# Adoption of the Transiently Non-culturable State – a Bacterial Survival Strategy?

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## ABSTRACT

Microbial culturability can be ephemeral. Cells are not merely either dead or alive but can adopt physiological states in which they appear to be (transiently) non-culturable under conditions in which they are known normally to be able to grow and divide. The reacquisition of culturability from such states is referred to as resuscitation. We here develop the idea that this "transient non-culturability" is a consequence of a special survival strategy, and summarise the morphological, physiological and genetic evidence underpinning such behaviour and its adaptive significance.



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## ABBREVIATIONS

| | |
|---|---|
| BCG | Bacille Calmette-Guerin |
| CFU | Colony-forming units |
| CTC | 5-Cyano-2,3-ditolyl tetrazolium chloride |
| DVC | Direct viable count |
| GFP | Green fluorescent protein |
| MPN | Most probable numbers |
| NIC | Not immediately culturable |
| Rpf | Resuscitation-promoting factor |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| TNC | Transient non-culturability/Transiently non-culturable |
| VBNC | Viable but non-culturable |

## 1. INTRODUCTION

Bacteria in natural environments are exposed to a wide variety of stressful conditions such as nutrient deprivation, extremes of temperature or pH, changes in oxygen tension, the presence of toxic compounds, light, etc. (Edwards, 2000). Various stress avoidance strategies have evolved that enable bacteria to cope with fluctuations in natural environments (Kaprelyants *et al.*, 1993; Neidhardt, 2002). Depending on the magnitude and combination of stresses experienced, different global stress responses ensure that cell integrity and the processes essential for viability (the ability to multiply) are maintained (Morita, 1990; Storz and Hengge-Aronis, 2000).

Bacteria that live in fluctuating environments are endowed with an array of two-component systems (Hoch and Silhavy, 1995; Hoch, 2000) that detect environmental changes and initiate the appropriate responses to such changes. Bacterial two-component systems are made up of a sensor histidine

kinase and a response regulator. The sensor kinase is activated by conformational change (that may result from ligand-binding), which leads to autophosphorylation (the γ-phosphoryl group of ATP is transferred to a conserved histidine residue). The response regulator then catalyses the transfer of the phosphoryl group from the phosphoryl-histidine residue to a conserved aspartate within its own regulatory domain. Phosphorylated response regulators usually bind to specific DNA sequences and activate the expression of particular genes. Sensors have been described that detect changes associated with several stress responses such as changes in oxygen tension, redox potential and osmotic potential, and altered concentrations of toxic heavy metals and volatile fatty acids. Other sensors detect the presence or absence of specific nutrients (e.g. sources of phosphate, potassium, magnesium, carbon or nitrogen). Genome sequencing projects have uncovered many more two-component systems that respond to as yet unknown signals (e.g. *Streptomyces coelicolor* contains some 85 sensor kinases and 79 response regulators, including 53 kinase-regulator pairs (Bentley *et al.*, 2002)). There are many variations on this basic theme, enabling different vital cellular processes to respond in an appropriate manner to a wide spectrum of environmental changes (Stock *et al.*, 2000). Phosphorelays represent a further refinement, in which additional proteins are recruited to form phosphorylation cascades sensitive to multiple sensory inputs. Both chemotaxis and spore formation, which may be regarded as stress avoidance mechanisms, are under the control of dedicated phospho-relays (Falke *et al.*, 1997; Hoch, 2000, 2002).

Once a stress has been sensed, immediate action may be taken. Arguably, the simplest of these is the negative chemotactic response that enables bacteria to move away from localised regions in their immediate environment where conditions are unfavourable for growth and/or survival. However, flight is not always an option. Most stress avoidance mechanisms require well-orchestrated processes of up- and down-regulation of specific genes (Hengge-Aronis, 1999; Storz and Hengge-Aronis, 2000). Some of these genes feature in the responses to many different stresses (e.g. *dnaK* and *groESL*, which encode chaperonins involved in protein refolding). These processes consume energy and, depending on the severity of the stress, accumulated resources may have to be mobilised. As well as synthesis of chaperonins, responses to stressful conditions may involve the production of specific enzymes or specialised protective compounds. Examples of the former include catalase and superoxide dismutase, which are up-regulated in response to oxidative stress (Hengge-Aronis, 1993). Examples of the latter include low molecular weight osmo-protectants such as trehalose and betaine (Hengge-Aronis, 1993; Strom and Kaasen, 1993). Universal stress

survival proteins play a significant role in the stress response and further survival of cells (Nystrom and Neidhardt, 1994). Synthesis of specialised DNA-binding (Dsp) proteins represents another important protective mechanism (Almiron *et al.*, 1992; Gupta *et al.*, 2002). These proteins co-crystallise with DNA in a metal ion-dependent process and protect it from degradation (Frenkiel-Krispin *et al.*, 2001).

After these relatively rapid responses have occurred, several different longer-term strategies may come into play, a common feature of which is a lowering of metabolic activity. If this lowering of metabolic activity is not very marked, nor very protracted, cells can emerge to re-initiate growth as soon as conditions become favourable again. However, if there is essentially metabolic shut-down, dormancy may ensue (Kaprelyants *et al.*, 1993; Kell *et al.*, 1998), with the production of highly specialised forms. Some bacteria produce spores in which metabolism is undetectable (Gould and Hurst, 1969). Others produce less specialised forms in which metabolism is very low (e.g. Kalakoutskii and Pouzharitskaja, 1973; Kaprelyants and Kell, 1993). In contrast to the first type of strategy, cells can only emerge from a dormant state after a protracted recovery period (in the case of spores, this corresponds to the period required for germination and outgrowth). In non-sporulating bacteria, metabolic shut-down is often accompanied by a loss of culturability (under conditions that support the growth of normal, viable cells). The process whereby culturability is restored is called resuscitation (Kaprelyants *et al.*, 1993; Kell *et al.*, 2003). Our present knowledge about dormancy in non-sporulating bacteria is very limited, although it is clear that "non-culturability" is only one of many specific properties of cells in this state. In this review we shall use the term "transiently non-culturable" (TNC) to describe this particular state, noting also that the definition of states such as this is purely operational (Kell *et al.*, 1998).

If the stress is extreme, or if the available energy supply is insufficient to permit avoidance, the cell will die. The death of some cells may permit the survival of others in the bacterial population, so death should not necessarily be viewed as a passive reaction to lethal stress (Postgate, 1976). Indeed, processes analogous to apoptosis (programmed cell death) in eukaryotic cells may also occur in prokaryotic cells. For example, it has been suggested that the death of the mother cell during endospore formation in *Bacillus* and the sacrifice of many cells during fruiting body formation in *Myxococcus* might be considered as examples of programmed cell death (Lewis, 2000). The plasmid-encoded toxin–anti-toxin systems found on bacterial plasmids provide another example (Yarmolinsky, 1995). Regulation of the expression of the genes encoding the toxin and the

anti-toxin, and hence the ratio of these proteins in individual cells, will control the proportion of live cells in a population of bacteria harbouring the plasmid. Under certain conditions, cell death can be regarded as a complex multi-step mechanism aimed at sacrificing some bacteria in the population so as to release nutrients permitting the survival of others (Finkel and Kolter, 2001).

This review will focus on the long-term strategies that bacteria employ to avoid stress, with special emphasis on those that involve the formation of (transiently) "non-culturable" cells.

## 2. BACTERIAL STRESS AVOIDANCE STRATEGIES

### 2.1. Starvation Survival

#### *2.1.1. The Longevity of Bacteria*

The question of how long an individual organism can survive (according to any sensible definition of that verb, which in microbes implies the ability to replicate upon transfer to favourable conditions) is a very vexing one. It is of course of great importance to our Culture Collections, as this determines how often strains must be sub-cultured, the kinetics of die-off, the benefits of low water activity, of low oxygen tension and of suitable additives in maintaining culturability, and so on, but this is not the place to discuss these matters. However, it is appropriate to discuss briefly some of the issues. Given the comparative recency of microbiology as a discipline – let us say a century or two – it is obvious that no laboratory study can shed serious light on the question of whether microorganisms can in fact survive (to produce daughter cells) for centuries, millennia or even beyond. The current record claim (Vreeland *et al.*, 2000) (see that article for references to earlier work) points at 250 million years for an organism isolated from a salt crystal. These authors also sequenced the rDNA of the organism with a view of establishing its lineage, which data in fact cast substantial doubt on such longevity (Nickle *et al.*, 2002). Of course, the main issue in such studies (as in the isolation of other microbes from complex present-day samples (Schut *et al.*, 1993) is to exclude contamination with modern organisms. This is extremely difficult (Keilin, 1959) (see other articles in the anthology edited by Crowe and Clegg (1973)), and the true (maximum) longevity of bacteria remains unknown.

However, if we assume exponential die-off kinetics, a starting population of interest of $10^{10}$ cells, and a half-life of even 10 years, we lose 90 percent of

the cells every 33 years; our $10^{10}$ cells have (obviously) then lost culturability in a rather modest 330 years. Even with this hefty inoculum of cells and a highly optimistic half-life of 100 years – well beyond anything ever measured in the laboratory – there are essentially no cells left after ca. 3000 years. The kinetics are simply against one here. The argument that extreme conditions *in vitro* may be tolerable under the unknown – and more organised – conditions *in vivo* certainly has merit, and the generally accepted record for thermophilic growth in the laboratory (at temperatures well above 100°C (Huber *et al.*, 2000)) challenges the textbook notion that such temperatures would be expected to cause the hydrolysis of nucleic acids at an alarming rate, inconsistent with the doubling time of such organisms. Such arguments have indeed equivalently been made in the context of claims of a much higher temperature for microbial growth near 'black smokers' (Baross and Deming, 1983; Trent *et al.*, 1984).

Actually, a subtler issue of longevity as it pertains to individuals in a particular population hinges upon whether the causes of 'death' are entirely endogenous (e.g. thermally induced protein denaturation or hydrolysis) or have a more or less controlling exogenous cause (cosmic rays, reaction of the cells with oxygen radicals, etc.). In reality it must be the latter, since this determines the effective size of the inoculum considered as the starting population for the purposes of the kinetic argument. Our present view is that the likely reasonable survival half-life under optimal conditions (in the absence of nutrient influx, turnover and regeneration) of a microbe is between $10^1$ and $10^2$ years, but not greater.

### *2.1.2. Experimental Starvation Survival*

Leaving aside these considerations based on a quasi-geological timescale, it is nevertheless recognised from experiment that microorganisms can survive starvation for protracted periods (Kjelleberg *et al.*, 1993), possibly with half-lives exceeding 30 years (Eisenstark *et al.*, 1992). This specific strategy, called starvation survival (Morita, 1990, 1997), has been documented in many different bacteria including *Escherichia coli* (Kolter *et al.*, 1993), *Salmonella typhimurium* (Spector, 1998), *Vibrio* spp. (Ostling *et al.*, 1997), *Listeria monocytogenes* (Herbert and Foster, 2001), *Staphylococcus aureus* (Watson *et al.*, 1998a) and *Mycobacterium tuberculosis* (Betts *et al.*, 2002). Starvation survival shows several important physiological features that are equally applicable to survival of other kinds of stress:

- Shut-down of metabolic processes related to cell multiplication and growth;

- Use of specialised compounds and structures to enable cells to maintain their integrity and protect important cell components;
- Maintenance of culturability (ability to grow in liquid and on solid media) so that cells can quickly recover and resume active growth as soon as nutrients become available or the stress is relieved.

The transition from active metabolism (characteristic of dividing cells) to maintenance metabolism is an important feature of starvation survival. Valuable resources are conserved by the cessation of growth, but organisms retain a relatively high level of metabolic activity to support core metabolism and specific repair processes, commensurate with maintenance of cell integrity and culturability (Morita, 1990). When initially discovered, the *stringent response* was thought to be a specific attribute of the protein synthetic machinery, allowing the shutdown of protein and RNA synthesis when amino acids are not freely available (Gallant and Cashel, 1967; Cashel, 1975). The stringent response is activated when ppGpp is synthesised as a result of the binding of uncharged tRNA molecules to the ribosome. A ppGpp-independent mechanism that reduces the level of translation during amino acid starvation has also been reported (Christensen *et al.*, 2001) and it is now accepted that ppGpp is a general stress *alarmone*, encompassing many diverse physiological processes. For example, ppGpp is produced when fatty acid synthesis is disrupted (Gong *et al.*, 2002) and it controls the complex system of "competition" between different sigma factors, depending on the environment (Jishage *et al.*, 2002). It also regulates the virulence of some bacteria (Bachman and Swanson, 2001) and is required for long-term survival of pathogenic mycobacteria under starvation conditions (Primm *et al.*, 2000). The stress alarmone, ppGpp, also regulates the production of colicins under conditions of nutrient deprivation (Kuhar *et al.*, 2001), which leads to death and lysis of some cells in the bacterial population, supplying others with nutrients.

Another regulatory mechanism characteristic of starvation survival is a general increase in the half-life of mRNA (Takayama and Kjelleberg, 2000). Moreover, during extended starvation some transcripts remain stable but silent; they are immediately available for translation when a suitable substrate for growth becomes available (Marouga and Kjelleberg, 1996).

Despite the fact that starvation survival consumes relatively little energy (as compared with the energy demands for growth and multiplication), prolonged starvation under these "low maintenance" conditions is or can be accompanied by the occurrence of irreversible changes to some cells, including some damage to important cellular components such as DNA,

RNA, proteins and lipids (Hood *et al.*, 1986). This may be the reason why the survival of some cells in a starving population is often accompanied by the death of others (so-called 'cryptic growth' (Postgate, 1967, 1976)). Nutrients released from dead bacteria can be used by other cells, permitting their survival. In a glycerol-limited culture of *Klebsiella aerogenes*, death by starvation of 50 individuals was necessary to permit the multiplication of one survivor in buffer lacking nutrients (Postgate and Hunter, 1962). In this study, it was shown that the ratio of multiplying/surviving/dying organisms depended on the particular conditions of starvation to which they were subjected. This population behaviour underlies the phenomenon of cryptic growth and may be considered as a kind of cannibalism (Postgate, 1976). Starvation survival must therefore be regarded as a temporary measure, at least under these conditions; owing to the gradual accumulation of damage, cells cannot remain in this state for protracted periods. When in suspension (as opposed to being dehydrated), they must either exit to resume active growth and multiplication, or they will die (i.e. lose culturability in an irreversible sense).

