of the cytological properties of the culturable cells, it has clearly been shown that substantial numbers of 'nonculturable' cells retain demonstrable metabolic activities.

What significance can we attribute to these activities? The cytological assays used range from the wholly empirical to those which are fully defined in terms of the physiological or molecular functions which must be retained to yield a positive response (Table 1). The application of such tests can reveal a great deal about the phenotypes of 'nonculturable' cells. Where activities are demonstrated it may be possible to establish the potential of such 'nonculturable' cells to contribute to specific environmentally significant processes such as nutrient cycling reactions and even pathogenicity. Moreover, the demonstrable capacity of some 'nonculturable' cells to respond to an external stimulus by specific gene expression shows that their activities are more than that of slowly degrading packages of preexisting enzymes. For example, nonculturable cells of pathogens may be capable of expressing virulence factors such as toxins and invasins in response to exogenous stimuli (Rahman et al., 1996).

Are any of the measures of activity listed in Table 1 reliable indicators of viability? Although increasing numbers of cytological assays are being established, and substantial advances have been made in microbial physiology, if viability is equated with culturability little can be added to the conclusions arrived at by Postgate and colleagues over twenty years ago that:

> *'At present one must accept that the death of microbe can only be discovered retrospectively: a population is exposed to a recovery medium, incu-*

Table 1. Cytological methods that have been used to estimate microbial 'viability' or activity

	Method	Minimum requirements for positive result	Comment
A.	Kogure, Direct Viability Count (DVC)[1,2]	Response to external stimulus, Transcription*, Translation*, Energy dependant	Mechanism not clear. Cell elongation in response to yeast extract and quinolone exposure assumed to be growth potential related
B.	Induced β-galactosidase production[3]	Response to external stimulus, Transcription*, Translation, Energy dependant, Retained enzyme activity	Well-defined genetic and biochemical pathway, access of substrate may be limited by permeability. Other genes / reporter genes can be used (e.g. luciferase [4])
C.	Energisation-sensitive probes[5,6] (e.g. oxonols, Rhodamine 123)	Energy dependant, Energised cytoplasmic membrane	Active labelling (or probe exclusion) reversible with uncoupling agents. Can be undermined by permeability barriers and changes in backgound labelling material
D.	Tetrazolium salt reduction (e.g. INT[7], CTC[8])	Energy dependant or Retained enzyme activity	Depends on the available energy source(s) which may be exogenous or endogenous and pathway(s) involved in their oxidation[9]
E.	Enzyme substrates[10,11] (e.g. fluorescein diacetate[12])	Retained enzyme activity, Intact permeability barrier	Depend on expression of the enzyme(s) involved in cells to be studied, access of reagents to enzyme and retention of reaction product
F.	Passive dye exclusion	Intact permeability barrier	Exclusion of nucleic acid labelling agents (e.g. propidium iodide, ethidium bromide, ethidium homodimer)[13]
G.	Nucleic acid staining[13]	Retained DNA, RNA or both	May be supravital (e.g. acridine orange or DAPI) or after fixation. Specificity and quantitative relationship to genomic content or physiological state rarely confirmed. Determinative rRNA-directed oligonucleotide probes may provide physiological information (ribosomal content)

* Likely but not specifically demonstrated.

Note: Methods A-E detect activity and are considered to indicate 'vitality'/ activity. Conversely failure of dye exclusion (F) indicates failure of an activity (maintenance of membrane integrity).

Sources: [1] – (Kogure et al., 1979); [2] – (Barcina et al., 1995); [3] – (Nwoguh et al., 1995); [4] – (Duncan et al., 1994); [5] – (Kaprelyants and Kell, 1993); al., 1995); [7] – (Zimmermann et al., 1978); [8] – (Rodriguez et al., 1992); [9] – (Gribbon & Barer, 1995); [10] – (Diaper & Edwards, 1994); [11] – (Manafi et al., 1991); [12] – (Mor et al., 1988); [13] – (Haugland, 1992)

> *bated, and those individuals which do not divide to form progeny are taken to be dead.' (p.5) ....'there exist at present no short cuts which would permit assessment of the moment of death: vital staining, optical effects, leakage of indicator substances and so on are not of general validity' (p.5) (Postgate, 1976)*

In agreeing with these earlier conclusions we inevitably reject the view that activity measurements are synonymous with 'viability assays'. Rather, we propose to classify such assays as indicators of(metabolic) *activity* since they demonstrate important aspects of cellular physiology. Although it is clear from Table 1 that the various assays reflect quite different levels of vitality (the converse of dormancy (Kaprelyants et al., 1993; Kaprelyants & Kell, 1992)), we take the view that, operationally, a positive result should lead to classification of the cell as *active* rather than, as in many published examples, as *viable*.

The apparent resuscitation of 'nonculturable' cells has been reported on many occasions. In general, samples from populations which failed to yield growth on solid media or *via* broth enrichment have been induced to yield colonies after special treatments. Animal passage, the gently graded addition of nutrients to starved cultures and temperature shift have all been used to achieve this (Table 2). The fact that cells have been cultured from populations that would traditionally have been described as nonviable is not disputed. However, in the majority of studies, recovery has been difficult both to produce and to reproduce. Moreover, the phenotype(s) responsible for the recovery of the cells, and the physiological basis for the processes involved, have not been defined.

The practical difficulties of determining the basis for activity and resuscitation phenomena associated with 'nonculturable' cells have been compounded by inconsistent use of the term 'viability' and by the view that this property can be assessed by some cytologi-

cal assays. In our view, the validity of any cytological assay can be confirmed only by correlation with culture assays for a specific mechanism of cell death in a single strain or possibly species and against a specific operational definition of viability. In this last respect, it is recognised that some authors appear to use demonstrable cellular activity (independent of culturablity) as their operational definition of viability (*i.e.* activity = viability). We are not able to support this view since validation of the assay then becomes dependent on a circular argument.

