Bacterial dormancy and culturability: the role of autocrine growth factors

Commentary

Douglas B Kell* and Michael Young

Addresses
Institute of Biological Sciences, Cledwyn Building, University of Wales, Aberystwyth SY23 3DD, UK
*e-mail: dbk@aber.ac.uk

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Abbreviations
MPN most probable number
Rpf resuscitation-promoting factor

Introduction and semantics

> "It is now well established that some micro-organisms can, under certain conditions, be deprived of all visible signs of life and yet these organisms are not dead, for when their original conditions are restored, they can return to normal life and activity." [1]
>
> "Counsel: I beg your pardon m'lud.
> Judge: Well, I mean, your witness is dead.
> Counsel: Yes, m'lud. Er, well, er, virtually, m'lud.
> Judge: He's not completely dead?
> Counsel: No, he's not completely dead m'lud. No. But he's not at all well."
>
> *Monty Python's Flying Circus, Episode 3, Court Scene*

It is sometimes hard to know if an organism is still alive, even when that organism is a human being [2]. However, the socio-legal importance of determining accurately whether an individual is dead (and thus irreversibly unable to return to a state of 'aliveness') has led to the development of operational indicators ('vital signs'), which are used, in a two-valued logic system, to classify individuals as dead or alive [3]. It is not so easy with microbes.

Were we to apply the same two-valued logic system to microorganisms we would have to pronounce them either alive ('viable') or dead ('non-viable'), and we would then have to decide precisely what are the vital signs for microbes. Classical microbiology equates viability with being able to multiply (i.e. viability = culturability). A consequence of this (since we must wait for any division to occur before scoring a cell as 'alive') is that we can never state that a given cell *is* alive, only that it *was* alive [4], a phenomenon mirrored in the famous Schrödinger's cat paradox of quantum mechanics [5,6]. Assuming, further, that we wish to maintain the concept of 'death' as having a physically irreversible meaning for microbes too, we must recognise the need for a separate word to describe organisms that are not culturable at a given time, yet may either revert to a state of culturability later, or manifest culturability when incubated under different circumstances. The usual convention for describing cells that have reversibly lost the ability to proliferate is to refer to them as being 'dormant' or 'anabiotic'. Thus, and given that the phenomenon of dormancy necessarily admits the concept of resuscitation, we shall use the words 'dormant' and 'dormancy' to describe a state of low metabolic activity in which cells are unable to proliferate without a preceding resuscitation phase [7]. Dormancy (e.g. of seeds and buds) is of course commonplace in the plant world [8], and even tumours may enter a state of dormancy or nonproliferation [9–11], a subject to which we return later.

There is, in addition, a substantial literature that refers to some (usually Gram-negative) bacteria as entering, or being in, 'a' (or even 'the') 'viable-but-nonculturable' (VBNC or VNC) state. If we equate viability with culturability (see above) the concept of VBNC is an oxymoron [12,13]. The available data [6,13,14] suggest that though they may be metabolically active, most or all of such cells are not dormant as defined above but are either injured or genuinely unculturable ('dead'). Since these issues have been discussed recently [6,13] we do not deal with them here. We focus instead on some particular features relating to the true dormancy of Gram-positive microbes. An overview of the definitions we are using is given in Table 1, and a broader analysis of physiological macrostates is in Figure 1.