Many laboratory models of nutrient starvation are based on the sudden transfer of copiotrophic organisms from a nutrient-rich environment, to one that is nutrient-poor. The organisms respond to this sudden stress using previously accumulated resources such as stored sources of energy (Dawes and Senior, 1973; Dietzler *et al.*, 1979) and phosphate (Rao and Kornberg, 1999; Kim *et al.*, 2002). In natural environments, most cell populations exist under the pressure of a "f(e)ast and famine" existence (Koch, 1971; Poindexter, 1981), with continuous exposure to environmental fluctuations and, frequently, multiple stresses. Cells in nature have adapted to a "low metabolic cost" existence, either genetically in the case of oligotrophs (Schut *et al.*, 1993; Morita, 1997; Schut *et al.*, 1997a,b), or physiologically in the case of copiotrophs. Both in the laboratory and in nature, organisms are preoccupied with accumulating resources for their next multiplication; this is generally more difficult in nature than in the laboratory. Moreover, under laboratory conditions the population density is usually very high, supporting cryptic growth or cannibalism, whereas when existing in a planktonic form in nature populations are either widely dispersed or, if localised, the effective population size may be comparatively small. In nature, there is therefore strong selective pressure for cells that have developed mechanisms permitting a low metabolic cost existence. Adoption of the TNC state in natural environments is easily understood within the conceptual framework of a low metabolic cost existence and may in fact be a necessary prerequisite for the successful colonisation of many natural habitats.

Bacterial endospores represent an extreme adaptation to a low metabolic cost existence, i.e. profound dormancy (Gould and Hurst, 1969). The low metabolic cost existence adopted by non-sporulating organisms in natural environments may be viewed as a state akin to dormancy, which has been defined as "a reversible state of low metabolic activity, in which cells can persist for extended periods without division" (Kaprelyants *et al.*, 1993). Both sporulation and dormancy are reversible states of metabolic shutdown, accompanied by loss of immediate culturability. We shall now focus on the TNC state.

## 2.2. Formation of "Non-culturable" Cells

It is well known that the number of cells which can be observed microscopically in natural environments often exceeds by orders of magnitude the number which can be recovered and cultured (e.g. Amann *et al.*, 1995; Torsvik *et al.*, 1996; Head *et al.*, 1998; Torsvik *et al.*, 1998; Barer and Harwood, 1999; Rondon *et al.*, 2000; Curtis *et al.*, 2002; Kell *et al.*, 2003). It is equally well documented that bacteria can enter states in which they have lost culturability but still retain measurable metabolic activity of some kind. These have been referred to as so-called "viable but non-culturable" (VBNC) forms (Roszak and Colwell, 1987). The terminology used to describe these forms is both confused and confusing; this, together with the heterogeneity of the cultures and the need for improved experimental designs – including in particular the use of 'dilution to extinction' methods – has been stressed before (Kell *et al.*, 1998; Barer and Harwood, 1999). We shall adopt the terminology proposed by these authors, using the term "non-culturable" in an operational sense. A more precise term would be "not immediately culturable" (NIC) (Kell *et al.*, 1998), but since the former is still in common use, we retain it here. The class of "non-culturable" bacteria is by now demonstrably a "rag-bag" of organisms displaying different physiological characteristics, which are discussed in turn in the following paragraphs.

Cell integrity is the most obvious characteristic. If the cell envelope or any other major cell components are irreparably damaged, then despite their apparent integrity, such injured cells are manifestly not viable and therefore they cannot be cultured. Cell integrity can be investigated using a variety of conventional techniques such as electron microscopy or by staining with propidium iodide (Davey and Kell, 1996) and there are even so-called "live/dead" kits available for this specific purpose. Luna *et al.* (2002) reported that only 30% of apparently intact cells, isolated from

sediment, had an intact permeability barrier. "Non-culturable" forms usually have an altered cell morphology, a reduced size, a coccoid shape and a thickened cell wall (Oliver *et al.*, 1991; Turpin *et al.*, 1993; Mukamolova *et al.*, 1995c; Shleeva *et al.*, 2002; Signoretto *et al.*, 2000, 2002). The size reduction plausibly confers a reduced maintenance energy requirement. These morphological changes, and the biochemical changes that underlie them, contribute to the increased resistance of "non-culturable" cells to different kinds of stress. "VBNC" cells of *Pseudomonas fluorescens* were more resistant to heating than were actively growing cells (Evdokimova *et al.*, 1994; van Overbeek *et al.*, 1995), and "VBNC" cells of *Vibrio vulnificus* were less sensitive to sonication than actively growing cells (Weichart and Kjelleberg, 1996). However, sometimes the opposite (i.e. increased sensitivity to a challenge) has been observed (Biketov *et al.*, 2000; Johnston and Brown, 2002). Moreover, it is not clear if "VBNC" cells really are more resistant to stress factors in natural environments, such as soil (Mascher *et al.*, 2000).

Another important criterion of the TNC state is that these cells are in an altered metabolic state with reduced respiratory activity. There are many controversial indications on this matter (Gribbon and Barer, 1995). It has been shown that "non-culturable" cells of *Micrococcus luteus* have a level of respiratory activity that is below the limit of detection using CTC (Kaprelyants and Kell, 1993). In the case of *Pasteurella piscicida* (Magarinos *et al.*, 1994) and in *Yersinia ruckeri* (Romalde *et al.*, 1994), the respiratory activity of "VBNC" cells was reduced by more than 80% compared with that associated with growing cells. However, in most reports concerning the (or a) "VBNC" state, detectable respiratory activity was used as a or even the criterion of "viability" (see, for example, Oliver and Wanucha, 1989; Garcia-Lara *et al.*, 1993; Morgan *et al.*, 1993; Boucher *et al.*, 1994; Ramaiah *et al.*, 2002). Since the products of CTC and tetrazolium reduction accumulate with time, these compounds are very sensitive indicators of respiratory activity. On the other hand, some cells referred to by the authors describing them as "VBNC" have metabolic activity similar to that of culturable bacteria and, in our opinion, such cells cannot be considered as being in a TNC state. Measurement of membrane energisation (often referred to as "the membrane potential") is another way to assess metabolic activity (Kaprelyants and Kell, 1992; Shapiro, 1995a; Davey and Kell, 1996; Davey *et al.*, 1999; Shapiro, 2000). Sometimes, cells stained with CTC have a significantly reduced level of membrane energisation and adenylate energy charge (Tholozan *et al.*, 1999).

The potential to (re)activate metabolism has often been considered as one of the major criteria for viability, especially when assessing microbes

in natural environments *in situ* where culturability is hard to observe. To this end, the direct viable count (DVC) method developed by Kogure and collaborators has been widely used to distinguish between viable and non-viable (dead) cells (Kogure *et al.*, 1979). After incubation in medium with a low concentration of yeast extract in the presence of nalidixic acid (to prevent multiplication), cells are stained with acridine orange. Non-culturable cells activated by yeast extract are stained orange (because of RNA accumulation) and they increase their size, sometimes producing filaments. Dead cells do not respond. It is worth mentioning that this technique does not simply show the metabolic activity of "non-culturable" organisms. It demonstrates their "resuscitation" potential, i.e. their ability to restore metabolism, and recommence growth when stimulated by the addition of nutrients. Because of the presence of nalidixic acid it cannot of course tell us whether such cells would have multiplied in its absence. The DVC method, developed originally for use with Gram-negative bacteria, has been adapted for use with Gram-positive bacteria. For example, ampicillin was used (instead of nalidixic acid) for *M. luteus* (Kaprelyants *et al.*, 1994) and ciprofloxacin was used for *L. monocytogenes* (Besnard *et al.*, 2000). The use of reporter gene fusions has also been proposed for estimating the "viability" of some "non-culturable" microorganisms (Bloemberg *et al.*, 1997; Cho and Kim, 1999a,b). As shown by Lowder and colleagues, GFP was quite stable in starving cells of *P. fluorescens* and its fluorescence could be related to the number of so-called "VBNC" cells (Lowder *et al.*, 2000). A modified, less stable version of GFP has also been used and a good correlation between metabolic activity and GFP fluorescence was noted (Lowder and Oliver, 2001).

PCR has also been employed to detect "non-culturable" cells of many organisms (Domingue and Woody, 1997), including e.g., *M. tuberculosis in vivo* (de Wit *et al.*, 1995; Hernández-Pando *et al.*, 2000), *S. typhimurium* under laboratory conditions (Romanova *et al.*, 1996) and *Campylobacter* spp. in water samples (Moore *et al.*, 2001). DNA stability is evidently one of the key criteria for viability. However, PCR products can be obtained from partially degraded DNA released from dead cells (Weichart *et al.*, 1997) and this method must therefore be employed with great care. Since prokaryotic mRNA generally has a very short half-life (and certainly compared with that of DNA), RT-PCR represents a less equivocal method. It has been employed to detect "non-culturable" cells of *E. faecalis* (del Mar Lleo *et al.*, 2000), *Helicobacter pylori* (Nilsson *et al.*, 2002), *M. tuberculosis* (Hernández-Pando *et al.*, 2000; Pai *et al.*, 2000) and *V. vulnificus* (Fischer-Le Saux *et al.*, 2002).

As is implied by their name, "non-culturable" organisms cannot be cultivated using conventional media and techniques. They should not be grouped together with injured organisms, which can grow under these conditions, but not in the presence of selective agents, such as detergents (Ray and Speck, 1973). An extremely important feature of (transiently) "non-culturable" cells, which serves to distinguish them from dead cells, is reversibility, i.e. their ability to convert to normally dividing cells under conditions permitting growth. This process, called "resuscitation" is discussed later. Finally, (transient) "non-culturability" as a survival strategy cannot simply be inferred from an inability to produce colonies or cultures. A convincing case can be made only when organisms are shown to conform to the various criteria mentioned above (i.e. cell integrity, metabolic shutdown, and reversibility/ability to resuscitate).

"Non-culturable" bacteria can be found almost everywhere in nature (soil, air, sea, estuarine waters, etc.), in other living organisms and in foods. They can also be obtained in the laboratory. It has been suggested that the "non-culturable" forms of pathogenic bacteria that are found in natural environments are perfectly able to cause disease when re-introduced into their host (Oliver and Bockian, 1995; Colwell *et al.*, 1996; Rahman *et al.*, 1996; Chaiyanan *et al.*, 2001) and this issue can obviously give cause for serious concern (Kogure and Ikemoto, 1997; Islam *et al.*, 2001). However, all of these experiments have been done under conditions in which the ostensibly "non-culturable" organisms have in fact been a mixture of culturable and non-culturable cells. Only when the experiment is done under what amounts to MPN conditions of dilution to extinction (i.e. one pathogenic cell as the inoculum into the host) can this conclusion be considered unequivocal (Kell *et al.*, 1998; Barer and Harwood, 1999). On the one occasion to date in which such an important experiment has been performed (Smith *et al.*, 2002), the conclusion was that such "unculturable" cells did not retain pathogenicity. We now consider what may be learned about the TNC state from analyses of "non-culturable" forms in these different environments.

### *2.2.1. "Non-culturable" Cells in Natural Environments*

The vast majority of the microbial biota in natural environments has never been brought into culture, as revealed initially by the renaturation kinetics of DNA isolated from such environments (Torsvik *et al.*, 1990) and subsequently by sequencing of PCR products obtained using primers corresponding to their 16S rRNA (Torsvik *et al.*, 1990; Ward *et al.*, 1990).

For example, it has been estimated that the diversity of the total community in a particular soil was at least 200 times higher than the diversity of bacterial isolates cultured from the same soil (Torsvik *et al.*, 1998). Many of the dominant organisms found in such studies belong to new species and even genera (Kalmbach *et al.*, 1997). The reasons for this discrepancy are being widely discussed (Ward *et al.*, 1990, 1998; Amann *et al.*, 1995; Gobel, 1995; Kell *et al.*, 2003). It has been suggested that appropriate cultivation conditions have not been found for these organisms. Very recently, significant strides have been made in several laboratories, using co-culture (Kaeberlein *et al.*, 2002), gel microdroplets (Zengler *et al.*, 2002), diluted media (Janssen *et al.*, 2002) and polymeric growth substrates (Sait *et al.*, 2002) to coax previously uncultured organisms into laboratory cultivation. In our view, the main difficulty is to understand whether (i) the "missing" organisms are fastidious, with highly specialised growth requirements, or (ii) they are simply surviving in a "non-culturable" state, occasionally "breaking" it to produce progeny when environmental conditions permit. Of course, both hypotheses are likely to contribute to the truth in different circumstances.

The first hypothesis is supported by the existence of oligotrophs, whose cell form, size, metabolism and life cycle are adapted to extremely nutrient-poor conditions (Hirsch, 1986). They have specialised, high-affinity uptake systems for scavenging nutrients from environments in which they are extremely scarce, enzymes with extremely high affinity for their substrates, and a minimal complement of *rrn* operons and ribosomes (Fegatella *et al.*, 1998). Oligotrophs are widespread and abundant in natural environments and can be found in water sources, soil and even within other living organisms (Wainwright *et al.*, 1991; Tada *et al.*, 1995). Because of their sheer abundance and their metabolic capabilities, they play a cardinal role in global recycling of nitrogen, sulphur and other inorganic materials (Wainwright *et al.*, 1991). It has been suggested that some of the so-called 'ultramicrobacteria' (i.e. tiny bacteria that pass through 0.45 μm filter) may also be oligotrophic organisms (Anderson and Heffernan, 1965; Torrella and Morita, 1981). However, more recent evidence (Schut *et al.*, 1993; Morita, 1997; Schut *et al.*, 1997a,b; Fegatella and Cavicchioli, 2000) indicates that at least some such 'ultramicrobacteria' are not 'small forms of normal (i.e. copiotrophic) bacteria' but 'normal forms of small bacteria' (Kaprelyants *et al.*, 1993). A recent investigation of one ultramicrobacterium showed that it had a very diverse protein complement, despite having a small-sized genome (only 1.5 Mb), suggesting that gene expression in these organisms may involve substantial co- and post-translational modifications of proteins (Fegatella and Cavicchioli, 2000). But in many cases,

it is still not clear whether filterable cells are specialised forms of known bacteria, or a degenerate stage in their life cycle, or whether they represent a group of microorganisms that has not previously been described (Zweifel and Hangstrom, 1995; Haller *et al.*, 2000). Finally, some copiotrophic organisms, such as *Vibrio* spp., can produce filterable cells under starvation conditions (Novitsky and Morita, 1976).