The conceptual problems of bacterial viability determinations are stretched to the limit in the case of the expression 'viable-but-nonculturable'. Apart from the difficulties inherent in arriving at a clear view of what it means, use of the definite article to describe cells in 'the' VBNC state implies that such a state has been defined (presumably in physiological terms). Use of 'VBNC' in this manner appears to provide a unitary explanation for what are in practice a series of perplexing phenomena. Whether there is a single physiological 'VBNC' state, a range of distinct states underpinning 'VBNC' phenomena, or whether the term is a misnomer, remains to be determined.

Finally, it is worth stressing that 'dormancy', as usually defined, refers to cells with negligible activity but which are ultimately culturable. The so-called VBNC cells are often claimed to have exactly the opposite properties: they are (metabolically) active but 'nonculturable' (ABNC). Operationally, we may therefore define *dormant* cells as those which fail to give positive reactions in vital assays, such as those outlined in Table 1, but which are nevertheless (ultimately) culturable.

The need to resolve these issues

The incentive to establish the basis for VBNC-related phenomena comes from both fundamental and applied issues. If some bacteria can differentiate into 'nonculturable' forms in response to certain stimuli, this undermines our interpretation of studies based on colony counting. How can we tell whether cells have been killed or have differentiated into a 'VBNC' state? It seems most unlikely that we will have to discard our interpretations of all colony count-based work since it has produced a largely coherent body of information. Nonetheless, the possibility that a fall in colony counts may reflect transition to a (dormant or) 'VBNC' state rather than death cannot be excluded. This at least raises the possibility that 'VBNC'-related phenomena might be *in vitro* quirks provoked by mild injury and therefore of little practical significance.

It would be relatively easy to sustain this view were it not for a number of serious, unresolved bacteriological public health problems where transition to and from a 'nonculturable' state appear to be implicated. Principally, these concern aspects of the epidemiology and natural history of infective diseases which cannot be reconciled with the sample pattern from which the known causal organisms can be isolated. Foremost amongst the epidemiological mysteries are cholera and campylobacteriosis where the failure to isolate *Vibrio cholerae* and *Campylobacter jejuni* from clearly implicated sources or reservoirs of infection could be accounted for on the basis of their being present in a 'VBNC' state. For both these organisms, environmental investigations have provided evidence for the presence of 'nonculturable' cells in appropriate samples (Brayton, 1987; Pearson, 1993) while *in vitro* studies have demonstrated their capacity to form metabolically active cells which could not be grown immediately (Rollins and Colwell, 1986; Xu et al., 1982). The list of organisms for which similar phenomena have been described (albeit less extensively) is substantial (Oliver, 1993). However, it must be stressed that these environmental/epidemiological and *in vitro* studies only constitute circumstantial evidence which is (at best) consistent with a rôle for 'VBNC' cells.

Further medically significant areas where transition to and from putative 'VBNC' states would have potential relevance include bacterial infections which have a clinically dormant or latent phase and the effects of antibiotics. Tuberculosis (Gangadharam, 1995; Wayne, 1994; Young & Duncan, 1995) and melioidosis (Dance, 1991) provide examples of the former. However, 'nonculturable' forms have not been directly demonstrated to have a pathogenic rôle in either of these diseases. Indeed, it is a reflection of the terminological problems that we seek to address here that mycobacterial dormancy and the clinical latency of tuberculosis are not clearly defined from a bacteriological perspective. Thus dormant cells in the Wayne model system do not make DNA or RNA but retain substantial enzyme activity and do not lose culturability. In contrast, in the Cornell mouse model of dormancy (De Wit et al., 1995; McCune et al., 1966), culturability is lost after treatment (albeit by measures that would not satisfy our MPN criteria – see later) but is subsequently regained in immunosuppressed hosts. In spite of these issues of detail, 'nonculturable' or

dormant cells of pathogens could provide explanations for latent bacterial infections and indeed for lack of a clinical response to antimicrobial agents shown to be effective against growing cells *in vitro*. From this point of view, it is worth noting that bactericidal antibiosis normally requires that the target organisms be growing, and that dormant (or at least non-growing) cells are thus resistant to the effects of antibiotics.

Thus there are many practical issues bearing on food and water safety, the distribution and influence of bacteria in the environment, the effects of antibiotics and the significance of declining colony-forming unit (cfu) counts which cannot be assessed until the authenticity of the putative VBNC states have been confirmed or refuted and, if the former, the presence of 'VBNC' cells unequivocally determined in natural samples. If some bacteria can differentiate into a 'nonculturable' state, the results of studies based on colony counting are difficult to assess unless unambiguous means of counting 'VBNC' cells are available (Barer et al., 1993).

How do bacterial cells become 'nonculturable'?

