The viable but non-culturable phenomenon explained?

The ability of a bacterium to cause disease and yet fail to respond to enumeration through classical culture procedures has led to the concept of a viable but non-culturable (VNC) state. In the environment of the human intestine, for example, previously non-culturable vibrios can regain the capacity to multiply (4). Such observations support the proposition that VNC bacterial enteropatogens pose a potential threat to health and dictate a pressing need to understand the basis of the failure to culture such organisms in current resuscitation protocols. This need acknowledges that the term VNC is an oxymoron (2); such cells are not unculturable, we are simply failing to provide appropriate conditions to support culture.

Recently, the VNC concept has been extended to cover the vast majority of environmental microbiology. PCR approaches suggest that, in reality, the diversity of species selected from the environment by selective enrichment is less than 1 % of that actually present in many niches. Significantly, evidence suggests a far greater microbial diversity, even amongst human pathogens, than currently appreciated with culture-dependent methods (19). Even in 'pure' laboratory cultures, there may be a fraction of cells which are VNC under conditions normally suited to their cultivation. We would accept physiological variation in asynchronous populations, so why should we be surprised if, due to some transient physiological difference from the remainder of the population, some variants fail to form colonies on nutrient agar?

The concept of a VNC state strikes at the heart of microbiology as the ability to recover organisms on agar medium remains its cornerstone. This paper proposes an underlying mechanism for non-culturability which provides a new appreciation of the limitations of current resuscitation protocols.

In a recent paper, we proposed (5) that some of the lethal effects associated with iminical processes (physical or chemical agents) actually result from a process of 'self-destruction' by the cells themselves (a suicide response). We propose that self-destruction in exponentially growing cells is caused by an oxidative burst which occurs when cells are growth-arrested following an iminical treatment. Protection against self-destruction can be provided by reducing the oxygen tension, or by adaptive responses associated with the stationary phase which protect the cell against DNA damage, free-radical damage and protein denaturation. The sensitivity of exponential phase cells is due to the production of intracellular free radicals rather than the direct action of the iminical process. What should be a sublethal injury becomes lethal because, through growth arrest, cell division is decoupled from metabolism. This results in overactive and perhaps futile metabolism which produces intracellular free radicals and the lethal outcome. Consistent with this view, Nystrom et al. (14) suggest that reduced production of electron donors (ArcA-dependent), and decreased levels and activity of the aerobic respiratory apparatus during growth arrest are integral parts of a defence system specifically aimed at avoiding the damaging effects of oxygen radicals and controlling the rate of utilization of endogenous reserves. From this we propose that a proportion of the increased resistance to iminical processes which develops in stationary phase populations can then be explained on the basis of the switch from respiration to substrate-level phosphorylation (hence lower rates of superoxide generation).

We now wish to propose that the concept of cell suicide could also provide an explanation for the failure to culture cells from environmental sources or from starved or cold-stored laboratory microcosms such as those described for Escherichia coli (13), Micrococcus luteus (10) and Vibrio vulnificus (16). Transfer of any population from one environment to another which is substantially different must pose a challenge to its adaptive capacity. Phenotypic adaptation requires time for protein expression. We propose that sudden transfer of cells to nutrient-rich agar at temperatures optimal for enzyme activity initiates an imbalance in metabolism, producing a near instantaneous production of superoxide and free radicals. In the absence of phenotypic adaptation the cells are not equipped to detoxify superoxide and, as a result, a proportion or all of these cells die.

Some insight into biochemical mechanisms that could contribute to 'metabolic imbalance' comes from the work of Imlay (9). A study of the E. coli respiratory chain components identified fumarate reductase as the key (and possible only) source of endogenous superoxide during respiration. This despite the paucity of this anaerobic terminal oxidase in the aerobic cell. The
potential for fumarate reductase to play a key role in the culturability of cells under resuscitation from different redox environments is clear. Inlay (9) states that "the propensity of fumarate reductase to generate superoxide could conceivably deluge cells with superoxide when anaerobic cells, which contain abundant fumarate reductase, enter an aerobic habitat." Given that some fumarate reductase is present even under fully aerobic conditions, a key metabolic balance may be the functional levels of enzymes such as fumarase, succinate dehydrogenase and fumarate reductase. The implication of fumarate as a metabolic signal for transducing bacterial chemotaxis (12) places this metabolite under particularly close scrutiny.