However, there is also substantial support for the second hypothesis. There are many examples in which "non-culturable" forms of known copiotrophic bacteria have been found in natural environments (Magarinos *et al.*, 1994; Kalmbach *et al.*, 1997; Kozdroj and van Elsas, 2001). These cells could not be cultivated but showed reactivation in the presence of nalidixic acid and yeast extract (by DVC). Non-culturable cells of *Yersinia pseudotuberculosis* have also been detected in soil by PCR (Troitskaia *et al.*, 1996).

The mechanism of production of "non-culturable" cells in nature, and the factors influencing it, are still unknown. Like sporulation, the formation of "non-culturable" cells can be induced by various stress factors (pH, toxic compounds, lack of oxygen and nutrient limitation). Sometimes, a combination of different factors unique for a particular ecological niche determines the strategy of bacterial survival. For example, *P. fluorescens* cells produced "VBNC" forms in some soils, but not in others (van Overbeek *et al.*, 1995). It is also possible that there is a seasonal dimension to the cycle of culturability/non-culturability. For example, it has been claimed that *Vibrio* cells produce only "non-culturable" cells when the water temperature in lakes and rivers drops below 10°C (see Oliver *et al.*, 1995; Weichart and Kjelleberg, 1996). Complicated interactions between different species, including host–parasite interactions (Romalde *et al.*, 1994), symbiotic associations (Lee and Ruby, 1995; Islam *et al.*, 1999) or co-existence (Didenko *et al.*, 2002), have great impact on bacterial survival strategies. Human activity can also influence significantly the diversity and physiological state of environmental bacteria. The introduction of heavy metals, disinfectants and other polluting compounds into natural environments inevitably influences bacterial culturability (Mascher *et al.*, 2000; Pitonzo *et al.*, 1999). In view of the uncertainty of the state of such complex environments, it is not possible to take into account all conceivable factors that may influence cell culturability in Nature. Therefore, defined laboratory models have been developed to simplify the problem and permit analysis of the relevance of different possible factors and mechanisms leading to "non-culturability". In the laboratory, pure bacterial cultures (or simple mixtures) are usually studied (although we note encouraging "large-scale" developments in post-genomics experiments (Giaever *et al.*, 1999)).

This imposes a serious limitation, since pure cultures of individual microorganisms are seldom if ever found in nature. On the other hand it provides a distinct advantage, since individual organisms can be properly characterised physiologically.

### *2.2.2. "Non-culturable" Cells in Aqueous Laboratory Microcosms*

Microcosms have been widely used for studying environmental bacteria under laboratory conditions. In some cases, growth media have been formulated using a defined mixture of components to mimic as closely as possible the natural environment (artificial microcosm). In others, water is simply taken from natural environments (and in the better experiments is filter-sterilised). Autoclaving of the natural substrates, which may result in the release of readily available nutrients, often prevents the formation of "VBNC" cells (Garcia-Lara *et al.*, 1993). A similar effect has also been observed in relation to sporulation of streptomycetes in soil (Mayfield *et al.*, 1972). A very important feature of these microcosm experiments is that a low inoculum of organisms should be employed, effectively excluding cryptic growth. Many different conditions have been used to produce "non-culturable" organisms. In many cases, starvation *per se* in a microcosm does not cause loss of culturability on a timescale of weeks. For example, in *V. vulnificus*, incubation temperature is the most important factor. Starving cells do not lose culturability at 24°C but similar cells incubated in the same buffer at 4°C lose viability rapidly, and produce an ostensibly homogeneous population of "VBNC" cells in 3 weeks (Weichart *et al.*, 1992). Similar results have been reported for many organisms, including *Vibrio anguillarum* (Eguchi *et al.*, 2000), *E. faecalis* (Lleo *et al.*, 2001), *Aeromonas hydrophila* (Wai *et al.*, 2000; Mary *et al.*, 2002), *Salmonella enteritidis* (Chmielewski and Frank, 1995), *H. pylori* (Shahamat *et al.*, 1993) and *Campylobacter* spp. (Thomas *et al.*, 2002). Remarkably, in the case of *V. vulnificus*, temperature but not starvation was the factor that "triggered" the transition to non-culturability (Oliver and Wanucha, 1989). *E. coli* cells incubated in a microcosm exposed to visible light also produced "VBNC" forms (Arana *et al.*, 1992). A combination of desiccating conditions and starvation induced the formation of "VBNC" forms in *S. typhimurium* (Lesn *et al.*, 2000). In some cases, mild stress did not cause a transition to the "non-culturable" state, whereas a stronger stress did. For example, "non-culturable" cells of *P. fluorescens* were produced when they were either exposed to a combination of low redox potential and oxygen limitation or a high

level (1.5 M but not 0.7 M) of NaCl (Mascher *et al.*, 2000). Addition of $CuSO_4$ to starving bacteria was also claimed to have induced a "VBNC" state (Ghezzi and Steck, 1999; Grey and Steck, 2001a), but (given the well-known bactericidal effects of heavy metals) it is more likely that the cells were simply killed, as has been found in other cases where mixtures of cells in different physiological states are studied indiscriminately (Bogosian *et al.*, 1998, 2000; Kell *et al.*, 1998; Nystrom, 2001). We remain puzzled by the apparent unwillingness of experimenters to perform these studies of culturability under conditions of dilution to extinction. As will be clear from the foregoing, it is not possible to define a universal procedure for induction of the "non-culturable" state. The precise conditions required for its establishment vary from one organism to another, presumably reflecting their different genetic, biochemical and physiological characteristics. Generally speaking, bacteria seem to lose culturability under conditions when they cannot initiate a starvation survival program. This may occur if a severe stress is encountered, or if they are simultaneously subjected to a combination of several different stresses, or even as a result of prolonged exposure to conditions that are not ideal for bacterial survival. Moreover, if the starvation survival program has already been initiated (for example, by pre-starvation at 24°C before "cold induction", in the case of *V. vulnificus*), cells do not then lose culturability (Weichart *et al.*, 1992). We still do not know if the examples described above represent specialised forms or simply dying cells. Exposure to a sudden stress without pre-adaptation, poor recoverability and, often, a lack of metabolic reorganisation, may suggest that "VBNC" (or dead) forms frequently arise as a result of a process of gradual cellular degradation.

### *2.2.3. Stationary Phase Culture as a Model for Induction of "Non-culturability"*

Compared with the microcosms discussed above, the stationary phase of batch culture in the laboratory is a very different model for studying the physiological processes that induce "non-culturability". It allows bacteria to be studied under complex conditions in a multicellular community, in which cell–cell interactions occur. The stationary phase in laboratory batch culture does not have a real counterpart in natural environments because in the laboratory, homogeneous populations of cells of a single species reach a comparatively high population density (often under conditions of nutrient excess). Many environmental factors (oxidative stress, low or high pH, accumulation of toxic compounds, lack of nutrients) are changing as

bacteria enter stationary phase, to which they are forced to adapt simultaneously. Most of these stresses arise as an indirect result of the metabolic activity of the cells themselves. The "artificial" stationary phase laboratory model makes it possible to explore the complete adaptive potential of different organisms.

Entry into stationary phase is therefore a gradual process of adaptation to an increasingly hostile environment. The so called "transition state" from exponential to stationary phase has been extensively characterised in model organisms, such as *Bacillus subtilis* and *E. coli* (Kolter *et al.*, 1993; Siegele and Kolter, 1993; Strauch and Hoch, 1993), from which it is clear that it represents a highly regulated, genetically programmed, developmental process during which the organisms change from one physiological state to another. By definition, bacteria cease to multiply when they enter stationary phase; however, as we shall see, the reason for this is not always obvious. In some cases, growth arrest is an irreversible process and cells cannot divide normally when re-inoculated into fresh medium (e.g. Weichart and Kjelleberg, 1996; Bogosian *et al.*, 1998; Kell *et al.*, 1998; McDougald *et al.*, 1998; Bogosian *et al.*, 2000) and this too remains largely enigmatic. We shall restrict our further consideration of stationary phase to this phenomenon of (ostensible) "non-culturability". As is our custom (Kaprelyants *et al.*, 1993; Kell *et al.*, 1998; Kell and Young, 2000), as well as the longstanding convention in microbiology (e.g. Postgate, 1969, 1976), we again equate the terms 'viability' and 'culturability' (the ability to multiply). We also recognise that these are not innate properties of the microbes themselves but a conflation of such properties (which are themselves inaccessible) with the experiment that is actually performed to assess them (Kell *et al.*, 1998), a phenomenon equivalent to the "Schrödinger's Cat" paradox in quantum mechanics (Primas, 1981). The phrase "viable but non-culturable" is thus an oxymoron (Barer *et al.*, 1993; Barer and Harwood, 1999).

During stationary phase, the proportion of surviving cells (which may or may not retain their culturability) and of lysing organisms, depends on the medium used and on the culture conditions. In *E. coli*, the richer the growth medium, the more rapidly the cells lose their ability to produce colonies on plates (Vulic and Kolter, 2002). Oxygen availability is also an important factor. For instance, a *sigE sigS* double mutant of *S. typhimurium* lost culturability (measured by cfu) very quickly when it was cultured with shaking, but not when it was cultured without shaking (Testerman *et al.*, 2002). The simplest explanation for this behaviour is the damaging influence of oxygen in shaking culture, but it is also possible that cells need oxygen for initiation of the "death" program and in its absence,

they use a different survival strategy. Unfortunately, in many cases it is difficult to understand whether cells are dying or whether they are surviving as "non-culturable" cells.

One of the approaches frequently used is to study the stationary phase that is provoked by starvation for a known substance. In this type of model, cells are inoculated in medium containing a limiting amount of a particular nutrient (usually a carbon, phosphate or nitrogen source). Cells grow until they almost exhaust the limiting compound and they then enter stationary phase. Cells sense that the nutrient is about to become limiting before its complete exhaustion (Smeulders *et al.*, 1999), so adaptation to starvation is a more or less gradual process. Depending on the nature of the limitation, cells can adopt different survival strategies, using different metabolic pathways. In a nitrate-limited culture of *P. fluorescens*, dormant (i.e. reversibly "non-culturable") cells were observed, whereas they did not arise when the organism was carbon- or phosphate-limited (Evdokimova *et al.*, 1994). In the case of *Mycobacterium smegmatis*, significant differences in population behaviour under carbon-, phosphorus- or nitrogen-starvation were not observed (Smeulders *et al.*, 1999). Unfortunately, these authors did not specifically study the "non-culturable" cells (which were the majority class in their mixed populations) and the reason for their lack of culturability was not established.

The fact that bacteria are simultaneously exposed to multiple stresses and have to adapt to the gradual environmental changes that occur during stationary phase, may explain the dramatic difference observed in population behaviour in stationary phase as compared with that observed during starvation survival in buffer. For example, "non-culturable" coccoid forms were produced when *H. pylori* cells were exposed to starvation in either phosphate-buffered saline at 8 °C, or under prolonged incubation in stationary phase (Nilsson *et al.*, 2002). Moreover, in both cases, transcription of some genes (*vacA*, *ureA*, *tsaA*) could be detected by RT-PCR. But in contrast to cold-starved bacteria, a significant proportion of stationary phase cells revealed "degenerative" changes according to electron microscopy. Moreover, they also showed a more rapid decrease of their ATP pool. The authors concluded that cells subjected to cold starvation can survive in a "non-culturable" state for a longer period than those exposed to prolonged stationary phase (at least 3 months in the first case and up to 40 days in the second). Production of any such "non-culturable" cells in stationary phase is presently not known to occur readily in other species. For example, neither *S. typhimurium* (Turner *et al.*, 2000) nor *E. coli* (Weichart and Kell, 2001) nor *S. aureus* (Watson *et al.*, 1998b) produced "non-culturable" forms in prolonged stationary phase. Interestingly,

Ericsson and colleagues (Ericsson *et al.*, 2000) reported that fewer than 10% of the total number of cells in a stationary phase culture of *E. coli* maintained cell integrity. However, as was mentioned previously, both species can produce "non-culturable" forms under other conditions.

A difference in adaptation strategy for cells incubated in stationary phase, as compared with cells suddenly transferred to phosphate buffer, has also been reported for *M. tuberculosis* (Betts *et al.*, 2002). Under conditions of buffer starvation, activity of the respiratory chain was reduced dramatically over the first 96 hours, whereas it continued unchanged over the same period in stationary phase. These experiments showed that the "sudden" stress occasioned by transfer to phosphate buffer led to the adoption of a starvation survival strategy, in which cells did not lose culturability. In this altered metabolic state there was a slowdown of the transcription apparatus, energy metabolism, lipid biosynthesis and cell division, in addition to induction of the stringent response. These cells could survive in the apparent absence of major nutrients (i.e. they adopted a typical starvation survival strategy) and they were not sensitive to rifampicin. In contrast, in the so-called "dormancy" model of *M. tuberculosis*, developed by Wayne and defined by him as adoption of a state of "non-replicating persistence" (Wayne and Sohaskey, 2001), the state of the cell population is determined by oxygen unavailability (Wayne, 1976). Cells are cultivated in sealed tubes filled with medium (almost no head space). After gradual consumption of nearly all the available oxygen, cells transit to a synchronised non-dividing state, i.e. stationary phase (Wayne, 1977). They become resistant to rifampicin, but sensitive to metronidazole (Wayne and Sramek, 1994), indicating that they develop an "anaerobic" type of metabolism, using the glyoxylate pathway and lipids as source of carbon for energy production. Protein synthesis is also shut down in these cells (Hu *et al.*, 1998). However, they do not lose culturability on plates and resume growth immediately after oxygen input. In contrast, cells "suddenly" depleted of oxygen do not develop this "anaerobic" persistence strategy, but simply die (Wayne and Diaz, 1967). This shows again that cells need time and adequate resources successfully to implement a survival strategy. *M. tuberculosis* populations incubated in stationary phase for prolonged periods of time can also enter a state of "non-culturability". After growth without apparent limitation of oxygen or nutrients, a significant proportion of cells lose the ability to form colonies on plates. Some of these cells may be dead/lysed; others can be recovered after incubation in liquid medium in the presence of supernatant obtained from early stationary phase cultures (Zhang *et al.*, 2001; Shleeva *et al.*, 2002). A diagrammatic representation of the culturability of these bacteria in different physiological states is shown in Figure 1.