Bacterial cells may become 'nonculturable' as a consequence of several fundamentally different processes. For example, damage to, or lack of, an essential cellular component may lead to loss of the ability to divide, either temporarily (sublethal injury) or permanently (lethal injury). DNA damage is undoubtedly an important mechanism; however, there is little information on the degree of damage required to prevent replication in the short term. Similarly, little is known of the minimum or 'threshold' concentrations of components such as ribosomes, transcription factors and so on that are required for (re)growth. The ability of the cell to cope with starvation or stress, to maintain essential processes and to repair damage will obviously depend on conditions prior to and during recovery. The phenomenon of 'substrate-accelerated death' (Calcott & Postgate, 1972; Postgate & Hunter, 1963; Postgate & Hunter, 1964) is particularly interesting in this respect, as it shows that inclusion of certain substrates in the recovery medium which had been limiting when starvation was initiated, may actually lead to the growth of substantially lower numbers of colonies compared to recovery media free from those substrates. Loss of culturability may also result from the activation of lysogenic phages or 'suicide' genes such as *sok/hok* or autolysins (Aizenman et al., 1996; Franch & Gerdes, 1996; Jensen & Gerdes, 1995; Joliffe et al., 1981). In these cases, a defined biological event, encoded by specific genes, is deleterious to the survival potential of the cell.

Although the examples of damage, deficiency and self-destruction help us to understand why a cell may become 'nonculturable', they do not account for why such a cell may grow on a specific medium at one point in time but not at another. Is it because critical genes can no longer be expressed, key resources have fallen below a threshold value or can it sometimes reflect a more deliberate process? Is it possible that there are discrete determinants whose expression instructs the cell not to replicate *in vitro*?

The 'VBNC' hypothesis leads us to consider the evidence for the last of these possibilities. In this context, loss of culturability in non-sporeforming bacterial cells could reflect a terminal differentiation pathway resulting from an intrinsic, genetically determined and regulated, developmental programme (Dow et al., 1983), analogous in some respects to that of sporulation. While there is good evidence that many non-sporulating bacteria have genetically determined programmes for *maintaining* culturability (Hengge-Aronis, 1993; Kolter et al., 1993; Matin, 1994; Östling et al., 1993), and there are occasional references to cognate processes involved in sporulation (DeMaio et al., 1996), there is no direct evidence for the development of 'nonculturable' cells as a means of starvation survival. It should also be stressed that studies on starvation survival normally do not, nor do they set out to, deal with cases of dormancy.

If the observed loss of culturability is suggested to be part of an active, adaptive response (as in sporulation), leading to a 'differentiated' phenotype specialised for stress survival, then it should be possible to find direct evidence for the programme involved. We suggest criteria for such evidence in the final section.

How might 'nonculturable' cells become culturable?

Any processes required to return 'nonculturable' cells to culturability will depend on the underlying basis for 'nonculturability'. The central issue here is whether increases in colony or MPN counts from below to above the threshold of detection stem from cells changing their phenotypes from 'nonculturable' to culturable or from regrowth of a small, previously undetected 'culturable' component of the population. Before

addressing this issue we identify a number of processes/physiological states which may be involved:

- *Regrowth* is the return to an actively growing state of cells that had ceased growth but had not lost culturability. Here growth is a combination of biomass accumulation and fission sufficient for detection of the organism concerned.
- *Injured cells and recovery/repair processes.* Some cells may respond to specific forms of damage by entering a physiological state in which specific reparative processes are necessary before (re)growth on their usual range of media is initiated. In one widely used operational definition, 'injured' cells will not form colonies on a selective medium but will do so on a rich medium, it being taken that the rich medium allows recovery before regrowth. Such cells are therefore culturable, albeit not under all circumstances. In Gram-negative bacteria, such injuries are often associated with the cell envelope (Ray & Speck, 1973). Some forms of injury (*e.g.* sub-lethal DNA damage) may render cells 'nonculturable' by any available means until the recovery process has returned them to culturability. However, it is also recognised (i) that even a single mutation can be lethal as judged by colony-forming ability, without in the short term having any effect on most measurable activities, and (ii) that mutations can occur in non-growing cells (Cairns et al., 1988; Zambrano et al., 1993). More recently it has been shown that non-growing bacteria can enter a hypermutable state (Bridges, 1996; Hall, 1995), so that recovery (in terms of regaining the ability to multiply) could, in some cases, involve DNA repair. We are not, however, aware of any detailed studies of the relation between culturability and DNA damage in typical starvation experiments.
- *Dormancy* can be defined operationally as a *reversible* state of metabolic shutdown (Kaprelyants et al., 1993). It reflects an absence of vitality or activity, as measured in a particular assay system (*e.g.* methods A-E in Table 1), which may persist for an extended period. Quite independently, dormant cells may be 'nonculturable' in that they require specific stimuli before they become active and culturable. For example, *Bacillus subtilis* spores will not germinate unless they are exposed to specific triggers (germinants) and conditions are conducive to outgrowth (Moir et al., 1994). Spores are therefore 'nonculturable' in a very restricted sense (and would not be recognised as such on most media since these contain appropriate germinants) and well-established germination processes can serve as paradigms for other examples of resuscitation or regrowth (see below). A critical feature is that the process is not necessarily a simple reversal of the pathway(s) that led to dormancy or 'nonculturability'. Further, dormant bacterial cells are characteristically more resistant to environmental insults than cells in any other recognised physiological state.
- *Resuscitation.* We use this term to denote transition of cells from 'nonculturable' to culturable states with respect to a given medium. For example, in the case of substrate-accelerated death outlined above, recovery of cells on media free from the 'lethal' substrate effectively resuscitates the organism's ability to grow on that substrate. A similar view could be taken with respect to cell envelope-damaged Gram-negative cells and their recovery on non-selective media. In contrast, in the case of dormancy in *Micrococcus luteus*, there is clear evidence that successful resuscitation requires the presence of viable (culturable) cells or of a pheromonal factor in the medium derived therefrom (Kaprelyants & Kell, 1993; Kaprelyants et al., 1994; Votyakova et al., 1994). Similar processes may occur in biofilms of *Nitrosomonas europaea* (Batchelor et al., 1997).
  As with germinating spores, such signals may play a triggering rôle in breaking dormancy, although ecological reasoning suggests they should normally exhibit a fair degree of species specificity (Kell et al., 1995) and they may also act as growth-stimulating substances with some properties analogous to cytokines (Kaprelyants & Kell, 1996). Thus two distinct forms of resuscitation are identified here, one in which the organism replicates in a medium which enables it subsequently to grow on a medium which was temporarily unable to support its growth and another in which specific extracellular signals appear necessary before growth is possible. Either explanation may be relevant to the phenomenon of resuscitation via animal passage where exposure of susceptible animals to 'nonculturable' preparations of pathogens by natural or parenteral routes of infection has been used to recover bacteria in their immediately culturable form and to determine infectivity. Animal systems have traditionally been used to isolate newly recognised pathogens before suitable culture media have been developed (e.g. with *Legionella* (Meyer, 1983)). They provide