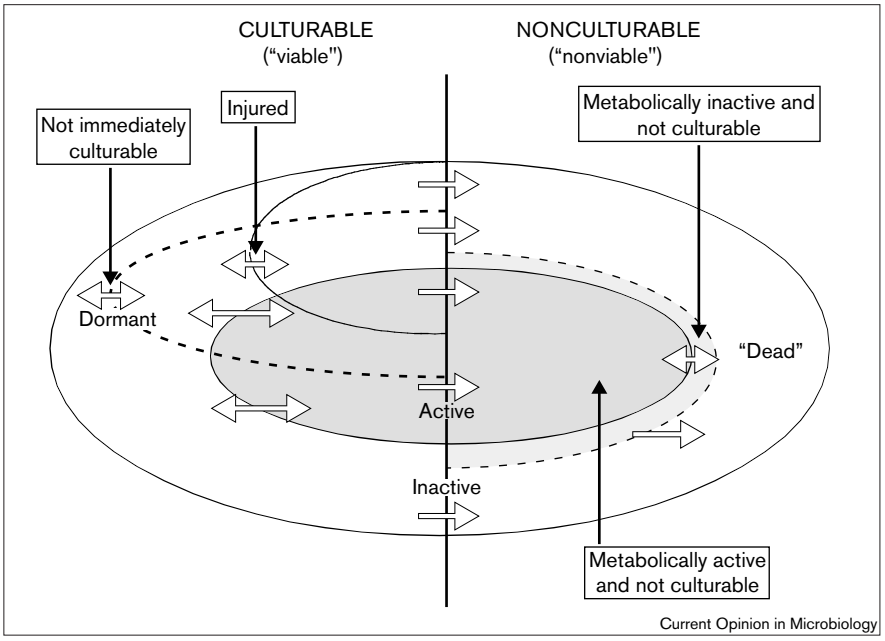
Table 1

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

Physiological state	Phenotype
Viable (culturable)	Capable of division; will form a colony on an agar plate or proliferate observably in liquid medium
Dormant	In a state of low metabolic activity and unable to divide or to form a colony on an agar plate without a preceding resuscitation phase
Non-viable (non-culturable)	Incapable of division; will not form a colony on an agar plate nor proliferate observably in liquid medium

*We use the phrases 'starvation' or 'starving cells' to refer to environmental conditions under which cells may be incubated, rather than to a physiological state [7]. 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.

Figure 1



Some major physiological states that may be exhibited by an individual cell. At the grossest level we discriminate the states of culturability (left and right sides of the figure) and metabolic activity or inactivity – defined according to a quantitative criterion – (inner and outer parts of the figure). All definitions are operational [6], and open arrows describe possible transitions between states. No route permits an operationally 'dead' organism to return to culturability. Some organisms may appear nonculturable but are not terminally so; it is appropriate to refer to them as 'not immediately culturable'. 'Injured' cells are operationally related but are more likely to be observed under conditions in which they have never lost their metabolic activity.

In this review, we explore the relationship between activity and culturability before concentrating on the recent discovery, in the high-GC Gram-positive bacteria (Actinomycetales), of a family of proteins that act as autocrine growth factors (cytokines). We then cover their relevance to cell cycle studies and prokaryotic development generally, and finally we point up the significance of this phenomenon to the search for novel, as-yet-uncultured bacteria whose bioactive, secondary metabolites may be of industrial significance.

We begin with a disease perspective.