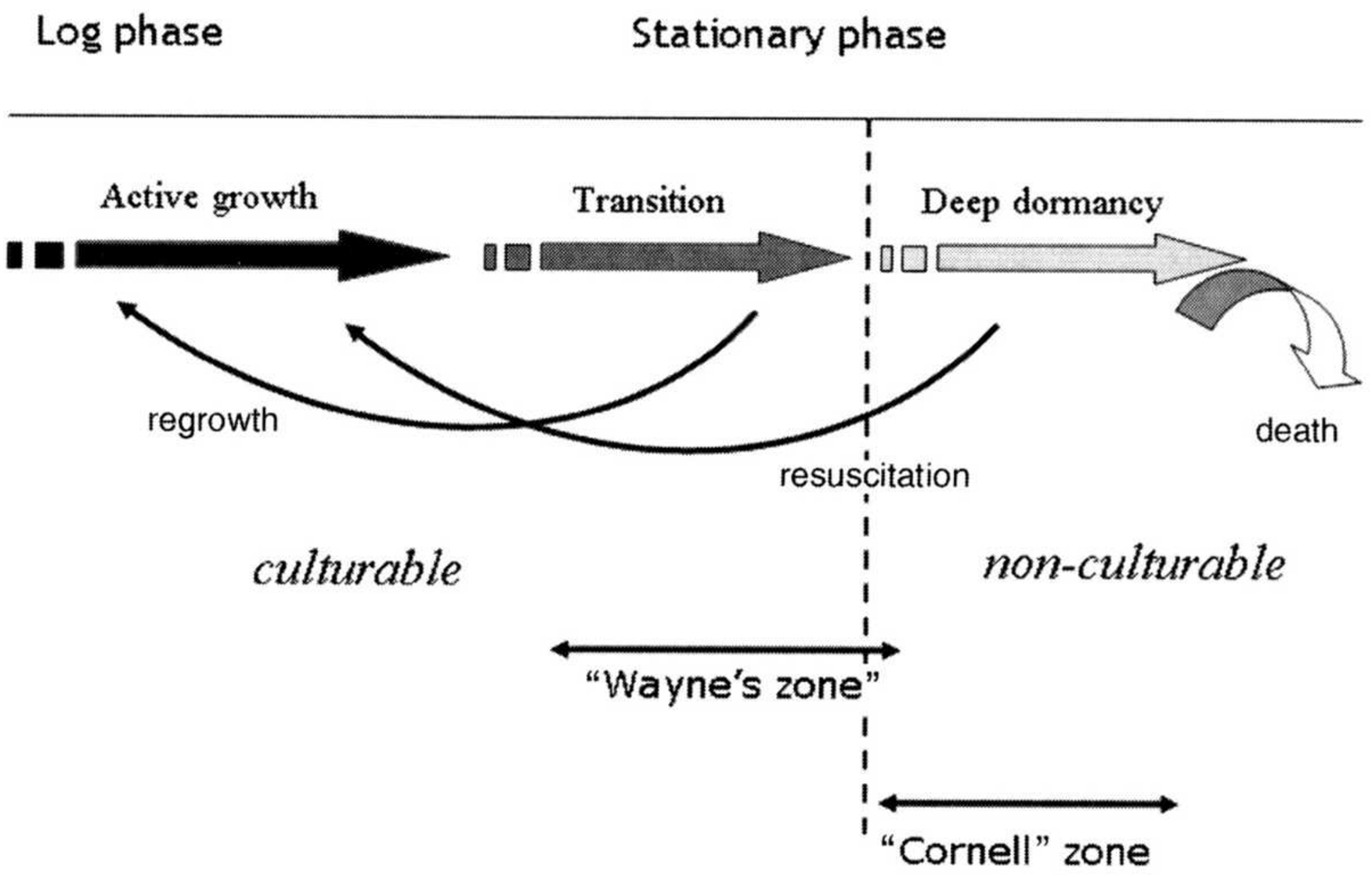


*Figure 1* A model depicting the transition of actively growing, viable *M. tuberculosis* to dormant forms during prolonged stationary phase. The intensity of shading of the arrows reflects the intensity of metabolic activity; small arrows indicate reversibility of transitions. The term 'non-culturable' is operational (Barer *et al.*, 1998) and refers, in this figure, to the inability of the bacteria to form colonies. Reproduced from Shleeva *et al.* (2002), with the kind permission of the publishers.

Most bacterial populations probably contain a mixture of culturable/non-culturable and viable/dead cells and the proportions of the different types will vary according to the culture conditions. This may depend on a single factor or on a combination of several different factors. For example, cultures of *M. tuberculosis* and *Mycobacterium bovis* (BCG) incubated in prolonged stationary phase (two months) in a sealed flask contained at least 99% of "non-culturable" cells, whereas similar cultures in "unsealed" flasks contained 99% of culturable cells. Variations in pH, medium composition, and airspace/liquid ratio again influenced the proportion of culturable versus "non-culturable" cells (Shleeva *et al.*, 2002; O.A. Turapov and D.I. Young, unpublished data). In summary, stationary phase is an example of a dynamic environment in which many complex stresses are being generated and responded to over an extended period of time. We suggest that this constellation of conditions favours a survival strategy that results in a loss of immediate culturability of surviving cells.

Another very important question is how stable are these "non-culturable" cells and how long can they exist in a reversible, "non-culturable" state? The answer depends on the organism under consideration. Some bacteria can survive in a "non-culturable" state for weeks or months, others for just a

few hours. This may be determined, in part, by the life style of the organism. Thus *M. tuberculosis* has a generation time of ca. 20 h and can survive in a non-culturable state for months (Sun and Zhang, 1999; Shleeva *et al.*, 2002) and possibly even years (Lillebaek *et al.*, 2002). However, the period of "non-culturability" is time-limited; after surviving for some critical period, at least in these planktonic conditions, the cell must eventually divide or die. It is possible that the fluctuations of culturability sometimes observed in stationary phase cultures (e.g. Ekweozor *et al.*, 1998; Shleeva *et al.*, 2002, and many unpublished observations) are determined by this necessity for division to occur after the survival time has reached its limit. In the case of *M. tuberculosis* after incubation for 4 months in sealed tubes, the population consisted entirely of "non-culturable" cells, but during the next few months, CFU numbers increased by up to 5–6 orders of magnitude (Shleeva *et al.*, 2002), usually accompanied by the lysis of some cells. The cells then started to lose culturability once again. This cyclical behaviour may permit populations to survive without added nutrients for many years (Corper and Cohn, 1933).

Any attempt to make general conclusions about the conditions that lead to the formation of "non-culturable" cells in stationary phase faces obvious problems. Indeed, it seems that each species probably represents a unique case and many conditions need to be explored to find those that are suitable. This can be illustrated by consideration of some detailed studies of the stationary phase behaviour of three fast growing Gram-positive bacteria, *viz. M. luteus*, *Rhodococcus* sp. and *M. smegmatis* (Kaprelyants and Kell, 1993; Mukamolova *et al.*, 1995b; Shleeva *et al.*, 2002). These organisms did not produce significant numbers of "non-culturable" cells in stationary phase under optimum conditions of cultivation (temperature, medium composition, aeration, etc.). However, if cells of *Rhodococcus* sp. were grown in a sub-optimal minimal medium, they transited quite rapidly to a "non-culturable" state in stationary phase. This was maintained for a period lasting only a few hours that occurred some 90 hours post-inoculation. During this period, the bacteria could not produce colonies or grow in liquid medium unless it was supplemented with culture supernatant obtained from actively growing cells. Several hours later, culturability was spontaneously restored. Inoculum age, medium composition, speed of flask agitation and culture volume in relation to the flask capacity significantly influenced the formation of non-culturable bacteria. For each culture, there was a window of several hours in stationary phase, during which *ca.* 99.9% of the bacteria lost culturability (Shleeva *et al.*, 2002). Similar results were obtained for *M. smegmatis* cells where a narrow window of "non-culturability", sensitive to medium composition and other parameters, has also been observed

(M. Shleeva and A.S. Kaprelyants, unpublished data). In contrast to *M. smegmatis* and *Rhodococcus* sp. cultures, where "non-culturability" was a transient phenomenon in stationary phase, the majority of cells of *M. luteus* persisted in a "non-culturable" state during a prolonged stationary phase lasting several months (Mukamolova *et al.*, 1998b; Kaprelyants and Kell, 1993). This timescale is intermediate between those of *M. tuberculosis* and *Rhodococcus* sp. "Non-culturability" in *M. luteus* is a particularly well-characterised example, in which some limited information is now available concerning the molecular basis of the loss and gain of culturability (see later). Again, like the two examples already mentioned, induction of the "non-culturable" state was achieved if cells were grown under sub-optimal conditions (in a lactate minimal medium). "Non-culturable" cells were not formed under starvation conditions (whether nitrogen-, carbon- or phosphate-starvation) for any of the three species tested. Possibly, cells grown in sub-optimal media have some sort of pre-adaptation to a "low cost metabolism", and at some stage adaptation reaches its extreme in the production of "non-culturable" cells. It is not yet clear whether lysis of some *M. smegmatis* or *Rhodococcus* sp. cells occurs during the phase of minimal culturability, which might serve as a signal for the observed spontaneous "resuscitation", or whether there are some other triggers that control the remarkable behaviour of these cells. It is also possible that "non-culturability" is a direct consequence of their continued existence under very poor growth conditions, i.e. the available nutrients are insufficient to support the normal metabolism and multiplication of all cells in the population. This situation can be modelled in chemostat culture. Loss of culturability has been reported, for example, in chemostat cultures of *Cytophaga johnsonae* (Hofle, 1983) and *Aerobacter aerogenes* (Tempest *et al.*, 1967). Under such conditions there is a tendency for cultures to 'differentiate', i.e. the more metabolically active cells are increasingly more effective at scavenging nutrients as they enter the chemostat such that the chemostat effectively becomes heterogeneous. Significantly, cells lost culturability much more quickly at a very low dilution rate. Similarly, in lactate-limited chemostat cultures of *M. luteus*, only 40% of cells were culturable, at a very low dilution rate of 0.01 $h^{-1}$ (Kaprelyants and Kell, 1992).

The inability to produce "non-culturable" cells in a rich medium can be explained by an imbalance between anabolism and catabolism (see Fig. 2). Cells in very rich medium grow at a maximal rate, supported by a maximal rate of metabolic activity. In a growing culture, catabolism and anabolism are balanced. When cells reach stationary phase, they shut down their main anabolic processes, mainly due to imposition of the

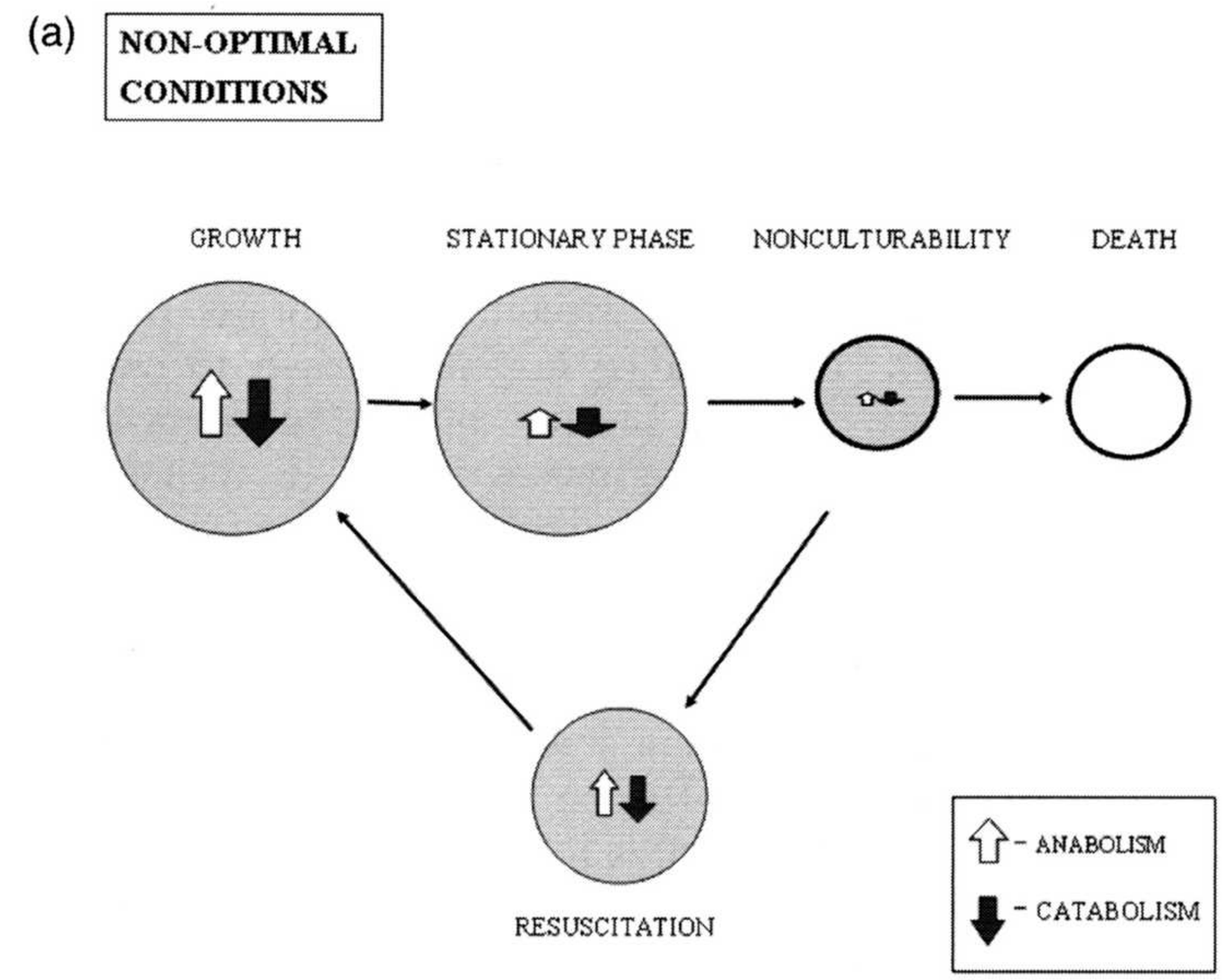


*Figure 2* Different mechanisms of loss of culturability of microbial cells operate under conditions that normally support slow (a) versus rapid growth (b). For detailed discussion, see the text.

stringent response, and induction of the RpoS-regulated global network (Cashel *et al.*, 1996). But these cells cannot reduce catabolic processes to the same extent as anabolic processes (Nystrom *et al.*, 1996), leading to the intracellular production of free radicals (Bloomfield *et al.*, 1998). If this unbalanced state is not rapidly rectified, free-radical scavenging mechanisms cannot cope adequately and cell structure deteriorates (Dukan and Nystrom, 1999; Nystrom, 2001), leading in extreme cases to lysis. (There is, perhaps, a parallel here with the phenomenon of "acid crash" that is seen when solventogenic clostridia growing in a rich medium fail to activate the metabolic switch from acidogenic to solventogenic metabolism, which would permit their long-term survival via the production of dormant endospores (Schuster *et al.*, 2001).) Such cells are "non-culturable" but metabolically active. Their "non-culturability" is a consequence of cell injury but not a low-metabolic cost existence, and they cannot therefore be considered to be in a TNC state.