a complex and dynamic nutritional environment, the essential features of which may be difficult to determine or to replicate *in vitro*. When apparent recovery occurs, it is often assumed that this is due to the resuscitation of previously 'nonculturable' cells in the animal. However, in the present context, each animal should really be considered analogous to a single tube in the MPN method and the results subjected to a similarly rigorous statistical analysis.

- *Limited cell division*. The growth of cells capable only of a limited number of divisions can only be detected by microscopy, flow cytometry or other sub-macroscopic means. The phenomenon could be more widespread than is generally appreciated (Kaprelyants and Kell, 1996; Mukamolova et al., 1995), and certainly the observation of microcolonies on agar plates is commonplace. Assays based on the formation of directly visible colonies (which typically require 25–30 generations), or the MPN method in which directly visible turbidity from a single bacterial cell would require at least some 10^6 cells.ml^{-1} or ca. 20 divisions, will score such cells as nonviable, whilst direct microscopy or slide culture over a very small number of generations would score it as culturable. Limited division is regularly observed for cells from environmental samples (Binnerup et al., 1993; Button et al., 1993; Hattori, 1988) and may be due, for instance, to dilution of an essential resource present at the time of sampling but absent in the isolation medium. The resource itself or its end-product(s) are therefore diluted out by successive rounds of fission. However, the recent isolation of marine oligotrophs capable of growth only to non-turbid cell densities in liquid media but apparently indefinite laboratory passage (Button et al., 1993; Schut et al., 1993) also indicates that limited cell division can result from cell density regulation. It would be valuable to know whether organisms isolated from environmental or laboratory viability studies as microcolonies could be passaged in this form or whether their growth potential was truly limited. In any event, the phenomenon indicates circumstances where mis-classification of cells as culturable or nonculturable may occur.

Some quantitative aspects of recovery

In a typical resuscitation experiment, starved or otherwise stressed cells are maintained in appropriate liquid medium and periodically sampled for viable (cfu) counts as judged by their behaviour when incubated on agar plates. When the count falls below the detection limit (often operationally referred to as 'zero viability'), samples (or in some cases the entire microcosm) are subjected to a recovery/resuscitation process prior to plating. Even a few cells that are immediately culturable when the 'resuscitation' procedure is applied have the potential to regrow prior to plating and the subsequent cfu count cannot differentiate resuscitation from regrowth. It is imperative, therefore, to determine, as accurately as possible, the probability that a given sample contains *any* culturable units prior to resuscitation. The precision of any determination of zero viability will depend on the number of samples taken and their volumes. To demonstrate that the contribution of regrowth has been excluded, it is necessary to decide on the statistical limits that are acceptable; for example, $p<0.01$ that a single viable cell was present in any *individual* sample taken through the recovery/resuscitation procedure. Some workers have attempted to circumvent this problem by estimating the maximum contribution that regrowth could make to their viability estimations. Unfortunately such arguments are not applicable because the duration of any lag phase and the extent of any logarithmic growth for such cells in stressed cultures are unknown.

An appropriate example to illustrate these remarks is the 'resuscitation' of *V. vulnificus* starved at 5°C. Cultures with fewer than 0.1 viable cells per ml could apparently be resuscitated after a temperature upshift to room temperature, accompanied by an increase in viable counts up to 10^6 per ml within 3 days (Nilsson et al., 1991). However, in more recent experiments based on the MPN assay, Weichart and colleagues concluded that this 'resuscitation' was likely to have resulted from the regrowth of a very small number of initially viable bacteria (Weichart & Kjelleberg, 1996). Therefore the results of experiments in which resuscitability has been tested on low dilutions of a culture where the possible presence of some culturable cells cannot be excluded should be accepted only with extreme caution.

The MPN assay, in which the conclusion is statistically-based, has major advantages in this context since it is not limited by the arbitrary definition of zero viability imposed by colony counting assays. Moreover, results from the two methods can useful-

ly be compared and, where the MPN assay gives higher counts, this constitutes preliminary evidence for a resuscitation process. A disadvantage with this approach is the inherent low precision of the MPN assay; the coefficient of variation is about 40% for 10 parallel tubes (Koch, 1994). In our experience, differences of at least 1.5–2 orders of magnitude are required before they can be considered significant.

In experiments in which apparent resuscitation has been carried out *in vivo* (by animal or human passage – see Table 2), the presence of a small number of culturable bacteria in a 'VBNC' population is of critical importance. In some cases, a single cell may be sufficient to cause infection, while in others the infective dose (ID_{50}) may not have been determined with sufficient precision to differentiate the relative contributions of culturable and 'nonculturable' cells to infection. Therefore the results of such studies can only be qualitative since, in the case of a positive resuscitation after passage of 'nonculturable' cells through an animal, it is not possible to determine how many cells were actually resuscitated. Moreover, even when the precision of infectivity assays can be assessed, the effects on ID_{50} of including nonculturable cells (either sense) has not been assessed. These problems are further compounded by the tendency to use low numbers of animals or human volunteers and rather large inocula (Colwell et al., 1996).