A disease perspective

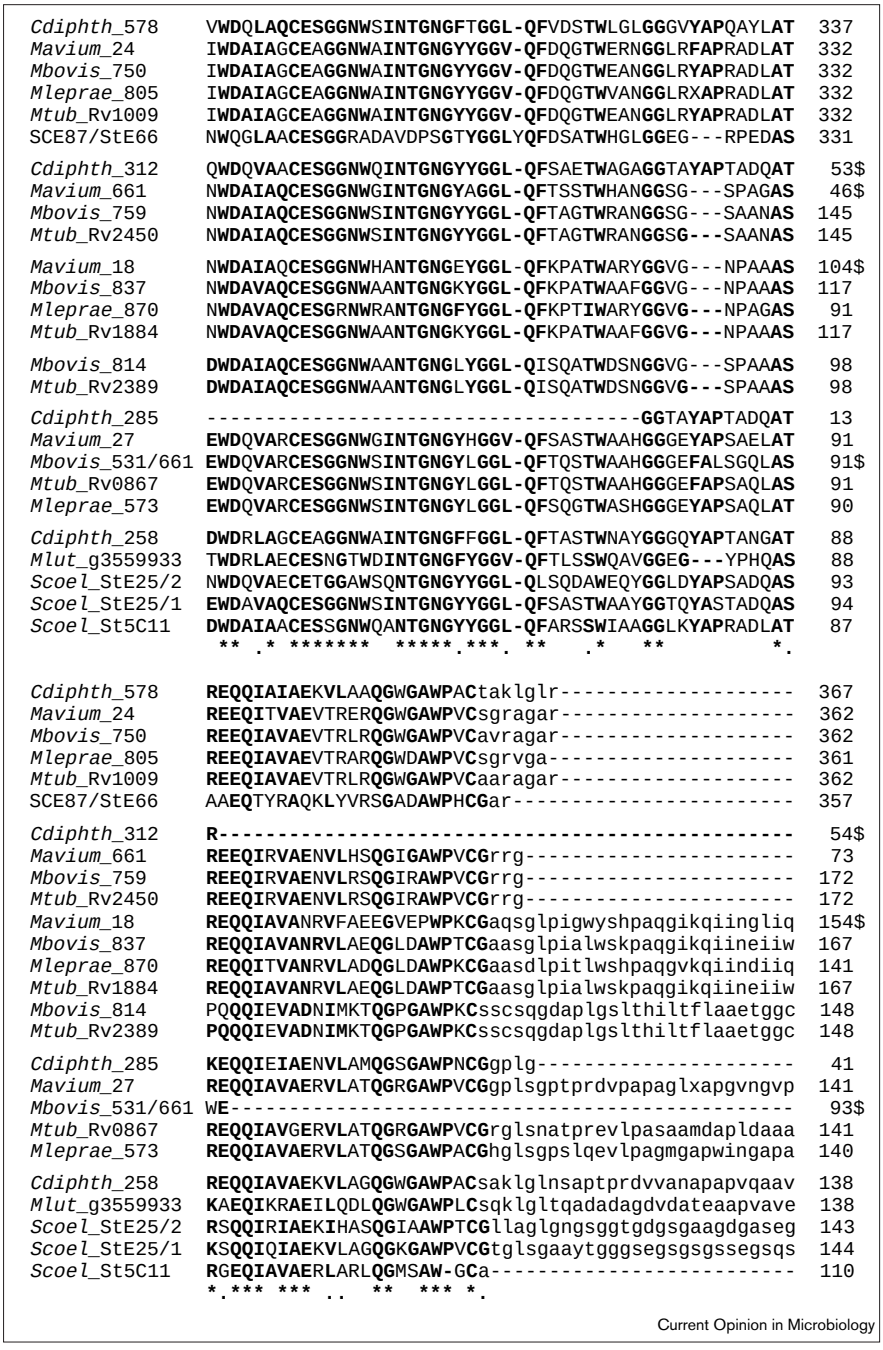
Apart from its intrinsic scientific interest, the significance of microbial dormancy becomes particularly obvious in the case of disease-causing microorganisms. If we wish to assess the microbiological load in an environmental sample, we are normally interested in the number of cells that can multiply, and this is assessed using assays that rely on proliferation, such as plate counts or the most probable number (MPN) assay [15,16]. This follows rather naturally from Koch's postulates. Obviously, dangerously wrong conclusions would be reached if (say) a sample contains only one culturable cell in 10^5 and the rest are dormant. The potentially culturable microbial load will be grossly underestimated (and, of course, an organism may produce a toxin even under conditions in which it cannot multiply). In addition, we now know with some certainty that many diseases are caused by organisms that may be cultured only with the greatest difficulty or (to date) not at all, and whose presence may be inferred only by molecular methods [17–22]. Indeed, it is more than likely that the number of diseases whose aetiology is recognised, at least in part, to have a microbial component will continue to increase as we bring the organisms involved into routine culture (e.g. as occurred for Legionnaire's disease with *Legionella* [23] and with peptic ulcers for *Helicobacter pylori* [24–26]). Whipple's disease provides another example [27,28]. It is associated with an organism (in this case an actinomycete) whose presence may be detected routinely by molecular methods but has still apparently not been brought into axenic culture.

In a related vein, the fraction of culturable cells present in non-clinical, environmental samples is often very small [13,29–37], as is the fraction of culturable species [38]. In some cases [39], the organisms detected by such molecular methods have close affinities with taxa we have learned to culture (and indeed that grow straightforwardly on common laboratory media), suggesting perhaps that the problem is not so much of culturing them *per se* but rather of bringing them into culture for the first time.