The requirement for a medium that is not optimised for growth in order for a particular species to achieve a TNC state may explain why some bacteria do not produce "non-culturable" cells in stationary phase models. Such bacteria (e.g. *E. coli* and *Vibrio* spp., which can grow in almost any poor or rich medium) may have an extremely flexible metabolic potential, being able to adjust their metabolism to many different types of medium. In these organisms, low temperature serves to induce "non-culturability". The requirement for growth under sub-optimal conditions for transition to the "non-culturable" state could explain why, in some cases, mutant strains (e.g. auxotrophs) cultivated under conditions appropriate for the wild type, can display temporary "non-culturability". For instance, *purF* mutants of *M. smegmatis*, defective in stationary phase survival, revealed a transient decrease in culturability followed by an increase (Keer *et al.*, 2000; Keer *et al.*, 2001). The authors suggested two possible explanations of this effect: (i) re-growth (cryptic growth) of viable cells and (ii) formation of "non-culturable" (and possibly dormant) bacteria in stationary phase and their subsequent resuscitation. According to our studies, the second suggestion is correct, as such cells could be resuscitated (M. Shleeva and A.S. Kaprelyants, unpublished results). *M. smegmatis* is therefore a good example of an organism in which different experimental conditions result in the formation of the same "non-culturable" phenotype. Indeed, it is possible that an essentially identical mechanism underlies the production of "non-culturable" cells in all these cases. However, more work is required to verify this hypothesis (see below).

Finally, another important point should be considered. Populations in stationary phase are very heterogeneous, often containing a mixture of

different cells doing different things. Some may be in a state of starvation survival and others may be in a TNC state; some may be actively growing and others may be dying. This makes it difficult to design incisive experiments and is a source of considerable confusion in the scientific literature. Flow cytometric cell sorting of subpopulations (Davey and Kell, 1996) coupled to, for instance, microarray measurements (Schena, 2000; Betts *et al.*, 2002), should soon help to set out the magnitude of this type of problem, i.e. how differentiated such cultures actually are.

### *2.2.4. "Non-culturable" Cells in Vivo*

"Non-culturability" is probably one of the major strategies of bacterial survival *in vivo*. The environments encountered by bacteria *in vivo* are very complex and they face several lines of host defence. These include general stresses like exposure to low pH and oxidising agents, as well as the influence of specific antibacterial compounds produced by specialised cells (Deretic and Fratti, 1999; Flynn and Chan, 2001). Lack of nutrients and low oxygen availability are additional challenges that they must face and overcome if they are to survive (Wayne and Sohaskey, 2001). There are many examples of latency, in which bacteria adopt a "non-culturable" state *in vivo*. The mechanism of this latency has been discussed extensively, but remains poorly understood (Young and Duncan, 1995; Parrish *et al.*, 1998; Honer zu Bentrup and Russell, 2001; Wayne and Sohaskey, 2001). The organisms present in latent infections represent a reservoir of bacteria that could, in principle, reactivate and hence they have important implications for infectious disease and public health. It is still not clear if these persisting bacteria in latent infections are specialised forms, or if they are injured, or if they are simply "normal" bacteria that have been suppressed by the host immune system. The available evidence is controversial. The best-known example is afforded by the "non-culturable" forms of *M. tuberculosis* that have been found in lung lesions, sometimes in substantial numbers, but there is no evidence that these forms could be converted to normal multiplying cells (Wayne, 1960). Another important factor is the response of the bacteria following antibiotic therapy. The Cornell model of latency is based on the treatment of *M. tuberculosis*-infected mice with a mixture of antibiotics. After treatment, mice reach a so-called "sterile" condition during which no culturable bacteria are detectable. However, during the next 3 months without antibiotics, about one third of the mice go on to develop tuberculosis (McCune *et al.*, 1966a, b). There is substantial variation on this model, depending on the precise experimental procedure

that is employed (Scanga *et al.*, 1999). During the "sterile" state, when culturable organisms are absent, DNA equivalent to ca. $10^5$ bacteria per organ (lung or spleen) can be detected using PCR (de Wit *et al.*, 1995). This might represent residual DNA resulting from the lysis of dead cells, but recently the presence of "non-culturable" organisms has been confirmed by RT-PCR (Pai *et al.*, 2000). This is the first evidence that the "non-culturable" cells of *M. tuberculosis* present in the tissues are actively expressing genes and are thus metabolically active (and note too that even ostensibly "normal" lung tissue may contain mycobacterial DNA (Hernández-Pando *et al.*, 2000)). Since metronidazole had no activity, either in the initial sterilising phase or in the subsequent sterile state in the Cornell model (Dhillon *et al.*, 1998; Brooks *et al.*, 1999), the bacteria present (or at least some of them) do not have an anaerobic type of metabolism. Reactivation of *M. tuberculosis* was prevented by treatment with a combined course of isoniazid, pyrazinamide and rifampin (Dhillon *et al.*, 1996), but not by rifapentine monotherapy (Miyazaki *et al.*, 1999). It is possible that tuberculosis bacilli in the Cornell model are injured cells or L-forms, resulting from the specific action of the antibiotics used in this model, as has been suggested for human patients undergoing anti-tubercular chemotherapy (Khomenko *et al.*, 1980). These cells may require special recovery conditions. In fact, the presence of L-forms and non acid-fast bacilli *in vivo* has been reported (Khomenko and Muratov, 1993). Interestingly, cells of *M. tuberculosis* from late stationary phase treated with rifampin showed zero culturability on agar plates and about $10^2$ cells per ml in liquid culture (Hu *et al.*, 2000). It also has been shown that some *M. tuberculosis* cells recovered from macrophages were extremely sensitive to freezing/thawing and treatment with detergents (Biketov *et al.*, 2000). The current practice of decontamination for recovery of bacteria from sputum samples, involves harsh alkali treatment. Many culturable bacteria may become "non-culturable" as a result of injury during this procedure and they may need special stimuli to restore culturability. Significantly, the addition of a resuscitation-promoting factor increased the number of culturable cells by one or two orders of magnitude (Biketov *et al.*, 2000).

There are other examples of an apparent association between "non-culturable" bacteria and disease states. *H. pylori*, the causative agent of stomach ulcers (Marshall and Warren, 1984; Decross and Marshall, 1993), is detectable by PCR of biopsy samples, but not by other methods (culture, urease test, histology), for a substantial period of time after patients have been treated with antibiotics (Loginov *et al.*, 1999). Moreover, BALB/c mice orally infected with coccoid (non-culturable) forms of *H. pylori* developed gastric inflammation with the same severity as animals infected with spiral

(culturable) forms of this organism (Wang *et al.*, 1997). "VBNC" uropathogenic bacteria have also been found in the mouse urinary tract after chemotherapy (Rivers and Steck, 2001) although, again, the important "dilution to extinction" tests (see Kell *et al.*, 1998) were not performed. At all events, these types of reports suggest that some bacteria can survive *in vivo* as "non-culturable" cells.

Moreover, there is the additional complication posed by the existence of some infectious bacteria that have never been cultured *in vitro*. Apart from the well-known case of *M. leprae*, with its degenerated genome (Cole *et al.*, 2001), *Tropheryma whipplei* (or *whippelii*) which has never been cultured *in vitro*, is now considered as the causative agent of Whipple's disease (Lynch *et al.*, 1997; Fredricks and Relman, 2001; Maiwald *et al.*, 2001). Even for organisms that have been cultured from some sources, there are diseases in which such organisms have been implicated but from which they have not been brought into culture. Sarcoidosis may be related to infection by *M. tuberculosis* (Khomenko *et al.*, 1994) or *Propionibacterium* (Eishi *et al.*, 2002) and Eales disease may be caused by *M. tuberculosis* (Madhavan *et al.*, 2002). The main problem in all of these diseases is an inability to isolate the infectious agent in pure culture from patients and, hence, Koch's postulates cannot be fulfilled (Fredricks and Relman, 1996). The presence of bacteria has been indicated by PCR but, except in the case of Whipple's disease, there is the attendant risk of sample contamination. Another problem is the possibility of secondary infection. In some cases, the microorganisms isolated from patients appeared to be very specialised forms. For example, "dense bodies" were isolated from a patient with interstitial cystitis (Domingue *et al.*, 1993). These "bodies" have a unique morphology with multilayer protective envelopes. The authors suggested that these structures could be resting forms of bacteria. L-form bacteria have also been reported as the causative agents of severe urological diseases (Domingue and Woody, 1997). It is still not clear if some of these organisms are truly specialised forms, or if they are simply injured cells suppressed by the host's immune system. Nevertheless, two clear facts show the importance of atypical/"non-culturable" bacteria in medicine. One is that the causative agent of infection can still be found in patients even after strong chemotherapy and the apparent clearance of infection (Velayati *et al.*, 2002). The other is the occurrence of reactivation disease, which is well known in the case of tuberculosis (Dubrow, 1976; Kuznetsova, 1985; Flynn and Chan, 2001; Lillebaek *et al.*, 2002) and other diseases (Domingue and Woody, 1997). Despite ongoing controversy, it seems very likely that adoption of a TNC state does truly represent a survival strategy exploited by several pathogenic bacteria *in vivo*. It effectively makes them "invisible" to the immune system, resistant to

most (probably all) antibiotics, and since it is a low energy cost strategy, bacteria can survive for protracted periods in this state.

## 3. ARE THERE SPECIFIC CHEMICAL INDUCERS OF "NON-CULTURABILITY"?

In the preceding discussion we have developed the idea that "non-culturability" represents a survival strategy employed by bacteria as they gradually adapt to various stressful environments. By analogy with spore formation in bacilli, streptomycetes and myxobacteria (Losick and Kaiser, 1997), we may surmise that "non-culturability" is induced in response to the perception of specific chemical stimuli generated by cells as a result of the prevailing environmental conditions. For example, specific microbially produced peptides regulate the development of competence for transformation and sporulation in bacilli (Kleerebezem *et al.*, 1997; Lazazzera and Grossman, 1998; Kleerebezem and Quadri, 2001; Lazazzera, 2001; Morrison, 2002) and *N*-acyl-homoserine lactones are involved in density-dependent signalling in Gram-negative bacteria (e.g. Salmond *et al.*, 1995; Fuqua *et al.*, 2001; Whitehead *et al.*, 2002). Such signals act as pheromones (Stephens, 1986; Kell *et al.*, 1995). They induce a wide spectrum of different physiological processes including, for example, bioluminescence in *Vibrio harveyii* (Cao and Meighen, 1989; Meighen, 1994), virulence in *Pseudomonas aeruginosa* (Winson *et al.*, 1995), antibiotic production in *Erwinia* (McGowan *et al.*, 1995), biofilm development in *P. aeruginosa* (Davies *et al.*, 1998) and the cell division cycle in *E. coli* (Huisman and Kolter, 1994; Garcia-Lara *et al.*, 1996; Sitnikov *et al.*, 1996). It has been proposed that an *N*-acyl homoserine lactone may serve as a signalling molecule under starvation conditions in *E. coli*, leading to activation of the *sigS* gene (Huisman and Kolter, 1994). Later it was reported that the level of *sigS* expression in *E. coli* is positively controlled by a homocysteine thiolactone during stationary phase (Goodrich-Blair and Kolter, 2000), providing a possible link between production of this molecule and the growth arrest that occurs under these conditions. Homoserine lactones may activate the starvation-survival response in *Vibrio* sp. (Srinivasan *et al.*, 1998). These authors showed that halogenated furanones, which are putative antagonists of acylated homoserine lactones (Manefield *et al.*, 1999, 2002; Hentzer *et al.*, 2002), inhibited the synthesis of proteins specifically induced upon carbon starvation. Of course many other compounds, for which no signalling role has yet been clearly established, are known to be excreted from

microbial cells, especially under conditions of slow or zero growth and multiplication rates. These include uracil and xanthine (Rinas *et al.*, 1995), furanones (Slaughter, 1999) and the numerous bioactive molecules of both "primary" (e.g. Wittmann and Heinzle, 2001) and "secondary" (e.g. Abel *et al.*, 1999) metabolism, which are often of applied interest (Devlin, 1997). Indeed, the "global" measurement of culture supernatants using modern mass spectrometric procedures shows that a vast and largely uncharacterised panoply of substances are normally excreted, and the application of numerical deconvolution methods to such data allows one to discriminate strains which differ only in the presence of a single gene (Allen *et al.*, 2002).