It should be appreciated that quantitative analysis of microbial enumeration has received extensive and rigorous attention in the past (e.g. as reviewed in Meynell and Meynell, 1970). A central feature of these considerations is low precision intrinsically associated with low colony counts (irrespective of technical errors). This again emphasises the difficulties and uncertainties involved in assigning a value of zero to culturability.

Resuscitation versus recovery of injured cells

Injury to bacterial cells may result in loss of viability, as judged by plate counts, and therefore in the formation of 'nonculturable' phenotypes. The injury phenomenon is not new, but has been less well studied (Ray and Speck, 1973). Operationally, the benchmark criterion for the discrimination of injured cells is their ability to grow on non-selective but not on selective plates. This view is based on studies in which the outer membrane of Gram-negative cells or the cell wall of Gram-positive cells were damaged (e.g. by freezing (Ray & Speck, 1972) or by starvation in natural water environments (Bissonnette et al., 1975)). It has also been shown that, after starvation in seawater, coliforms may grow only on agar media made from seawater (Dawe & Penrose, 1978). The high plating efficiency on non-selective media can make injured cells distinguishable from nonculturable cells (in either sense). Note, however, that surface growth could itself be considered a stress (high surface tension and oxygen concentration) that could result in poor growth of significantly injured cells.

It might be argued that cells starved for long periods of time or kept at low temperatures represent, at least partly, those 'injured' cells which can not grow on even nonselective plates. Indeed, McFeters and Singh cite a number of studies where variations in conditions including the agar medium composition, temperature, use of chelators etc., influenced the recovery of injured bacteria (McFeters & Singh, 1991). If we accept the last suggestion, it is at least possible that the 'resuscitation' of non-culturable cells may actually represent their recovery from injury. It was shown that *E. coli* injured with chlorine could be 'resuscitated' in ligated ileal segments of mice (McFeters and Singh, 1991), whilst Weichart and Kjelleberg (Weichart and Kjelleberg, 1996) mentioned that populations of *V. vulnificus* starved at 5°C contain some injured cells which are sensitive to the concentration of agar on the plates. They proposed that injured subpopulations may partly explain the reported cases of resuscitation of these bacteria (see Table 2). We also found that an important event in the resuscitation of starved *M. luteus* cells is the repair of the membrane barrier in a majority of the cells, although it was not immediately followed by an increase in their culturability (Kaprelyants et al., 1996).

Such examples indicate that stressed populations are not necessarily homogeneous, and may contain a mixture of dormant and injured cells; moreover, dormant cells could be injured at the same time. From a practical point of view it is very difficult to discriminate between the contributions of cells in these different states to a colony or MPN count since the activity of the cells responsible for the detected growth must be explicitly identified prior to culture. This can be done when the fate of individual cells is followed or when cells with particular metabolic phenotypes are separated by fluorescence activated cell sorting (FACS) (Kaprelyants et al., 1996). However, if the proportion of injured cells is low it is not easy to assess their contribution and this can lead to misinterpretation (Weichart and Kjelleberg, 1996). It is also worth noting

Table 2. A summary of some studies in which resuscitation of 'dormant' or 'nonculturable' bacteria has been attempted

Organism	Conditions for formation of 'VBNC'" state	Resuscitation[1]	MPN or dilution culture[2]	Remarks	Reference
Aeromonas salmonicida	starvation in sea water, 15 °C	+	–	usage of rich medium (TSB) for resuscitation	Husevag, 1995
Aeromonas salmonicida	starvation in sea water, 4 °C	–	–	various media and conditions have been used for resuscitation	Ferguson et al., 1995
Aeromonas salmonicida	starvation in water, 10 °C	–	+	various resuscitation media have been used	Morgan et al., 1991; Morgan et al., 1992
Aeromonas salmonicida	starvation in sterilised river water, 15 °C	+	–	Allen-Austin et al., 1984	
Aeromonas salmonicida	starvation in sterilised river water, 15 °C	–	+	usage of rich medium for resuscitation	Rose et al., 1990
Campylobacter jejuni	starvation in physiological saline solution, 20 ∘C	–	–	resuscitation in simulated stomach, ileal and colon environments rich media)	Beumer et al., 1992
Campylobacter jejuni	starvation in sterilised pond water, 4 °C	+	–	resuscitation of some strains via passage in mice	Jones et al., 1991
Campylobacter jejuni	starvation after stationary phase 4–6 weeks	+	+	resuscitation under MPN conditions	Bovill & Mackey 1997
Klebsiella pneumoniae	starved bacteria in phosphate buffer	+	–		Lappin-Scott et al., 1988
Legionella pneumophila	starvation in pure water, 30 °C	–	–	resuscitation in co-cultures with *T. pyriformis*	Yamamoto et al., 1996
Legionella pneumophilia		+	–	resuscitation via chick embryo yolk sac	Hussong et al., 1987
Micrococcus luteus	long storage in stationary phase, room temperature	+	+	resuscitation factor supernate taken from active culture required	Kaprelyants et al., 1994
Pasteurella piscicida	starvation in seawater, 6 and 20 °C	+	–		Magarinos et al., 1994
Salmonella enteritidis	starvation in salt solutions, 21 °C	–	+	usage of lactose broth Difco for resuscitation	Chmielewski & Frank, 1995
Salmonella enteritidis	starvation in sterilised river water, 25 °C	+	–	resuscitation by nutrient addition after 4 but not 21 days after culturability lost	Roszak et al., 1984
Pseudomonas fluorescens	starvation in soil, 24 °C	+?	+	only several divisions of 'VBNC'" cells during resuscitation were found	Binnerup et al., 1993
Pseudomonas fluorescens	N-starvation in minimal medium, 25 °C	+	–	usage of medium lacking a carbon source for resuscitation	Evdokimova et al., 1994
Pseudomonas aeruginosa	starvation in stationary phase	+	see text	resuscitation of individual cells on filters in anaerobic conditions	Binnerup et al., 1995
Vibrio cholerae Escherichia coli	starvation in autoclaved water	+	–	usage of passage through rabbit ileal loop	Colwell et al., 1985
Vibrio cholerae	starvation in autoclaved artificial sea water, 4 °C	–	+	usage of nutrient-free medium for resuscitation	Ravel et al., 1995