Activity and culturability

Because culturability assays take time, many attempts have been made to establish rapid, optical tests that might give an indication of the physiological state of individual cells. It is essential to study cells individually because bulk measurements of metabolic activity cannot, even in principle, reflect culturability, which is a property of individual cells. If bulk activity drops by 50%, have half the cells lost all activity, or have all the cells lost half their activity, etc. [17,40,41]? Both flow [17,42,43] and image [44–46] cytometry have been applied, often from the perspective that nonculturable cells will have leaky membranes, which permit the

Figure 2



Partial sequence alignment of Rpf-like gene products from *Corynebacterium diphtheriae*, *Mycobacterium tuberculosis, Mycobacterium leprae*, *Mycobacterium avium*, *Streptomyces coelicolor* and *Micrococcus luteus*. Conserved (*) or conservatively substituted (.) residues are in bold. $ denotes partial sequence. Predicted gene products from *M. tuberculosis* (Rv0867, Rv1009, Rv1884, Rv2389, Rv2450) *M. luteus* (g3559933) and *M. leprae* (g2440090) are compared with predicted gene products from several genome sequencing projects currently in progress at the Sanger Centre (*M. bovis* contigs 531/661, 750, 759, 814, 837; *M. leprae* contigs 573 [= g2440090], 805, 870 [= MSGB38COS]; *S. coelicolor* cosmids SCE87/StE66, StE25/2, StE25/1, St5C11 and *C. diphtheriae* contigs 578, 285, 258, 312) [http://www.sanger.ac.uk/Projects/Microbes/] and at TIGR (*M. avium* contigs 18, 24, 27, 661) [http://www.tigr.org/tdb/mdb/mdb.html] have been aligned with respect to their Rpf-like domains. The complete version of this figure may be consulted at http://biomednet.com/cbiology/mcr.

influx/efflux of substrates/products that are normally membrane-impermeant. Barer's group in particular [44–46] has been able to relate cytochemical activities to the culturability or otherwise of pathogenic bacteria, although the patterns of activity differ greatly between organisms. It has long been known that growth (multiplication) and metabolism can be uncoupled, and it is thus very reasonable to effect a clear discrimination between bacterial activity and culturability [6,13] (Figure 1). Importantly, therefore, some 'unculturable' bacteria ('as-yet-uncultured' organisms) did display substantial metabolic activity when tested in this way. This said, truly dormant cells do not, by definition, display significant metabolic activity.

Dormancy in Gram-positive organisms and the role of bacterial cytokines

We have recently shown, using the high G + C Gram-positive bacterium *Micrococcus luteus*, that starved organisms can enter a dormant state [7,47,48], in which their

culturability may be as little as 10^{-5}, but from which they can be resuscitated in the presence of sterile, filtered supernatant [49–51]. Use of the MPN method (i.e. dilution to extinction [6,52–55]) served absolutely to exclude regrowth as the source of the return to culturability. The resuscitation-promoting factor (Rpf) is a protein, which has been purified to homogeneity and is active at picomolar concentrations [56]. As well as increasing the viable cell count of dormant *M. luteus* cultures, it is also necessary for the growth of viable cells. It therefore has the properties of a cytokine [57,58]. Rpf also stimulates the growth of several other high G + C Gram-positive organisms including *Mycobacterium avium*, *Mycobacterium bovis* (BCG), *M. kansasii*, *M. smegmatis* and *M. tuberculosis*. Genes encoding similar proteins are apparently ubiquitously distributed among (and restricted to) the high G + C Gram-positive bacteria (actinomycetes) [56,59]. Genome sequencing has uncovered examples in *M. leprae*, *M. tuberculosis*, *M. bovis*, *Streptomyces coelicolor* and *Corynebacterium diphtheriae* (Figure 2) and others have been detected by hybridisation in *M. smegmatis*, *Corynebacterium glutamicum* and a variety of *Streptomyces* spp. Most organisms (unlike *M. luteus*) have several *rpf*-like genes, but we do not yet understand why.

It is ironic that even in the post-genomic era we can barely solve the most fundamental physiological question we might ask of a microbial cell — is or was it alive or not at a given time?

