

Relationships between cellular activity and culturability

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ABSTRACT

While the inadequacies of culture as a means of describing the distribution and influence of specific bacteria are well recognised, the capacity of cellular activity measurements to overcome these problems remains to be established. Central to these issues is the question of whether any measured activities can be rigorously proven to be predictors of culturability. Unless such relationships can be established for readily cultured bacteria, the viability of any observed bacteria which have yet to be cultured cannot be concluded from cellular activity measurements. Work in this laboratory has centred on development of methods for the measurement of cellular activity in bacteria, and the relationships of these measurements to culturability of organisms exposed to a variety of stresses. Using substrate enhanced tetrazolium reactions, rhodamine 123 uptake, induced Beta-galactosidase activity, rRNA *in situ* hybridisation and the Kogure cell elongation (DVC) method, all assessed by digital image analysis, we have studied these relationships across a broad range of bacteria. No consistent pattern has emerged regarding the capacity of any of these measurements (or combination thereof) to predict the culturability (including post-animal passage) of the cell populations observed. None of these methods may therefore be considered adequate predictors of culturability or viability for environmental investigations.

Introduction

The detection and measurement of cellular activity in bacteria provides opportunities to determine behavioural, physiological, and biochemical features of cells and populations without growing them. The obvious attractions of applying such methods to characterise nonculturable (NC) cells should not be allowed to obscure the difficulties involved in interpreting their results. Here we discuss work that illustrates some of the points at issue in the current debate about NC cells.

Many of the cytological assays that have been extensively applied to NC cells were explicitly developed as tests of viability. In Table 1, we consider them in terms of the specific activities for which there is available evidence.

Regarding the Kogure cell elongation test, it seems reasonable that the changes observed in cell length must involve cell growth with all the attendant metabolic reactions that it implies. Indeed we have observed inhibition of the reaction by chloramphenicol and rifampicin, indicating that transcription and translation must be involved (LT Gribbon, unpublished observations). The Kogure and related procedures may be seen, therefore, as

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Table 1. Some cellular activity assays applied to bacteria.

Activity	Example	Biochemical Basis
Cell elongation	Kogure test [9]	Uncertain
Energisation of cytoplasmic membrane	Rhodamine 123 uptake [6], Oxanol exclusion.	Intact proton pump
Enzyme activity	Tetrazolium salt reduction [4, 15] Fluorescein diacetate hydrolysis [10]	Presence of enzyme substrate (+ coenzymes where required)
Response to external stimulus	Induced β -galactosidase [11]	De-repression, transcription, translation.
Precursor incorporation	^3H -leucine incorporation detected by micro-autoradiography [5]	Probably translation, possibly catabolism and incorporation of ^3H by another route.

indirect tests of these processes although the specific nature of the stimulus provided by the quinolone reagent remains to be established.

The enzyme methods clearly show that cells have sufficient organisation to have retained the enzymes required for the reactions demonstrated. However, exactly which proteins and coenzymes are required for positive reactions is far from clear. In the case of tetrazolium salt reactions, much is made of the capacity of reagents such as INT (p-Iodonitrotetrazolium violet) and CTC (cyanoditotyl tetrazolium chloride) to indicate “respiration”. Yet, respiration is essentially a series of redox reactions involving oxygen or other inorganic compounds as electron acceptors. Pure preparations of many of the enzymes involved, combined with appropriate coenzymes and completely devoid of any living system can reduce tetrazolium salts. Although elegant studies (e.g. [13]) have suggested that intact electron transport chains can be involved in tetrazolium reduction *in vivo*, their participation is currently inferred rather than proven. Similarly, there are significant uncertainties about the relative contributions of substrate sources, endogenous and exogenous to the cell concerned, contributing to the reaction product. These issues are regularly addressed by careful and extensive control reactions in histochemical reactions [1], but it is unusual for such controls to be performed in bacterial studies.

Evidence Relating Cellular Activity and Culturability in Experimental Monocultures

Cellular activity assays provide qualitative and, in some cases, quantitative descriptions of phenotype. In practical terms such descriptions are related to culturability in two distinct ways, statistically and historically. In the statistical process, cells with particular phenotypes are identified in samples from a mixed culturable/NC population and, at the same time but with a separate sample from the same population, a quantitative test of culturability is performed. The culturable cell count may then be observed to be consistent with or discrepant from the active cell count. In the historical relationship the assays of activity and culturability are performed on one sample population. Thus the growth potential of cells with a particular phenotype is explicitly identified.

In practice, the statistical relationship has been addressed most often. Indeed, the validity of the activity assay as a measure of viability is generally established in this way by mixing cells inactivated by formaldehyde or heat treatment with actively growing cell populations, and confirming that the cellular assay identifies active and inactive cells in the right proportions. Given the fact that these treatments directly disrupt the biochemical elements identified as responsible for the reactions (Table 1), this outcome is hardly surprising. However, even if we accept the potential of these assays to discriminate between viable and non-viable cells, in the statistical relationship, unless the active and culturable cells are in the majority, there is no direct evidence that the culturable cells and the active cells are the same population. It is often the case that total, active and culturable cell counts are separated by several orders of magnitude. Here, the culturable cells are in such a small minority that there is no simple basis on which to determine whether they come from the active or inactive populations.