Table 2. Continued

Organism	Conditions for formation of 'VBNC'" state	Resuscitation[1]	MPN or dilution culture[2]	Remarks	Reference
Vibrio, *Aeromonas*, *Pseudomonas*, *Alcaligenes* spp	non-culturable ultramicrobacteria from estuarine waters	+	–	resuscitation was found for a narrow range of nutrient concentrations	MacDonell & Hood, 1982
Vibrio parahaemolyticus	starvation in minimal medium under 3.5 °C	–	+	usage of rich medium for resuscitation	Jiang & Chai, 1996
Vibrio cholerae	starvation in salt solution, 15 °C	+	[4]	Conversion to the colony-forming cells was effected with a short heat shock	Wai et al., 1996
Vibrio cholerae	starvation in buffered saline, 4 °C	+/–	+/–	Resuscitation in intestine after ingestion of non-pathogenic vaccine strains by volunteers. 2 sets of experiments were done; only one claimed resuscitation. Dilutions were probably not great enough to exclude presence of some viable cells	Colwell et al., 1996
Vibrio vulnificus	starvation in defined media, 5 °C	–	+	usage of fully supplemented medium MMMglucose for resuscitation	Weichart et al., 1992
Vibrio vulnificus	starvation in defined media, 5 °C	+	–		Nilsson et al., 1991
Vibrio vulnificus	starvation in defined media, 5 °C	+	–	usage of natural estuarine environment for resuscitation	Oliver et al., 1995
Vibrio vulnificus	starvation in defined media, 5 °C	+	+[3]	*in vivo* resuscitation injection in mice	Oliver & Bockian, 1995
Vibrio vulnificus	starvation in defined media, 5 °C	–	+	wide range of conditions for resuscitation *in vitro* were used	Weichart & Kjelleberg, 1996
Vibrio vulnificus	starvation in defined media, 5 °C	–	+	resuscitation in artificial sea water by temperature upshift	Biosca et al., 1996
Yersinia ruckeri	starvation in sterile river water, 6 or 18 °C	+	–	usage of rich medium for resuscitation	Romalde et al., 1994

[1] A + sign means that resuscitation was attempted and indeed claimed as judged by the appearance of increased numbers of culturable organisms, whilst the – sign means that no recovery or resuscitation was observed.
[2] A + sign means that the authors diluted the samples before performing resuscitation, in an attempt to remove genuinely viable cells present at the start of the resuscitation experiment, whilst the – sign means that they did not.
[3] The growth of cells on the medium used here may have underestimated the culturable fraction, since there is evidence that this system contains an injured fraction Weichart and Kjelleberg, 1996.
[4] Dilutions *per se* were not done but at one stage *no* viability was observed, although the resuscitation yielded 1000 colonies again the total count of bacteria at the onset of resuscitation is not shown.

that for medical and environmental studies, 'nonculturable' injured cells may have the same significance as dormant cells.

Studies in which true resuscitation of 'nonculturable' cells has been claimed

From the above it follows that in a culture exhibiting a significant difference between total and viable (culturable) counts, it is not clear whether the 'nonculturable'

cells represent *specific* 'VBNC' cells, cells in any of the states defined above, or nonviable cells. Moreover, the distinction between some of these states cannot be realised until reversibility to 'normal', culturable bacteria has been proved.

Therefore the central point of discussion in this area is now focussed on the results of recovery or resuscitation experiments, almost all of which were done by cultivation or maintenance of nonculturable cells in liquid media followed by plating on agar plates.

Although some early experiments have purported to show the ability of 'nonculturable' bacteria to grow on agar following resuscitation in appropriate liquid media (see Table 2), we again stress that a limited number of operationally viable cells in the starved population could have been responsible for the growth which occurred. In this regard, the MPN assay, which provides an estimate of viable cell numbers *via* their cultivation in liquid medium at high dilutions (Postgate, 1969), can be useful in overcoming this uncertainty (Kaprelyants et al., 1994). Of course the monitoring of *individual* cells by any method is equally acceptable, and Binnerup et al. have described a method for monitoring individual cells during resuscitation in a special chamber (Binnerup et al., 1995).

Table 2 illustrates the range of conditions that have been used to obtain nonculturable cells (either sense) and attempt resuscitation. The evident absence of a common defined set of conditions which can produce entry into and exit from a 'nonculturable' state make comparisons between studies and further development of work in this area difficult. Nevertheless, Table 2 clearly shows that, in almost all cases, where the populations of nonculturable bacteria were diluted to an extent which might have been sufficient, statistically (perhaps $p < 0.01$), to remove any viable cells, resuscitation was not successful.