There is as yet no really direct evidence for the existence of chemical inducers of "non-culturability", but there are indications that such molecules may exist. The accumulation of compounds in the external environment that inhibit bacterial growth during the transition to the "non-culturable state" has been reported (Weichart *et al.*, 1992) (as have the presence of molecules stimulating regrowth following transfer of *E. coli* cells from stationary phase to growth medium (Weichart and Kell, 2001)). Accumulation of toxic compounds could also be responsible for the transition to "non-culturability" in the case of *M. tuberculosis* (Sun and Zhang, 1999; Zhang *et al.*, 2001; Shleeva *et al.*, 2002), *Rhodococcus* sp. (Shleeva *et al.*, 2002) and *M. luteus* (Mukamolova *et al.*, 1995a). Indeed, so-called 'staling factors' have been known for many years (e.g. Barker *et al.*, 1983). These toxic compounds may be implicated in adoption of the TNC state. In *M. xanthus*, production of bactericidal derivatives of unsaturated fatty acids causes lysis of the majority of cells in the population during the formation of fruiting bodies and the release of spores (Varon *et al.*, 1984). Accumulation of some specific low molecular weight compounds is considered responsible for the "programmed" loss of culturability of *E. coli* cells in stationary phase (Vulic and Kolter, 2002). It is interesting that ethanol interferes with this process, since it also interferes with endospore formation in *B. subtilis* (Vulic and Kolter, 2002). The accumulation of low molecular weight compounds that suppress growth in stationary phase has also been reported in *S. typhimurium* (Barrow *et al.*, 1996). Alkylresorcinols are involved in cyst formation in *Azotobacter vinelandii*, suggesting that these compounds may act as regulators of "non-culturability" in this organism (Reusch and Sadoff, 1979). The addition of alkylresorcinols to growing cells of *Bacillus cereus* caused the formation of "refractile" spore-like "non-culturable" forms (Duda *et al.*, 1982). However, there is no clear evidence that these "non-culturable" forms can be resuscitated.

Alternatively, the toxic compounds listed above may provoke a "non-specific" form of chemical stress (like exposure to heavy metals, for example), which is known to cause the formation of "non-culturable" (or dead) cells (Grey and Steck, 2001a, b). These effects are observed only in high-density cultures. There is no evidence of accumulation of inhibitory compounds or of inducers of "non-culturability" in the diluted microcosm experiments discussed previously (although we note that non-planktonic bacteria in nature may co-exist at concentrations that are locally (effectively) very high). It is not clear whether these substances are natural physiological "waste" products of overflow metabolism (Neijssel and Tempest, 1976) or specific regulators of the culturability to "non-culturability" transition. Therefore, the important question of chemical induction of "non-culturability" – which has substantial implications for public health – remains unanswered, and more experimental research is required.

## 4. IS "NON-CULTURABILITY" GENETICALLY CONTROLLED?

We now consider whether adoption of the TNC state is associated with the expression of specific genes. Currently all that we can (or tend to) measure is the end result, i.e. "non-culturability". Until the processes that underpin the transition to, the maintenance of, and the exit from, the "non-culturable" state are better understood, we shall remain unable to provide a definitive answer to the question. Nevertheless, the simple fact that "non-culturable" cells show altered morphology, biochemistry and physiology, suggests that the TNC state is genetically controlled.

During the alternative process of starvation survival (see above) some genes are switched on and others are switched off, suggestive of control by a general starvation-induced program (Kjelleberg *et al.*, 1993) and it has also been argued that cell death can be genetically controlled (Vulic and Kolter, 2002). In addition, the adoption of several other physiological states is also genetically controlled (transition to a non-dividing state in stationary phase or under starvation conditions, development of resistance to different stresses). For example, an *arcA* mutant of *E. coli* was unable to reduce respiration and total metabolic activity in stationary phase (Nystrom *et al.*, 1996) and, as a result, it was severely impaired in its ability to survive prolonged periods of carbon starvation.

There are many reports showing induction of the synthesis of specific proteins depending on conditions of starvation. For example, cultures of *V. vulnificus* pre-starved at 24°C and then shifted to 5°C maintained

culturability at low temperature in a starvation-condition-dependent manner (Paludan-Muller *et al.*, 1996). This is probably related to initiation of the starvation survival program. A null mutation in the *smcR* (*luxR*) gene prevented starvation survival under these conditions (McDougald *et al.*, 2001). The physiological age of the cells also influenced the transition to "non-culturability" (Oliver *et al.*, 1991). There are many examples of changed biochemical and morphological characteristics of "non-culturable" cells. Moreover, it has been shown that some "non-culturable" cells can take up methionine and synthesize proteins (Rahman *et al.*, 1994). These processes presumably reflect the expression of particular genes. Additional evidence in favour of this interpretation has been obtained by Boshnakov *et al.* (2001) who identified several genes that are preferentially expressed in "VBNC" forms of *S. typhimurium*, using differential display technology. Very recently, different protein expression profiles have been found in starved vs. "VBNC" cells of *E. faecalis* (Heim *et al.*, 2002).

Further evidence for genetic control of "non-culturability" comes from analysis of other mutant strains. A mutation has been described that speeds up the transition of *V. cholerae* to some kind of "VBNC" state (Ravel *et al.*, 1994), although it is not clear what particular gene was disrupted in this mutant. In the case of *M. smegmatis*, a *purF* mutant lost culturability in stationary phase, whereas the wild type control did not (Keer *et al.*, 2001). The mutant cells subsequently regained culturability and it was established that this was not due to reversion. The intriguing behaviour of this mutant is not currently understood. Several mutants with an altered "VBNC" response have also been isolated in *S. typhimurium* (Romanova *et al.*, 1996). For example, an *lpf* mutant did not produce "VBNC" forms when incubated under conditions that normally lead to the adoption of a "VBNC" state. Disruption of *glgC* (glycogen metabolism) increased the period of survival in the "VBNC" state, whereas disruption of *pqi*, a paraquat-inducible gene that is induced under stress and controlled by the *sox*RS locus, and another gene of unknown function, altered the timing of the period of transition to the "VBNC" state.

One of the most difficult problems to be overcome in any study of gene expression in "non-culturable" cells is population heterogeneity. Cultures frequently contain mixtures of culturable and "non-culturable" cells, which can make it very difficult to ascribe quantitative changes to a particular cell type with any degree of reliability unless careful and quantitative arguments are made. Flow cytometry can be very helpful in this regard (Davey and Kell, 1996) but this technology is presently only applicable in cases where the genes under investigation are expressed at a comparatively high level. Homogeneous cultures (100% of cells in the population in the same

physiological state) are the best model for this type of study, but they are not readily obtained.

One example of such a nominally "homogeneous" culture is the so-called Wayne model of "dormancy" for *M. tuberculosis*, induced by gradual oxygen depletion *in vitro*, that was mentioned previously. Bacteria stop DNA replication and cell division in a synchronised fashion. However, they remain "culturable" because they resume synchronous division and growth immediately after oxygen input. These cells have an active but altered "anaerobic" type of metabolism, based on use of the glyoxylate pathway with fatty acids as carbon source. Microarray and proteomic analyses have confirmed that this "dormancy" model is more correctly viewed as an adaptive response to oxygen depletion. In cells from the Wayne model, there is strong up-regulation of genes encoding an $\alpha$-crystallin homologue thought to be a chaperonin, a putative response regulator Rv3133c, and two conserved hypothetical proteins Rv2623 and Rv2626c (Boon *et al.*, 2001). A similar set of genes is up-regulated during oxygen shift-down in actively growing cultures (Sherman *et al.*, 2001). The Wayne model therefore represents a clear example of bacterial adaptation to unfavourable growth conditions. *M. tuberculosis* cells in a starvation survival model showed a completely different profile of gene expression (Betts *et al.*, 2002). It has been suggested that during prolonged stationary phase *in vitro*, *sigJ*-regulated gene expression determines the survival strategy (Hu and Coates, 2001). It would not be surprising if yet another completely distinct strategy were involved in the survival of *M. tuberculosis in vivo*. Unfortunately, little is known about mycobacterial gene expression in the Cornell model of latency. Apart from the heterogeneity of cells, analysis is hampered by the poor availability of material.

It is equally important to understand the molecular basis of cell recovery/resuscitation from the "non-culturable" state. Recovery of *E. coli* after starvation was dependent on the expression of some specific genes (Siegele and Kolter, 1993) but, as yet, there is no clear evidence supporting the presumed existence of genes specifically required for restoration of culturability in other organisms.

## 5. RESUSCITATION OF "NON-CULTURABLE" CELLS

The important distinction between cells in the TNC state and cells that are "dead" is that only the former can be brought back into a culturable form, by a process called resuscitation. The distinction between cells that are "non-culturable" and those that are dead is, as with higher organisms

(Watson, 1987; Berger, 1992), purely operational (Kell *et al.*, 1998); there are undoubtedly many living organisms that we do not yet know how cultivate. (In these cases it is the microbiologist, rather than the organism, that would more correctly be regarded as "uncultured".) The main criterion for the presence of "non-culturable" cells is that under specialised conditions they can be coaxed back into a state where growth and division are resumed. The ability to resuscitate thus serves unequivocally to distinguish "non-culturable" cells from dead cells. Indirect criteria (metabolic activity, cell integrity, etc.) are not sufficient proof of viability (the ultimate ability to resume growth), and such measurements are a source of much confusion, especially when carried out in cultures consisting of mixtures of cells in different physiological states (Kell *et al.*, 1998). Resuscitation is almost certainly a very complex process, requiring restoration of many cellular functions, leading finally to the reacquisition of culturability (Kaprelyants and Kell, 1993), and the ability to restore one particular function does not necessarily imply competence to perform them all. We thus concentrate here on the end result of resuscitation, viz. the restoration of culturability.

The essential problem – assuming that the objects of study are not merely dead – is to find conditions that are suitable for resuscitation. Different physiological processes have to be taken in account when the medium is selected. First of all, the cells may be "metabolically injured" (Postgate, 1976); one or more components of a medium that is harmless for normal cells can be toxic for recovering cells. Substrate-accelerated death is a well-documented example (Calcott and Postgate, 1972). For isolation of bacteria from natural environments, a diluted broth is normally used (MacDonell and Hood, 1982). The optimal conditions for the recovery of "non-culturable" bacteria from lake water were very low substrate concentrations and an incubation atmosphere of 21% oxygen at 16°C for 4 weeks (Bussmann *et al.*, 2001). Consistent with this, a very rich medium did not permit the resuscitation of "non-culturable" cells of the copiotroph *M. luteus* (Mukamolova *et al.*, 1998b). It has been reported that addition of catalase or sodium pyruvate can significantly improve the recovery of some, but not all, "non-culturable" cells found in nature or obtained under laboratory conditions (Bogosian *et al.*, 2000). This observation supports the old idea that some cases of "non-culturability" are a direct consequence of the damaging action of free radicals (Bloomfield *et al.*, 1998). In some cases, restoration of culturability was observed when cells were plated in soft agar (Weichart and Kjelleberg, 1996). Sometimes cells can be cultivated successfully in liquid but not on solid media (Bovill and Mackey, 1997; Biketov *et al.*, 2000; Hu *et al.*, 2000; Shleeva *et al.*, 2002). A combination of

liquid medium and catalase was the best procedure for resuscitation of "non-culturable" cells of *A. hydrophila* (Wai *et al.*, 2000).

It is also possible that "non-culturable" cells cannot produce some important compounds required for the initiation of division. They have to be supplied in the medium. In the case of *M. luteus* and *Rhodococcus* sp., small amounts of yeast extract improved resuscitation dramatically and, moreover, the optimal concentration of yeast extract depended greatly on the age of the "non-culturable" cells (Mukamolova *et al.*, 1998b; Shleeva *et al.*, 2002). Improved recovery of cells of *C. jejuni* and *Campylobacter coli* has also been observed in media containing yeast extract (Moore, 2000). Addition of certain phospholipids in agar increased the number of CFU in populations of "non-culturable" *M. tuberculosis* cells by several orders of magnitude (Zhang *et al.*, 2001). These agents are known to promote the repair of damaged membranes (Ray and Speck, 1973) suggesting perhaps that the cells in these experiments were "non-culturable" as a result of injury. However, other explanations should not be ruled out. In the case of *M. luteus*, "non-culturable" cells pre-treated with cerulenin (an inhibitor of fatty acid synthase) did not resuscitate (Kaprelyants and Kell, 1993). Moreover, during the process of resuscitation of "non-culturable" cells of this organism, the disappearance of lysophospholipids correlated with restoration of the permeability barrier (*de novo* protein synthesis was not required) and permitted resuscitation of the majority of "non-culturable" cells in the population (Mukamolova *et al.*, 1995b).

The detailed cultivation regime (shaking speed, temperature, pH, etc.) can be an important factor for resuscitation. Sometimes a transition from one type of starvation to another can be helpful. For example, when "non-culturable" cells of *P. fluorescens* obtained under conditions of nitrogen starvation were transferred to complete medium, there was no resuscitation (substrate-accelerated death?) whereas they were resuscitated successfully when transferred to conditions of carbon (and energy) starvation before plating (Evdokimova *et al.*, 1994). The authors noted that metabolic activity was restored before culturability. In some cases, pre-incubation of "non-culturable" cells in liquid medium in the presence of antibiotics improved resuscitation dramatically (Mukamolova *et al.*, 1995b). However, in other cases, cells do not need special manipulation and culturability is restored after the stress has been relieved. For example, Nilsson and colleagues (Nilsson *et al.*, 1991) reported that "VBNC" cells of *V. vulnificus*, obtained by starvation at 4°C, could be resuscitated by simple temperature upshift. However, it has been pointed out by others that re-growth of a small number of culturable cells present in these populations could be responsible for the increased culturability that they observed (Weichart *et al.*, 1992; Bogosian

*et al.*, 2000). Similarly, Ohtomo and Saito (2001) reported the rapid resuscitation of saline-stressed cells of *E. coli* following a reduction in salt concentration. Some attempts have also been made to resuscitate cells by physical treatments. For example, sonication and heating in the case of *Streptomyces* (Hirsch and Ensign, 1976; Miguelez *et al.*, 1993), or high pressure and heating in the case of *Bacillus* (Paidhungat *et al.*, 2002), promotes spore germination. In two reported cases, heating of VBNC forms stimulated the restoration of culturability (Wai *et al.*, 1996; Kurokawa *et al.*, 1999), but high pressure did not similarly affect VBNC cells of *Vibrio* (Berlin *et al.*, 1999).