In the historical relationship the activity test must not compromise the culturability of the target cells, and there must be some means of separating cells with different results in the assay for subsequent culture. This has been achieved by flow cytometry and sorting by Kaprelyants and colleagues working on *Micrococcus luteus* [7]. Interestingly, these authors were able to enrich culturable cell populations on the basis of their rhodamine signal, however, identification of such cells was not precise since ~80% of the cells in their most enriched fraction remained NC.

Some Specific Changes in Patterns of Activities Observed in this Laboratory in Populations During Decline to and Established as Nonculturable

We start with some observations on *Helicobacter pylori* in work aimed at establishing whether NC coccoid cells of *H. pylori* had a similar phenotype to the coccoid cells formed

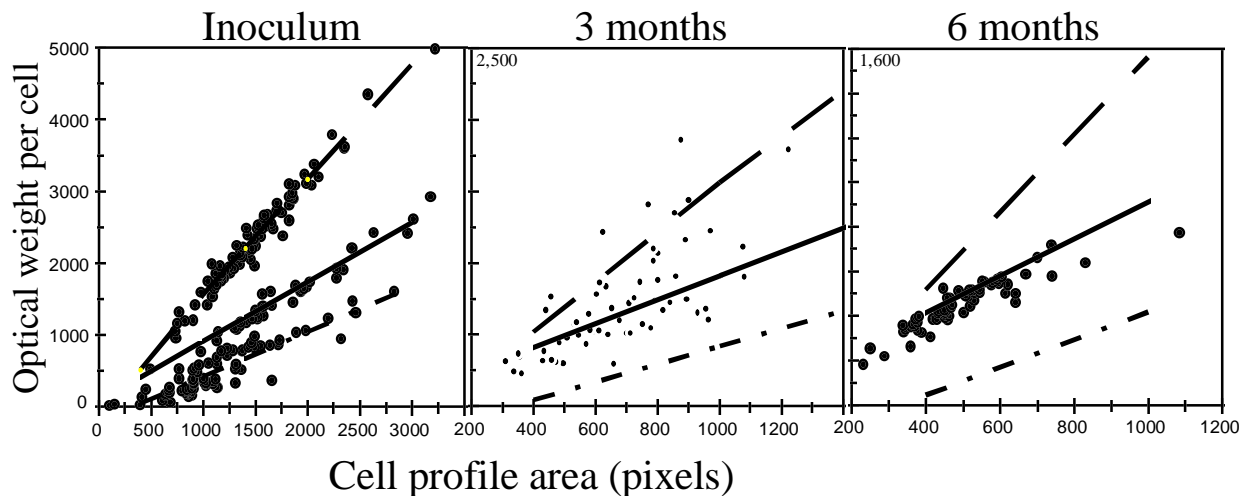


Fig. 1. Succinate enhanced INT reduction in *Helicobacter pylori* cells before and during maintenance in phosphate buffered saline at 4°C for the times indicated. Each point represents values obtained for a single cell as described previously [4,11]. Briefly, optical weight is an estimate of the integrated optical density of the object (i.e. the amount of tetrazolium reduced per cell) measured during brightfield illumination and cell profile area is the count of the pixels covered by the cell profile (the total area covered by a two dimensional image of the cell). Colony counts were below 10 CFU/ml within two weeks while total cell counts remained at 10^8 cells/ml [4]. The three lines drawn in each panel represent sub-populations identified by the ratio between optical weight (OW) and cell profile area (CA). Note the differences in scale between the panels.

during cold stress of *Vibrio vulnificus*, the organism for which evidence for the proposed “viable but nonculturable” state was and is strongest [12]. They did not and, in fact, carbon nitrogen stressed NC *H. pylori* cells maintained at 4°C retained a higher proportion of active cells than similarly maintained *V. vulnificus* cells [4].

Further image analysis of the succinate-enhanced tetrazolium reactions used in these studies revealed the additional features that are demonstrated in Fig. 1. Three sub-populations, defined by the ratio between their optical weight (OW) (amount of formazan per cell) and cell area (CA), were apparent at the time when cells were prepared for inoculation into the phosphate buffered saline microcosm. The *H. pylori* cells became NC within two weeks under the maintenance conditions and, during the subsequent five months, the population with the mid range of OW/CA ratio became dominant. Thus, while there may be no constant relationship between culturability and this specific activity, closer inspection of the results indicates the existence of physiologically distinct sub-populations in which the relationship between phenotype and culturability may be more informative.