Three aspects relevant to maintaining culturability in laboratory-generated microcosms are pertinent to our hypothesis. Firstly, a review of the conditions in which enhanced resuscitation of culturable cells from microcosms is most usually achieved (3) indicates that these typically correspond to nutritionally restricted conditions. These are the same conditions used empirically for the recovery of cfu from populations damaged by inimical agents (often with the addition of oxygen scavengers such as glutathione or catalase; 6, 7). Secondly, the phenomenon of 'substrate accelerated death' as described by Postgate (17) can be explained rationally by this hypothesis; Postgate & Hunter (18) showed that a substrate that permits the growth of certain Gram-negative species will accelerate death when added to starved populations. The fact that this does not occur with substrates not previously experienced by the bacteria suggests that adaptation to achieve utilization of the alternative substrate allows re-coupling of growth to metabolic activity and hence there is no cell metabolic imbalance. These workers observed that substrate accelerated death in suspensions of Aerobacter aerogenes (18) was inhibited by the uncoupler 2,4-dinitrophenol. Recent work (8) showing that E. coli generates superoxide when provided with substrate under non-growth conditions, and that the uncoupling agent carboxyl cyanide p-(trifluoromethoxy)phenylhydrazone substantially reduces the levels of peroxide production, provides a possible explanation for this finding.

Thirdly, conditions that favour retention of culturability in populations with an increasing VNC component correlate well with conditions that increase bacterial resistance to inimical agents, namely those which promote synthesis of protective hydroperoxidase enzymes or DNA-binding and repair systems. Oliver (15) showed that V. vulnificus starved at room temperature for 4 h prior to incubation at 5 ºC showed a decrease in the rate at which cells became non-culturatable. Furthermore, following 24 h or more incubation at room temperature they exhibited no decrease in culturability. Stationary phase cells required about twice as long to become non-culturable following transfer to 5 ºC compared with exponentially growing cells. Smith & Oliver (20) showed that 'nutrient downshift' (at 25 ºC) of a suspension of V. vulnificus for 12 h produced protection against oxidative challenge (H2O2, 0.2 mM). These observations can be accounted for by the induction of RpoS-mediated hydroperoxidase induced through stationary phase or starvation at ambient temperatures. This provides protection from oxidative damage. The important concept is that oxidative damage results during the resuscitation stage; only cells pre-adapted to deal with an oxidative burst can survive. The viability remains but culturability is lost through an inappropriate recovery protocol associated with transfer to nutrient-rich agar at 25 ºC.

There remains one further area of relevance to this discussion, namely the observation that growth of E. coli in high cell density batch fed cultures leads to accumulation of non-culturable cells (1). Because these cells remain effective 'factories' from a biotechnology perspective, i.e. the bioreactor continues to produce the recombinant product, the concept has emerged that cells in the bioreactor become VNC. Data on oxygen consumption in bioreactors indicate a fully dissimilatory metabolism with continuing respiration even at very low growth rates. Since an explanation for the loss of culturability remains elusive, we propose that the reason lies in oxidative damage. E. coli at low or zero growth rates – starvation or stationary phase – should not be respiring aerobically, rather it should be utilizing substrate-level phosphorylation. We propose that, in the bioreactor, the 'normal' functions controlling the regulated entry into the stationary phase are overridden, possibly due to the fed batch process (nutrient pulsing). This results in a significantly greater biomass (division beyond normal stationary phase) and the absence of a controlled shift from respiration into substrate-level phosphorylation. Respiration in the absence of growth 'kills' the culture via free-radical generation. Thus this is not a VNC state as the cells in the reactor are genetically damaged and, we predict, could not be recovered under any circumstances.

The above prompts some consideration of terminology. Metabolic activity is not an indication of viability. This is illustrated by the biorefermenter where the cellular productivity remains, but where we consider the cells to be unrecoverable through accumulated free-radical damage to the genome. Equally the absence of colonies on recovery medium does not constitute evidence for dead cells, as potentially viable cells can be killed by the very conditions prescribed for recovery. Of course the possibility of cell suicide associated with attempts to culture starved microcosms does not preclude the possibility of transition to a 'dormant' phenotype (11).

The value of this discussion resides in our proposal of a unifying mechanism for cellular destruction which is central to the failure to recover truly viable cells, the loss of viability in bioreactors, and the action of inimical processes. The central role of oxidative damage generated from within the cell itself provides for a rational approach to the design of recovery procedures. In conclusion there is every reason to accept the practical existence of a VNC state, but, if we are finally to remove the non-culturable label from what are truly viable cells, we must better understand the biochemistry and physiology of the interactions between growth, respiration and the devastatingly destructive power of oxidative damage. Since the basis of the VNC state is that recovery can occur when organisms are passages through the in vivo conditions of the mammalian host, such studies may shed additional light on the prevailing micro-environments associated with host-pathogen interactions.