Some "non-culturable" cells may require highly specialised environments for resuscitation. This may apply in particular to certain pathogenic bacteria, "non-culturable" forms of which can resuscitate and multiply only *in vivo*, or in artificial conditions that are very similar to those encountered *in vivo*. Restoration of culturability and virulence *in vivo* to previously "non-culturable" cells has been reported for *V. vulnificus* (Oliver and Bockian, 1995), *V. cholerae* (Colwell *et al.*, 1996), *Salmonella enterica* (Asakura *et al.*, 2002), *Ralstonia solanacearum* (Grey and Steck, 2001b) and *C. jejuni* (Cappelier *et al.*, 1999), though in none of these studies were the estimates of culturability done under conditions of dilution to extinction. Resuscitation of "non-culturable" cells of *Legionella pneumophila* within *Acanthameoba castellanii* has also been reported (Steinert *et al.*, 1997). "Non-culturable" cells of *Y. pseudotuberculosis* could be resuscitated by passage through an axenic culture of *Tetrahymena pyriformis* (Didenko *et al.*, 2002). Interestingly, *Bacillus* spores could germinate and colonise the mouse intestine for brief periods, indicating that this environment supports spore germination (usually requiring a specific chemical germinant) and multiplication (Casula and Cutting, 2002). It is possible that some specific compounds *in vivo* stimulate resuscitation of "non-culturable" forms. For example, growth of some bacteria is stimulated by hormones or lymphokines from higher organisms (reviewed by Kaprelyants and Kell, 1996) – and *vice versa* (Henderson *et al.*, 1996) – and Williams, Lyte and colleagues have shown the significance of mammalian neurotransmitters in the former regard (Lyte, 1992, 1993; Lyte and Ernst, 1992, 1993; Freestone *et al.*, 1999, 2000; Neal *et al.*, 2001). VBNC cells of *H. pylori* showed metabolic reactivation (increase of ATP level and transcription of some genes) when human erythrocyte lysates were added to the recovery medium (Nilsson *et al.*, 2002).

A crucial problem in any resuscitation experiment is distinguishing between resuscitation of "non-culturable" cells and re-growth of culturable cells, even if the latter represents only a very small proportion of the total

cells present. Sometimes a mixture of re-growth and resuscitation occurs (Dukan *et al.*, 1997). Several procedures have been employed to infer the occurrence of resuscitation, e.g. measurement of the kinetics of growth/resuscitation, following the ratio of "non-culturable"/culturable cells, etc. However, it is very difficult, if not impossible, to determine the percentage of resuscitating cells using these methods. For quantitative analysis, Most Probable Numbers (MPN, i.e. counts based on dilution to extinction) should be determined. Different dilutions of cells are inoculated into several replicate tubes containing liquid medium. By scoring positive/negative tubes and using statistical tables, it is possible to back-calculate the most probable number of culturable cells in the original sample. An important feature of this simple methodology is that the behaviour of "non-culturable" cells can be monitored at low dilutions where, statistically speaking, culturable cells are absent. This method is especially useful when different stimulatory (or indeed inhibitory) compounds are added to or present in the growth medium. If a substance stimulates the growth of culturable cells in the population, changing their growth rate, the kinetic argument (see above) is invalidated. For example, it was found that some low molecular weight compound stimulated re-growth but not resuscitation of starved *E. coli* cells (Weichart and Kell, 2001). If these experiments had not been done using the MPN method it would not have been possible to distinguish the re-growth observed by these authors from resuscitation.

Unfortunately, there are very few unequivocal demonstrations of the resuscitation of "non-culturable" cells (Kell *et al.*, 1998). In many cases, no resuscitation was observed under any of the conditions tested (Bogosian *et al.*, 1996; Eguchi *et al.*, 2000; Kolling and Matthews, 2001; Mary *et al.*, 2002). In others, resuscitation was possible only over a limited period of time following adoption of the TNC state (Roszak *et al.*, 1984; Lleo *et al.*, 2001; Grey and Steck, 2001a). There are many discrepancies and complications in the published literature describing the resuscitation of "non-culturable" cells. A further feature is that in some experiments "non-culturable" cells inoculated in fresh medium were able to make limited cycles of division, and then stopped. For example, VBNC forms of *P. fluorescens*, obtained from a soil microcosm, could only produce microcolonies resulting from two or three divisions when resuscitated (Binnerup *et al.*, 1993). The reason for such limited divisions is not known. For *M. luteus*, accumulation of toxic compounds has been observed during resuscitation in fresh medium and under such conditions, cells also made only a limited number of divisions (Mukamolova *et al.*, 1995a). Stationary phase populations of *E. coli* contain some cells capable of making just one division (Ericsson *et al.*, 2000). In a few cases, only one of the two daughter

cells could divide subsequently. The authors proposed that this behaviour might result from some kind of localised deterioration or damage, inherited by only one daughter cell. The phenomenon of limited divisions in eukaryotic cells is of course very well documented (Hayflick and Moorehead, 1961; Rubin, 2002). Finally, as previously mentioned, many of the VBNC cells described in the literature may be dying or metabolically injured. By definition, such cells cannot be "resuscitated".

One of the most intriguing questions is whether special resuscitation-inducing factors exist. Addition of some compounds (so-called germinants, such as alanine or adenosine) dramatically increases the rate and proportion of spores of various *Bacillus* spp. that germinate (Moir and Smith, 1990). Germinants bind to specific Ger proteins and stimulate ion transport, leading to the activation of lytic enzymes that degrade the spore envelopes, to rehydration of the spore core, and to the breaking of dormancy (Paidhungat and Setlow, 2000). Some components in resuscitation medium may have a similar effect to that of germinants. A change of growth medium may switch on the early stages of resuscitation, i.e. restoration of membrane energisation and respiratory activity, but in some cases this is not sufficient for restoration of culturability (Votyakova *et al.*, 1994). In the case of *M. luteus*, the addition of sterile culture supernatant was necessary for the restoration of culturability of "non-culturable" cells, suggesting that active cells could secrete compounds that promote resuscitation (Kaprelyants *et al.*, 1994). Interestingly, the resuscitation of undiluted populations containing small numbers of culturable cells did not require the addition of any specific factors (Kaprelyants and Kell, 1993; Votyakova *et al.*, 1994).

The resuscitation-promoting factor (Rpf) in the sterile culture supernatant of *M. luteus* was non-dialysable, heat-labile and trypsin-sensitive, suggesting that it was a protein and this was subsequently confirmed (Mukamolova *et al.*, 1998a). The presence of Rpf was necessary for the restoration of culturability to "non-culturable" (dormant) cells of *M. luteus*, although their metabolic activity could be restored in its absence (Votyakova *et al.*, 1994). In addition to promoting the resuscitation of "non-culturable" cells, Rpf caused a reduction in the apparent lag phase of cultures growing from small inocula. An additional assay was developed using washed vegetative cells, whose growth was dependent on supplementation with Rpf, which indicates that Rpf has a more general role as a growth factor in *M. luteus* (Mukamolova *et al.*, 1998a; Kaprelyants *et al.*, 1999) (Figure 3).

Rpf is a secreted protein, active at picomolar concentrations, suggesting that it may represent a signalling molecule. Disruption of the *rpf* gene was not possible in *M. luteus* in the absence of a second functional copy, indicating essentiality (Mukamolova *et al.*, 2002a). Moreover, the protein

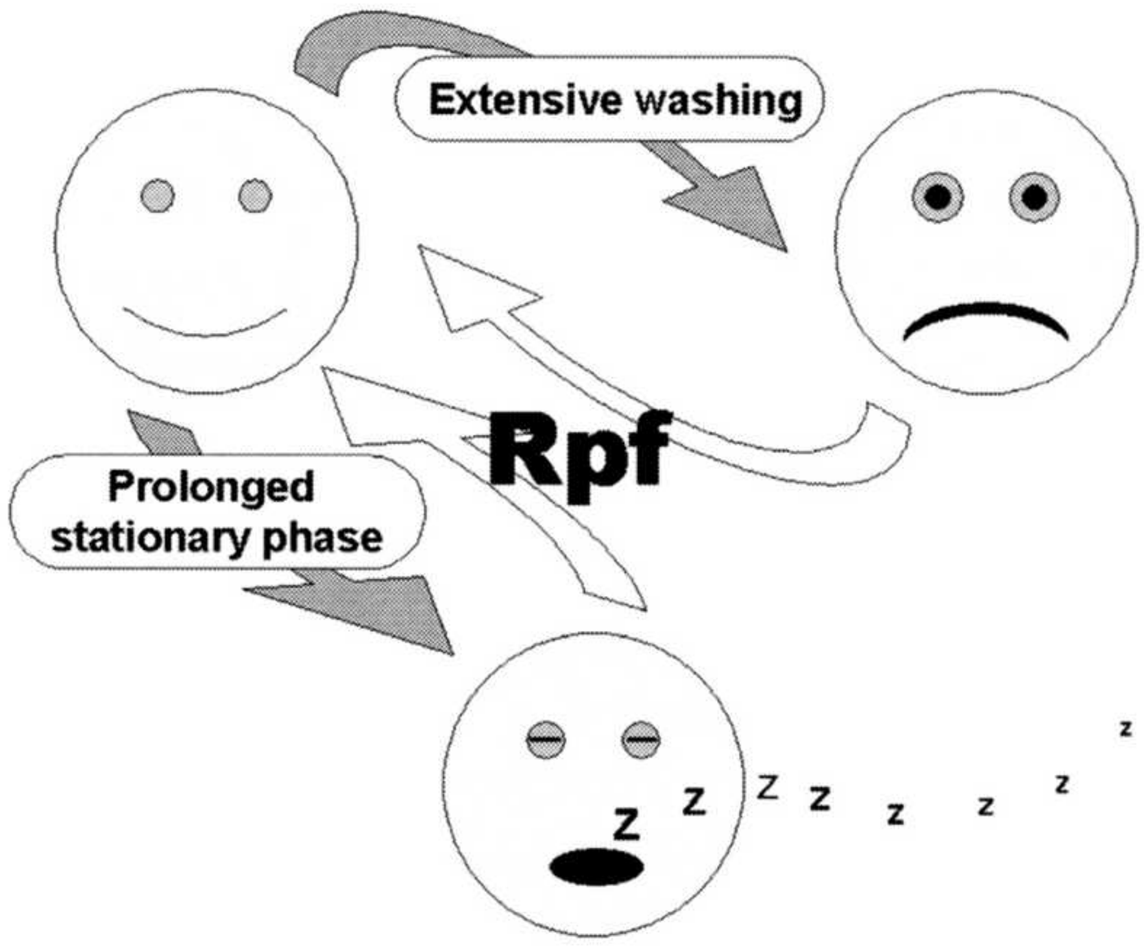


*Figure 3* *M. luteus* requires Rpf for growth. Growing cells make Rpf. During a protracted stationary phase, bacteria may enter a state of dormancy during which they are "non-culturable". Culturability is restored by the exogenous provision of Rpf. Normally, the source of Rpf is an actively growing neighbouring cell(s) but recombinant Rpf made in *E. coli* also suffices (Mukamolova *et al.*, 1998a). In the laboratory, actively growing cells can be made Rpf-dependent by extensively washing them, which removes pre-synthesised protein molecules from the cell surface, and inoculating them at low density into a minimal medium.

must be secreted (or provided exogenously) to be biologically active (Mukamolova *et al.*, 2002a). However, its mechanism of action remains unknown. Our working hypothesis is that Rpf, or a peptide derived from it, is the ligand for a surface-located receptor, but until the predicted receptor has been characterised this speculation is supported only by the extreme potency of the molecule – a truncated derivative encompassing only the Rpf domain (see below) was active at femtomolar concentrations (Mukamolova *et al.*, 2002a). It still remains entirely possible that Rpf has a catalytic activity of some kind. The schematic diagram in Figure 4 shows the relationship between Rpf production and the growth cycle of *M. luteus* in laboratory batch culture.

Rpf is the founder member of an extended protein family that is found throughout (and, so far as is known, exclusively within) the high G+C cohort of Gram-positive bacteria. There are several representatives in most organisms and the gene family now contains in excess of 30 members (Kell and Young, 2000; Matsuda *et al.*, 2001; Jang *et al.*, 2002). For example, *M. tuberculosis* (Cole *et al.*, 1998) and *S. coelicolor* (Bentley *et al.*, 2002) both contain five *rpf*-like genes. It has recently been established that recombinant versions of all five proteins from *M. tuberculosis* have similar

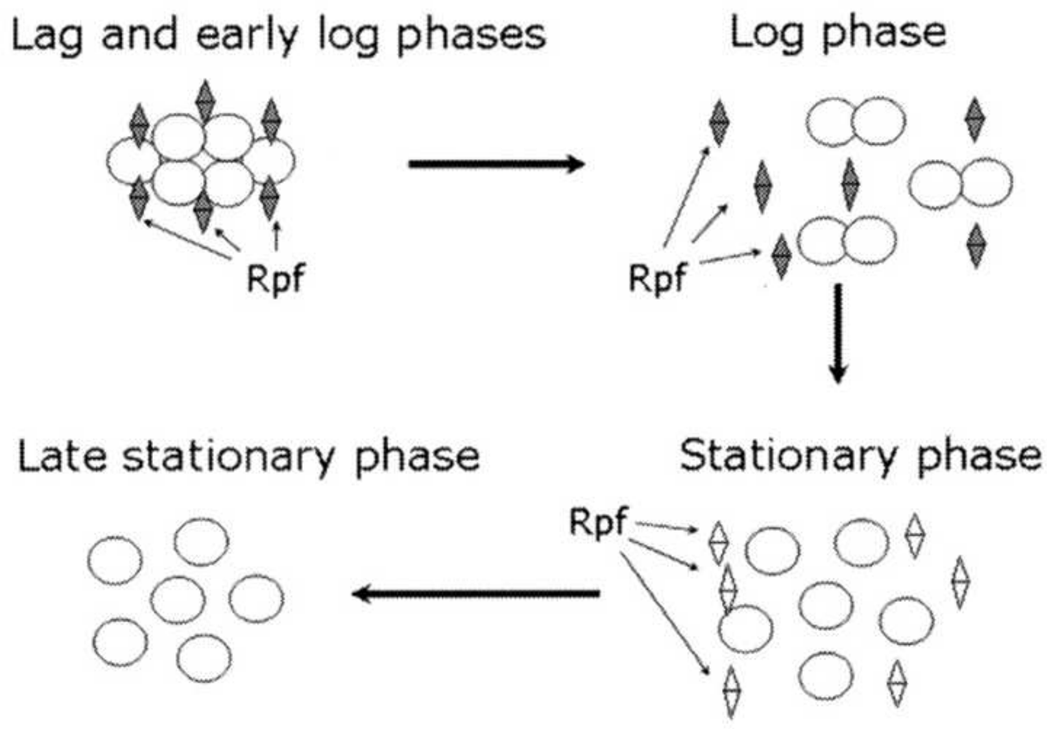


*Figure 4* The distribution of Rpf during different stages of growth of *M. luteus* in laboratory batch culture. Bacteria make and secrete Rpf during lag phase and early log phase (Mukamolova *et al.*, 2002a). Most molecules remain associated with the cell surface. As growth continues and clumps of cells start to break up, Rpf is released into the culture supernatant. In stationary phase, Rpf molecules in the supernatant lose biological activity, and eventually disappear (degradation). If cells spend a protracted period in stationary phase they enter the state of "transient non-culturability".

biological activity to that of *M. luteus* Rpf (Mukamolova *et al.*, 2002b). Moreover, Rpf-like biological activity has also been observed using recombinant proteins from *Corynebacterium glutamicum* (Jang *et al.*, 2002) and *M. smegmatis* (D.I. Young and M. Shleeva, unpublished data) and in culture supernatant from *Rhodococcus* sp. (Shleeva *et al.*, 2002). Most members of the protein family are predicted to be secreted, or membrane-anchored, and lysM modules (Bateman and Bycroft, 2000) promoting association with the cell wall peptidoglycan are a common feature of the Rpf-like proteins from the non-mycolate actinomycetes. Although there is some evidence for selectivity (see below), all of the Rpf-like proteins so far tested show cross-species activity (Mukamolova *et al.*, 1998a, 2002b; Biketov *et al.*, 2000; Shleeva *et al.*, 2002; A. Davies, unpublished data). In addition, *M. luteus* culture supernatant (presumably containing Rpf) has been reported to enhance the isolation of mycobacteria in liquid medium (Freeman *et al.*, 2002).