In our second example we address the question of how cell phenotypes change during the decline to nonculturability. In this case exponential phase *V. vulnificus* cells inoculated into nine salts medium and maintained at 4 °C were followed by INT assays until the colony counts fell below the limit of detection. On the basis of earlier work, we had envisaged that the decline phase would be accompanied by steady decline in INT reduction rates [14]. Fig. 2 shows the overall decline trend combined with a decline in cell size and a

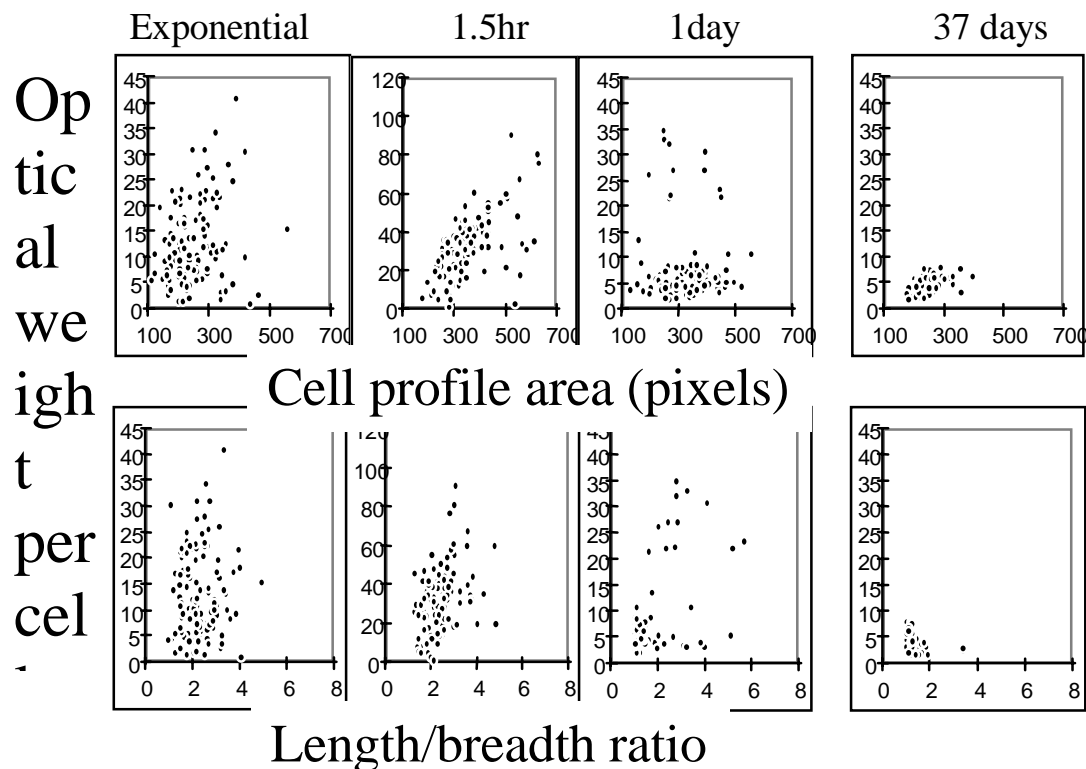


Fig. 2. Cellobiose-enhanced INT reduction in *Vibrio vulnificus* cells before and during maintenance in nine salts solution at 4°C. Total cell counts remained at approximately 10^8 cells/ml while the colony counts were below 10 CFU/ml at 37 days. Note the difference in scale between the 1.5 hr and other panels. See legend to Fig 1 for explanation of optical weight and cell profile area. Length/Breadth ratio provides an indication of coccoid transformation. Cells with a ratio <2 would be considered coccoid.

shift to coccoid morphology. However, the 1.5 hr panel reveals that cellobiose enhanced INT reduction was actually accelerated almost three-fold during the early phase of the combined nutrient and temperature downshift stress. From these and related studies we have concluded that there are no simple relationships between the cellular phenotypes demonstrated by activity assays and the physiological states of the target cells. In particular, “common sense” is a poor basis on which to predict the phenotypes that will be observed.

In these examples, although we have discussed dissociations between activity and culturability, it must be emphasised that we do not know whether the NC cells involved could have been resuscitated by application of appropriate conditions. However, these issues “cannot be addressed by any finite experiment” [3]. In our final example we have studied the question of whether an activity test, widely accepted as an indirect measure of viability, provides a basis on which to predict a resuscitation event that is of practical importance. We used the Kogure cell elongation test applied to NC cells of *Salmonella typhimurium* to prepare oral and intraperitoneal inocula for female balbC mice comprising elongation-positive cells in excess of the LD₅₀ defined for the specific pathogen-host combination. We found neither infection nor colonisation attributable to this elongation-positive population of active cells and concluded that the Kogure test cannot be used as a predictor of infectivity in salmonella infection studies.

Concluding Remarks

We have argued the view that results of established cellular assays of activity applicable to bacteria are not consistent predictors of culturability or, in one instance, of infectivity. Not only do we find the relationships between activity and culturability inconstant but, in those settings where culturability is restricted to cells with a particular observable phenotype, we find no reason to believe that the same relationship will hold when the starting population and the noxious insult are different. We have avoided discussing the potential relationships between activity and viability as these have been addressed elsewhere [8]. Nonetheless, since we subscribe to the view that the only adequate operational definition of bacterial viability is proof of replication (in most cases culturability), it follows that we consider activity assays unreliable indicators of viability. None of this excludes the possibility that so called “viable but nonculturable cells” may represent a genuine physiological state in bacteria [2, 12]. We simply take the view that the phenotype of such cells has not been demonstrated by cytological assay.

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