In only three cases do we feel there is sufficient evidence for the existence of a *reversible* state of 'nonculturability' in nonsporulating bacteria: resuscitation of *M. luteus* in the presence of a factor produced by viable bacteria and measured using the MPN assay (Kaprelyants et al., 1994) and the conversion of 'nonculturable' *Vibrio cholerae* to platable (surface culturable) cells *via* a short heat shock (Wai et al., 1996). Very recently, Bovill and Mackey (1997) resuscitated *C. jejuni* some 23-fold under MPN conditions. In a fourth case (Whitesides & Oliver, 1997), the resuscitation of *Vibrio vulnificus* from sea water microcosms, the evidence also appears strong. Nonetheless, in our own investigations with this organism, we have not obtained comparable results, using MPN counts as the criterion for nonculturability and would classify a sub-population of the cells analysed in this study as 'injured' rather than 'nonculturable' (Weichart and Kjelleberg, 1996). This general issue of replication of results in different laboratories remains an uncomfortable feature of investigations in this area and is particularly prominent in the attempts to resuscitate *C. jejuni* via animal passage.

One reason for this may be the specific nature of conditions and strains required to produce these phenomena. Based on our experience with *M. luteus*, we consider it highly unlikely that a single protocol will be devised for the resuscitation of 'nonculturable' cells generally; rather, it is probable that tailored protocols will need to be devised for different types of 'nonculturable' cell on a species- or even a strain-specific basis. For example, we found that resuscitation of *M. luteus* takes place only under a very narrow range of concentrations of yeast extract (ASK, DBK, N.D.Yanopolskaya and G.V. Mukamolova, unpublished observations; see also (MacDonell & Hood, 1982)). Sporadic cases (or at least claims) of successful *in vivo* resuscitation (e.g. by passage through animals or their organs, see Table 2) might also indicate the importance of the presence of growth-stimulating factors during resuscitation (Kaprelyants and Kell, 1996).

Finally, the duration of nonculturability prior to resuscitation should be considered. If the putative 'VBNC' cells represent a form in which cells can survive for extended periods under adverse conditions, it should be possible to achieve resuscitation after prolonged periods of 'nonculturability'. The published instances claiming recovery generally relate to relative short periods (days) and could therefore reflect a transitional period (to nonviability) during which cells can be 'rescued'. Where resuscitation has been achieved in mixed populations of culturable and 'nonculturable' cells, it is of course impossible to determine how long the resuscitated cells had been 'nonculturable'.

Proposals

We end with some proposals which we hope will clarify the issues. The proposals are of two types: (i) some suggestions concerning *operational* definitions, together with terms that are best avoided unless strictly defined, and (ii) some suggestions regarding experimental pro-

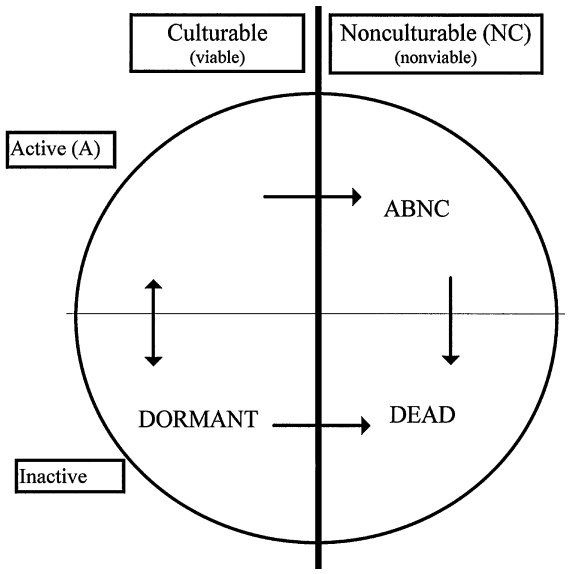

Figure 2. Diagram indicating the major physiological states of non-growing microorganisms discussed and their interrelationships. ABNC represents metabolically Active But Not Culturable. The view is taken that 'viable' is to be equated with 'culturable under any stated set of circumstances'. Arrows represent possible *transitions* between physiological states. Note that no arrow crosses from the nonculturable to the culturable zones. No zones are marked to indicate the sub-lethally injured or Not Immediately Culturable (NIC) cells discussed in the text. Cells which are inactive but remain culturable cells are identified as dormant.

tocols designed to discriminate between some of the major physiological states discussed.

We begin by recognising that in fact all cells may be assigned into four major categories as combinations of two alternatives: (i) culturable or nonculturable and (ii) (metabolically) active (A) or inactive . These are strictly operational definitions, and cells are assigned according to appropriately applied methods. A clear distinction needs to be made between cells which *were* Not Immediately Culturable (NIC) from those which were or are nonculturable. The former refers to our usage hitherto of 'nonculturable' while the latter describes cells that were nonculturable within the ultimate confines of the experiment. NIC cells may only be identified in retrospect since their return to culturability must have been demonstrated for them to be so recognised.

In Figure 2 we propose terms which retain the view that, in microbiology, viability (viable) and culturability (culturable) are *operationally* synonymous and that nonviable = nonculturable. The arrows represent possible *transitions* between states, and no arrow leads from 'nonviable' to 'viable'. Any cell which is *termed* 'nonviable/nonculturable' by an experimenter but which is subsequently *found* to be 'viable/culturable' is simply recognised as having been misclassified. It should be borne in mind that the *processes* involved in the transition to and from a particular physiological state are not necessarily the same (*e.g.* sporulation → germination → outgrowth). Both viable (culturable) and nonviable cells may be active or inactive.

Active but Not Culturable (ABNC/ANC) is suggested to describe cells which exhibit measurable activity but which fail to grow to a detectable level. In studies which include protocols capable of demonstrating NIC cells, the possibility of recognising active or inactive cells in this category arises.