## 6. SOCIAL BEHAVIOUR OF BACTERIAL POPULATIONS AND "NON-CULTURABILITY"

Over the last decade it has become abundantly clear that bacteria do not live in isolation one from another. Rather, they form structured communities in

which individual cells communicate with each other via signalling molecules (reviewed by Kaiser and Losick, 1993; Losick and Kaiser, 1997; O'Toole *et al.*, 2000; Shapiro, 1995b). Social behaviour of cells is undoubtedly of cardinal importance for many different cellular processes associated with multiplication, differentiation, survival in fluctuating environments and death. In this section we consider how multicellular bacterial communities respond to stress.

One good example is presented by the behaviour of bacteria growing in biofilm communities. Biofilms are complex structures (Rittman, 1999; Stoodley *et al.*, 2002) resulting from the expression of genetic information according to a defined developmental programme (Danese *et al.*, 2001; O'Toole *et al.*, 2000). They provide a niche in which growth conditions are optimised for communal living (Gilbert *et al.*, 2002) and polysaccharide production confers substantial resistance to several different stresses (Li *et al.*, 2001; Mah and O'Toole, 2001), including starvation (Kim and Fogler, 1999). In response to nutrient depletion, cells in the biofilm can adopt two alternative survival strategies. Organisms may enter a starvation survival programme *in situ*, or the biofilm may dissociate as its members adopt a motile free-living (planktonic) existence. Failure to find food in the latter case can lead to adoption of a TNC state.

There is an interesting parallel here with cells that normally grow as clumps or aggregates. During prolonged incubation of cells of *M. tuberculosis* or *M. bovis* (BCG) in stationary phase, a heterogeneous population arose containing "aggregates" composed of dead lysing cells and single short rods that were "non-culturable" and could be resuscitated (Shleeva *et al.*, 2002; O.A. Turapov and D.I. Young, unpublished data). Persistent cells inside murine macrophages are also disaggregated (Biketov *et al.*, 2000).

In denser populations, cells often survive using a strategy that has been likened to "cannibalism" (Postgate, 1976). Generally this allows some members of the population to survive and generate progeny in the future at the expense of the others and, hence, it may be regarded as an example of cooperative or social behaviour, permitting species maintenance. In stationary phase cultures of *E. coli* mutants arise showing an "evolutionary cheating" behaviour (Vulic and Kolter, 2001). These cells have *rpoS* mutations and cannot initiate a proper "stationary" phase response. They continue to multiply in conditions of stationary phase due to the development of amino acid-dependent catabolism (Zinser and Kolter, 2000). Moreover, they have a growth advantage in mixed culture with wild type bacteria and tend to overgrow them (Zambrano and Kolter, 1996). The survival advantage of the mutant is inversely dependent on its frequency in

the population, and a balance of normal cells and "cheaters" can be maintained. Moreover, these cells are more sensitive to various stresses than is the wild type, A similar phenomenon has also been described for *M. smegmatis* cells incubated in prolonged stationary phase (Smeulders *et al.*, 1999). Cells producing colonies with altered morphology had a "growth advantage in stationary phase" but not under normal growth conditions.

There is some evidence that cells can produce or release compounds that help other cells to survive under conditions of stress (Harrison, 1960; Rowbury, 2001) and adapt to new environments (Nikolaev *et al.*, 2000). These compounds are found in the culture supernatant of many different species and, apparently, there is no specificity in their action.

Usually, the population of surviving cells in a starving culture is very heterogeneous. In many organisms it consists of a mixture of dead (lysing) cells and survivors; the latter may include viable cells that will produce CFU and may even be slowly multiplying, and "non-culturable" cells, which may be dormant. This may result from the "initial" heterogeneity of the population due to unsynchronised growth. In the case of *M. luteus*, the surviving population always contained some residual culturable cells ($10^3$–$10^5$ cells/ml in populations containing $10^9$–$10^{10}$ cells/ml). These culturable cells promote the resuscitation and growth of the majority of "non-culturable" cells when inoculated into fresh medium (Votyakova *et al.*, 1994). This can be regarded as a particularly efficient survival strategy since it permits survival of the majority of the cells in the original bacterial population and is a clear example of the cooperative behaviour of surviving cells. In the case of *V. vulnificus*, a population of "non-culturable" cells, starved in buffer at 4°C, were restored to culturability when they were transferred to 20°C. However, this was not possible when the population was diluted 10- or 100-fold. The simplest explanation is that in this case, "resuscitation" is in fact re-growth of initially undetectable culturable cells. But it is also possible that culturability was restored to some cells more quickly than to others and that the cells that became culturable early on, secreted a growth-promoting substance. The experimental data on "cooperative" resuscitation of *Vibrio* cells are quite controversial: in some experiments active cells, mixed with VBNC cells, helped the latter to restore culturability (Whitesides and Oliver, 1997), whereas in other experiments no growth-promoting effect of active cells was detected (Bogosian *et al.*, 1998).

The resuscitation of "non-culturable" cells by compounds accumulated in culture supernatant was first observed in *M. luteus* (Kaprelyants *et al.*, 1994). It has also been observed in *C. jejuni* (Bovill and Mackey, 1997), *Nitrosomonas europaeae* (Batchelor *et al.*, 1997), *M. tuberculosis* (Sun and Zhang, 1999), *Rhodococcus* sp. (Shleeva *et al.*, 2002) and *M. smegmatis*

(M. Shleeva and A.S. Kaprelyants, unpublished). It is clear that cooperative behaviour is an important feature not only in populations during the transition to a non-dividing state but also during the reverse process of recovery or resuscitation. Interestingly, large inocula of *Bacillus megaterium* spores germinated at a significantly higher rate (in percentage terms) than did small inocula, suggesting that germinating spores may produce something that stimulates the germination of their immediate neighbours (Caipo *et al.*, 2002). Similar effects have been observed in *Clostridium botulinum* (Wong *et al.*, 1988). In contrast, inhibition of *Streptomyces* spore germination was observed at very high population densities (Polyanskaya and Triger, 1990; Triger and Polyanskaya, 1991). The discovery of endogenous germination inhibitors in *Streptomyces* may be significant in this context (Petersen *et al.*, 1993).

The resuscitation of "non-culturable" cells occurs in response to the perception of external factors and it is now becoming apparent that some of these factors can be produced by neighbouring cells. We have suggested that, in the high G + C cohort of Gram-positive bacteria, the active molecules are, or include, proteins of the Rpf family (Kaprelyants *et al.*, 1999). It has been argued elsewhere (Postgate, 1995) that adoption of the "non-culturable" state by the majority of cells in a population is of adaptive significance, since a small number of "sentinel" organisms can make best use of scarce resources during periods of starvation and then signal the onset of conditions favourable for growth to their genetically identical but quiescent neighbours. Should the entire population lose culturability, all is not lost, since the resuscitation-promoting factors show cross-species activity (Mukamolova *et al.*, 1998a, 2002b).

Finally, it has recently been demonstrated that *rpf* is an essential gene in *M. luteus* (Mukamolova *et al.*, 2002a). Why does this organism (and possibly others that make Rpf-like proteins) secrete a protein that is essential for its growth? Why have bacteria evolved such a precarious system for growth regulation? This is most readily understood if we adopt the view that the organism is not in fact the individual bacterial cell, but the entire bacterial population. The bacteria that we grow as isolated planktonic forms in laboratory shake flasks probably form structured communities in nature where they enjoy all the benefits of a multicellular existence.

## 7. CONCLUSION

There has been much discussion and controversy concerning the nature and biological significance of "non-culturable" cells. Nevertheless, it is generally

agreed that "non-culturable" cells are abundant in Nature and that they can be obtained under laboratory conditions and, therefore, they have importance for ecology (Roszak and Colwell, 1987), medicine (Barer *et al.*, 1993; Barer and Harwood, 1999) and industry (Millet and Lonvaud-Funel, 2000; Nebe-von-Caron *et al.*, 2000). In this review, we have developed the hypothesis that transient "non-culturability" is a consequence of a special survival strategy. The fact that "non-culturable" cells have altered morphology and are adapted for survival under stressful conditions suggest that they show some resemblance to the more specialised survival structures produced by sporulating bacteria. We have emphasised that only cells that satisfy several different criteria, i.e. integrity of all cellular components, low metabolic activity and, most importantly, the ability to be resuscitated – preferably in the absence of other culturable cells – should be considered as having adopted the TNC state. Unfortunately, in many cases in which "non-culturability" has been inferred, only one criterion (e.g. staining with CTC or tetrazolium) has been applied. Moreover, we still do not know whether VBNC cells, obtained under cold starvation, persisting cells in latent infections *in vivo* and "non-culturable" cells, arising in stationary-phase/chemostat culture, are different manifestations of the same underlying process, i.e. whether they represent similar surviving forms of different bacteria. We exclude "non-culturable" metabolically active cells from the category of special surviving forms. In order to adopt the TNC state, bacteria must first adapt to a low metabolic cost existence and as a consequence of this they then lose culturability. Initial loss of culturability, which may be followed by gradual metabolic decline, is indicative of injury, leading to death, and is quite distinct from entry into a TNC state.

One of the main potential criticisms of the idea that "non-culturable" cells are specialised resting forms, is that their state of "non-culturability" is temporary, and that it can have two very different outcomes, resulting in resuscitation on the one hand and death on the other. It could therefore be argued that it simply represents an intermediate state in a process of gradual decline towards death, rather then a programme of adaptation to unfavourable conditions (Barer and Harwood, 1999). Admittedly, the "non-culturable" forms under discussion here cannot survive for years, as is the case for profoundly dormant forms like bacterial endospores. However, *Streptomyces* spores (generally accepted as specialised survival forms) also have limited longevity, and are much more sensitive to environmental fluctuations than are the highly resistant bacterial endospores. In fact, actively growing cells, "non-culturable" forms, *Streptomyces* spores and endospores can be considered to represent different points on a continuum, where survival of inimical conditions becomes favoured as metabolic activity

is gradually reduced (see Fig. 1). Long-term survival of endospores is dependent on a complete shutdown of metabolic activity, resulting from the adoption of a state of low water activity (in the laboratory a similar state is achieved artificially by lyophilisation). The limited duration of the TNC state is not therefore a compelling argument against the view we have taken, which is that the TNC state should be regarded as a survival strategy.

Another point of general concern to people working in the field is the low reproducibility/high variability of experiments. We suggest that this is mainly related to the fact that there is very substantial heterogeneity of cells in bacterial populations. From our own experience, we know that a slight change of culture conditions can cause dramatic changes in population behaviour (Shleeva *et al.*, 2002). Sometimes these changes are very difficult to control. The best experimental models are uniform populations of "non-culturable" cells. Unfortunately, this ideal is seldom realised; "non-culturable" cells very often represent less than 10% of the total population. In our opinion, this fact does not challenge their existence, nor does it undermine their biological significance, but it certainly complicates attempts to investigate them.

The most severe criticism of the significance of the phenomenon as a general survival strategy (if such it is) is the fact that only in a relatively few instances has resuscitation been convincingly demonstrated. There can be many reasons why attempted resuscitation has failed. In some instances the organisms under investigation may be dead, whereas in others inappropriate conditions may have been employed. Finding the right conditions still remains one of the major challenges, since they vary from one organism to another and no generalisations can currently be made. "Non-culturable" cells are very different from culturable cells and finding the appropriate conditions for resuscitation is every bit as difficult as finding the appropriate conditions to support the growth of any previously unknown organism. Like endospores, "non-culturable" cells may be able to resuscitate only in the presence of specific chemical signals. Another parallel is that spore germination and resuscitation from the "non-culturable" state are both stochastic processes depending on many different factors. The production of such specialised forms does not automatically mean that the organism will survive and give rise to progeny at some time in the future; it merely increases the probability that this will happen.

The main factor that has to be overcome in order to clarify the status of "non-culturable" cells is ignorance. We still do not know how and why bacteria adopt a "non-culturable" form. Also, we do not know what is happening at the molecular level during the transition to "non-culturability", in the period of "non-culturability" itself, and during the

subsequent process of resuscitation. Unless we have a much fuller understanding of these matters we cannot decide whether "non-culturable" cells are dormant or whether they are forms of life that are merely undergoing a more or less graceful degradation. In spite of the fact that the phenomenon was first described 20 years ago (Xu *et al.*, 1982) and many experiments have been done since then, we still remain woefully ignorant. The application of the new powerful -omics methods (transcriptomics, proteomics and metabolomics) to organisms of known macro-physiological state may be expected to provide significant new insights in the future.

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