Operational dormancy as a failure to progress through the cell cycle

For higher and particularly differentiated organisms there are of course many tight controls on cell growth and division, mediated both 'internally' by molecules such as cyclins [60] and 'externally' via hormones and growth factors [57,58,61]. The particular role of at least some growth factors (e.g. epidermal growth factor) is to activate receptor tyrosine kinases, which carry proliferatory signals to the nucleus via one or more interacting phosphorylation cascades. Certainly, the malfunctioning of such regulatory circuits can lead to uncontrolled proliferation, neoplasia and tumours. Leaving aside the elements of metastasis, for which hydrolase (especially matrix metalloproteinase) activities are required [62–64], it is clear that for a primary tumour to grow it must have access to its normal complement of growth factors (or molecules with an equivalent effect) and thereby pass through all the relevant cell cycle checkpoints. Similarly, their removal would inhibit tumour (and normal cell) growth, leading to what is known as tumour dormancy [9,10] and in some cases affecting survival [65–67]. In one sense it is therefore a statement of the obvious that bacterial cells that are not able to multiply do not progress normally through their cell cycle. The question therefore arises as to whether dormant or washed cells become stuck in a particular phase or at a particular checkpoint (e.g. they might complete a round of replication but not initiate a new one, and thus be in the equivalent of G_0). Nothing is known about this currently.

Are the transitions to and from dormancy active, programmed processes?

While in differentiated eukaryotes the maintenance of survival [65] can depend on the continuing provision of external signals, we do not know whether the transition to true dormancy is part of an essentially ordered developmental programme, as is recognised for more obviously developmental processes such as sporulation [68,69] or the 'stationary phase' in Gram-negative organisms [70,71]. Alternatively, progression into a dormant state may simply reflect a gratuitous and graceful degradation from a state of normal activity and culturability, for which the loss of any number of different functions might be responsible [13,72]. Similarly, the return from dormancy to culturability could involve either a reproducible and ordered programme of gene expression or a more general and stochastic repair/recovery process. Thus far we have little information in the *M. luteus* system, although the comparatively coherent timings of the loss and gain of metabolic and biochemical functions [49,73] could suggest that dormancy and resuscitation, at least in this system, are both active and programmed.

Dormancy and the search for previously uncultured microbes of industrial significance

Actinomycetes are the high G + C, Gram-positive, filamentous organisms that produce two-thirds of the known antibiotics of microbial origin [74–76]. Nearly 8000 actinomycete antibiotics had been described by 1994, of which 80% were from *Streptomyces* species and 20% from other actinomycete genera. Many medically and economically important compounds are represented: antibacterials, including the tetracyclines, erythromycin, rifamycin, clavulanic acid, gentamicin, kanamycin, vancomycin and pristinamycin; antifungal agents such as candicidin and nystatin; anticancer drugs like daunorubicin and doxorubicin; the anti-parasitic agent avermectin; the iron chelator ferrioxamine; immunosuppressants such as FK506 and rapamycin, and insect control agents such as the spinosyns. There is little doubt that actinomycetes represent the microbial clade with the greatest potential to provide us with new useful bioactive molecules, and the question obviously arises as to what fraction of actinomycetes have already been brought into culture.

Most commentators recognise that, in general, the number of microbes in an environmental sample that may be observed microscopically is very much greater than the number that may be cultured therefrom [13,17,29–37]. Estimates of the fraction that have ever been cultured are often of the order 1%, but there is evidence from DNA reannealing kinetics in particular that the correct number may be even smaller [38,77,78]. Are these numbers also true for actinomycetes? McVeigh and colleagues [39] used generic actinomycete-selective PCR primers to isolate 16S rDNA from a temperate forest soil. All 46 examples that fell in the actinomycete radiation were apparently novel and had not been previously cultured, as judged by their rDNA sequences. Similar findings with Actinobacteria were made by Rheims, Felske and colleagues [79–82].

Is it more than possible [59] that the loss of Rpf activity in the hostile extracellular environment can account, at least in part, for the loss of culturability of actinomycetes generally, whose presence can be detected by molecular analyses.

Concluding remarks

Comparative genomics has revealed that horizontal gene transfer between bacteria is even more widespread than had previously been suspected [83–85]. This complicates both analysis and discussion of the evolutionary origins of pheromonal and proteinaceous signalling systems. What is now abundantly clear, however, is that signalling via proteinaceous growth factors is not the sole prerogative of higher eukaryotes. It is also a prominent feature of the lifestyles of invertebrates [86], of ciliates [87] and, as we have recently shown, even the lowly prokaryotes.

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