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As we accumulate DNA sequence information about an increasing number of bacterial genomes, new ways of looking at the information are required in order to understand the significance of this plethora of detail. The relatively small size of the minimalistic genomes of bacteria such as the mycoplasmas suggests that little more than a few hundred genes are necessary to specify bacterial structure and multiplication. Additional genes have accumulated in particular species to adapt them to specific niches. Ecological niches are not static, they are highly dynamic. This is not just because of the seasonal variations and the march of geological time, but also because of the action of the organisms themselves which inhabit the environment. The interplay between organisms continually generates new opportunities for variant offspring to do better than their parents.

The last 50 years have demonstrated, in the most dramatic way possible, the enormous potential of mobile genetic elements (plasmids, phage and transposable elements) to allow rapid evolution of bacterial populations. Antibiotics which were considered as the miracle of modern medicine have been rendered of limited use in many contexts because of the spread of plasmid-borne resistance genes. Detailed study of plasmids has identified the replication functions, the stable inheritance mechanisms and the conjugative transfer processes that they encode and these genes, especially the ones for conjugative transfer, are starting to make some sense of the apparent plasticity of the bacterial genome. Werner Pansegraf (Leiden, The Netherlands) reported that genes already associated with the conjugative transfer of plasmid DNA between bacteria and from bacteria to plant cells have now turned up in association with ‘pathogenicity islands’ in Helicobacter pylori. This provides further evidence for the underlying unity of genetic exchange/genome plasticity processes in bacteria. That bacteria can evolve very quickly, not just by plasmid transfer, was demonstrated by Larry Forney (East Lansing, MI, USA), who described how adaptation of Alcaligenes to degradation of 2,4-dichlorophenoxyacetic acid occurs rapidly and involves both genomic rearrangements and morphological changes. The genetic changes are likely to involve mismatch repair or recombination and create alternative ways of achieving the same growth advantage. To help understand the role of genetic exchange in this sort of bacterial evolution, when the environment changes was one of the main purposes of this workshop, the third in a series organized with ESF support. The aim of the ESF Network has been to bring together molecular biologists and microbial ecologists to create a much richer and broader context in which to explore this topic. A major success illustrated at the meeting was the disappearance of the boundary between these two disciplines.

One of the ways in which barriers have been broken down is by explanation of the fundamental techniques and intellectual approaches of each discipline, prompting dialogue. One of the key issues described by Liz Wellington (Warwick, UK) is the significance of the fact that more than 90 % of bacteria from the environment are unculturables. Does this mean that the genes they carry play no role in the gene flux or are they a source of genetic diversity by release or transfer of DNA to the actively growing sector? Techniques are needed to separate culturable from non-culturables and to then assess whether genes can transfer into or out of the non-culturables component. If there is no genetic exchange, then one may be justified in setting up model systems consisting of just the cultivable organisms. Monitoring the changes in these organisms without culturing them can be facilitated by total DNA extraction from environmental samples. Kory Smalla (Braunschweig, Germany) described a range of techniques which are now available for removing inorganic and organic material (such as humic acids) which interfere with most DNA manipulations, so that PCR can be used to detect the presence of specific mobile genetic elements. However, to isolate a mini replicon it is still important to purify DNA of the plasmid in question, create a library of DNA fragments and then determine which one is capable of self-replication in the appropriate host. Martine Couturier (Brussels, Belgium) presented a neat positive selection vector for cloning fragments which could then be screened for replicons or transfer origins. Since the transfer origins are normally dependent on many other genes it is best to screen them in the host carrying the parental plasmid. Ellen Zechner (Graz, Austria) showed how the nicks introduced into one strand at the replication or transfer origin can be detected in vivo by extension from a primer hybridized to total DNA so that the origin can be mapped under physiological conditions and the factors controlling origin activity can be determined. Such an extension of molecular techniques into more complex environments was also illustrated by Soren Molin (Lyngby, Denmark), who described the use of fluorescent probes for in situ identification of species, determination of growth rate and assessment of plasmid-mediated gene spread in mixed populations. Grazyna Jagura-Burdzy (Warsaw, Poland) and Guenther Koraimann (Graz, Austria) talked about techniques for studying protein–DNA interactions. A challenge will be to detect such interactions in more complex environments in vivo or to use the specific recognition of DNA sequences by these proteins as a means of probing events in the bacterial cell. Technical advances will surely develop from this dialogue.

Plasmids of Gram-positive bacteria were a major topic, which prompted examination of the concept ‘cryptic’ as applied to plasmids. From the molecular studies reported by Sierd Bron (Groningen, The Netherlands) and Jacques Mahillon (Louvain La Neuve, Belgium) was a major highlight of the workshop.