Dormant cells are metabolically inactive but capable of making a transition to a growing state. Although cells of organisms which have not yet been cultured axenically are clearly the progeny of viable cells, we have excluded them from our discussion because the central issue here is the proposed capacity for cells of readily culturable organisms to make transitions to and from an NIC state. Cells of 'as yet uncultured' bacteria may be found in samples from human infections (e.g. *M. leprae or Tropheryma whipelii* (Relman et al., 1992) and are abundant in environmental samples (Amann et al., 1995; Torsvik, 1996)). They are *operationally* nonculturable but in many cases they can be recognised by molecular and cytological methods such as rRNA analysis and *in situ* hybridisation (Amann et al., 1995). There is currently no available means by which cellular assays of activity applied to these nonculturable cells can be validated as indirect measures of viability; thus the viability of any individual cell in this category must be considered indeterminate.

We also suggest that, where possible, investigators avoid the use of terms such as 'viability', 'live' and 'dead' unless clear operational definitions are provided. In fact it is not generally necessary to use such terms, and more precise terms which indicate the method(s) applied (e.g. cfu count, MPN count, the proportion of (quantitatively) dye-positive cells etc.) are more accessible and less open to misinterpretation, particularly (Kell & Sonnleitner, 1995) where they are reinforced by statistical analysis.

How then should the term 'VBNC' be related to our proposals? Where studies have not demonstrated recovery, the cells investigated fall into our ABNC category, depending on their demonstrable activity. If cells are shown unequivocally to be ultimately culturable and the possibility of regrowth has been excluded, they should be placed in the NIC category. Nonculturable

able and NIC require operational definitions related to the culture methods and times applied. Although we recognise that many may not wish to accept our terminology, we are particularly concerned that it should be possible to relate studies to the framework we propose. To this end authors should, at the very least, state their working definitions for 'viability' and 'VBNC'.

With these considerations in mind, what experimental criteria might be applied to the characterisation of cells which have widely been referred to as 'VBNC'? First, we propose to exclude both dormant and injured cells from consideration since these can be identified operationally. The remaining group are uninjured cells which retain activity yet fail to grow on the standard media for the experiment without going through a resuscitation process. To be worthy of further investigation these cells should be shown unequivocally to be capable of recovery under conditions that *exclude* the possibility of regrowth. If such cells exist, their phenotype(s) should be definable in morphological or physiological terms. This could be achieved by microscopy, flow cytometry, or the mapping of gene expression and protein synthesis. Finally, the transition process should be energy-dependent and it should be possible to isolate and characterise mutants that are at least partially deficient in the process. Further, the gene(s) involved should be part of a definable stimulon and their expression should be related to the time when the response was initiated and dependent on the expression of other genes associated with recognisable regulons.

Given this, the experimental criteria for claiming a cell to be exhibiting dormancy, injury, resuscitation etc. follow from the definitions. We outline some of them in Figure 3.

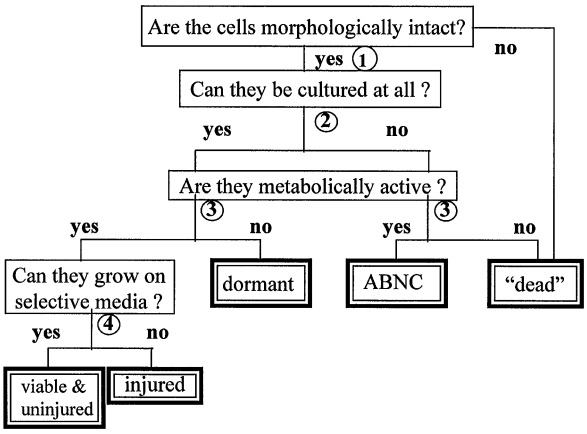

Figure 3. Decision tree for discriminating different physiological states of individual microbial cells. The numbers indicate some of the assays which might be used in an experimental study. 1. Intact morphology as viewed by microscopy or electron microscopy, assessing for example DNA content. Dye exclusion may also be used; for injured cells, however, the permeability barrier may be temporarily disrupted but upon recovery the barrier may be restored; 2. Plate count, microcolony assays including slide culture, liquid culture, may include resuscitation steps such as animal passage; *in vitro* and *in vivo*; 3. DVC, INT, CTC, respiration, membrane energization, uptake and/or incorporation of nutrients; 4. Growth on media containing for example bile salts or antibiotics; alternatively, injured cells can be distinguished by recovery on soft agar or by assessing the intactness of the membrane permeability barrier by fluorescent probes or osmotic behaviour.

Concluding remarks

Although one might prefer to avoid tackling some of these issues, there is such dissonance between the views in which (i) 'viable' is to be equated with 'culturable', and yet (ii) there is said to be a state termed 'viable-but-nonculturable', that one is forced to take a position on what constitutes an unambiguous operational definition of viability. For the reasons outlined, we conclude that, where tests of culturability can be applied, the more traditional view, wherein microbiologists regard the terms viable and culturable as operationally synonymous, is likely to remain the more useful.

We recognise and reiterate that this approach is applicable only to organisms which can be cultured and to the operational domain. Two further domains can also be considered: the conceptual, in which a variety of different properties may be attributed to cells to explain natural phenomena but without any direct means of confirming their veracity, and the pragmatic, in which viability is attached to an operational definition which has practical significance (*e.g.* the infectivity, pathogenicity or food spoilage capacity of the cells concerned). Providing the context used for any particular discussion is clearly assigned to one of these alternatives, further confusion should be avoided.

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