The ecological role of VBNC cells in the marine environment

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ABSTRACT

The quantification and identification of viable and active cells within natural marine communities is of great ecological interest. Although a large variety of techniques have been developed to characterise the physiological state of individual cells, the existence and nature of a viable or active but non-culturable state is still debated. The application of these techniques to natural marine communities suggests the existence of a large fraction of viable but non-culturable cells. The ecological role of viable or active but non-culturable cells within marine communities can be investigated by combining cellular and molecular techniques. Flow sorting of microorganisms from environmental samples allows the physical isolation of the cell populations of interest for subsequent molecular analysis. The sorting of active, productive and dead bacteria from natural marine waters may help to further understand the significance of active but non-culturable cells within natural communities.

Introduction

The viable but non-culturable state of bacteria in the marine environment has been extensively studied during the last 20 years as a result of important advances in the fields of fluorescent dye technology and cytometry. Studies have focused on characterisation of the physiological state of: (i) pathogenic bacteria submitted to stressful conditions (temperature, starvation, salinity and solar irradiation) and; (ii) bacterial cells within natural communities independently of their taxonomic affiliation. Both from physiological and ecological points of view, the significance of the viable but non-culturable state remains unclear. The ecological interpretation, in particular, is very difficult due to the presence of different classes of non-culturable cells within natural communities.

A first class of non-culturable cells consists of bacterial species that are fully culturable under 'normal' laboratory conditions, yet can enter a non-culturable state when exposed to stressful growth conditions (for reviews see [10, 13, 15]. Although these cells have lost their culturability, some of them can retain measurable metabolic activity and are commonly called " viable but non-culturable cells " or " active but non-culturable " cells [20].

A second class of unculturable bacteria is represented by bacteria forming colonies on agar plates or in liquid media with a low efficiency. For instance, Button et al. [3] isolated oligobacteria with doubling times of a few weeks or months *in situ* and one day to one week *in vivo*. Bianchi and Giuliano [2] used the microcolony assay and reported high counts of viable cells in surface and intermediate seawater masses when compared to traditional CFU counts. The authors suggested that the commonly observed low viable counts could be due to failure to correctly observe and identify cell division ability.

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A third class of unculturable marine bacteria are those unable to grow and reproduce under traditional cultural conditions due to unknown growth requirements. These bacteria include most of the symbiotic bacteria and have never been isolated. Their identification is only possible by cloning techniques.

All classes of non-culturable bacteria may exist within natural communities. Therefore, there is clear evidence that the viable but non-culturable state exists within marine communities and the reasons for failure of cells to form colonies may be as diverse as the species under consideration.

Concept and Definitions

Viability was defined by Oliver [16] as a cell that can be demonstrated to be metabolically active while being unable to undergo the sustained cellular division required for growth in or on a medium normally supporting growth of that cell. This is in contradiction to the definition proposed by Barer et al. [1], who defined a viable cell as a cell which is capable of fission to produce similarly viable progeny under realisable culture conditions. These authors have defined terms such as "active but non-culturable cells" (ANC) operationally as those which can retain certain assayable activities [9]. According to these definitions, ANC cells may be viable or non-viable. In contrast, active cells were defined from an ecological point of view by DelGiorgio et al. [4] as the fraction of metabolically active cells. These cells are considered as growing cells and may take part in the production of biomass. However, in this case the term activity is different from the "physiological" activity defined by Kell et al. [9]. One way to resolve this semantic debate would be to use the term "productive" cells for the active cells that take part in production of biomass in natural aquatic ecosystems.

The semantic debate is of little interest and, as stated by Kell et al. [9], each term should be defined in reference to a given method. For natural complex communities, we suggest the following definitions:

Culturable cells: cells able to reproduce under culture conditions as detected by any analytical tool used for observing cell division. This definition includes macro- and microcolonies as well as the dilution technique used for oligotrophic bacteria. These cells are viable cells.

Active cells: all cells able to retain any measurable metabolic activity and cellular integrity with preservation of at least one genome.

Productive cells: cells showing a metabolic activity related to growth and detectable without addition of any nutrients. Productive cells are not necessarily culturable cells.

Moribund cells: cells with depolarised membranes, intact cellular structures and containing at least one copy of the genome. This category is made to include cells that are not active, but not yet dead in regard to the definition of death (see below). This state is still very ambiguous.

Dead cells: cells with permeabilised membranes and/or damaged nucleic acids.

The Physiological Basis of the VBNC State: Application to Starvation Studies

Viability studies of selected organisms, mainly human and fish pathogens, have focused on culturable bacteria entering a non-culturable state. *Vibrio vulnificus* is probably one of the most commonly used marine bacteria in studies of the ANC cells response to temperature and starvation stresses (for reviews, see [14, 17]). However, the techniques used to

characterise the ANC state address different aspects of cell metabolism and it may consequently be difficult to compare the results obtained by the different methods.

Joux et al. [6] have shown a progressive physiological cell alteration throughout the starvation process for *Salmonella typhimurium* starved in seawater. The cells lost successively their culturability, metabolic activity, respiration, and membrane potential, and then membrane permeability, leading to the degradation of nucleic acids. Conversely, the absence of ANC forms during starvation in seawater has been reported for *Deleya aquamarina* [5]. In this case, most physiological functions were lost at the same rate suggesting that the ANC state is perhaps not so widespread in marine ecosystems.

The question of whether the ANC cells are able to exit this "dormant" state and return to a growing state when their environment becomes favourable is at the center of the actual debate on the ANC state [1, 14]. Demonstration of the reversibility of this non-growing state is based on resuscitation studies, but most of these are open to criticism. In most studies, it is generally uncertain whether the ANC cells are capable of resuscitation or whether the increase in cell numbers reported in a large number of publications on this issue is merely the result of growth of a few viable cells [14]. This is due to the existence of an important heterogeneity of physiological states within a population, and to our inability to provide the proof that a cell in a given active state, as determined by any given method, is able to grow and divide.

The actual tendency is to consider that, although the reversibility of the non-culturable state is not clearly demonstrated, cells which maintain at least one genome and an intact and polarised membrane cannot be considered as dead cells [8, 12, 14]. However, the methods used to characterise the DNA content of individual cells, as well as the integrity of membranes, should be considered with caution [11, 12]. The combination of a dye that

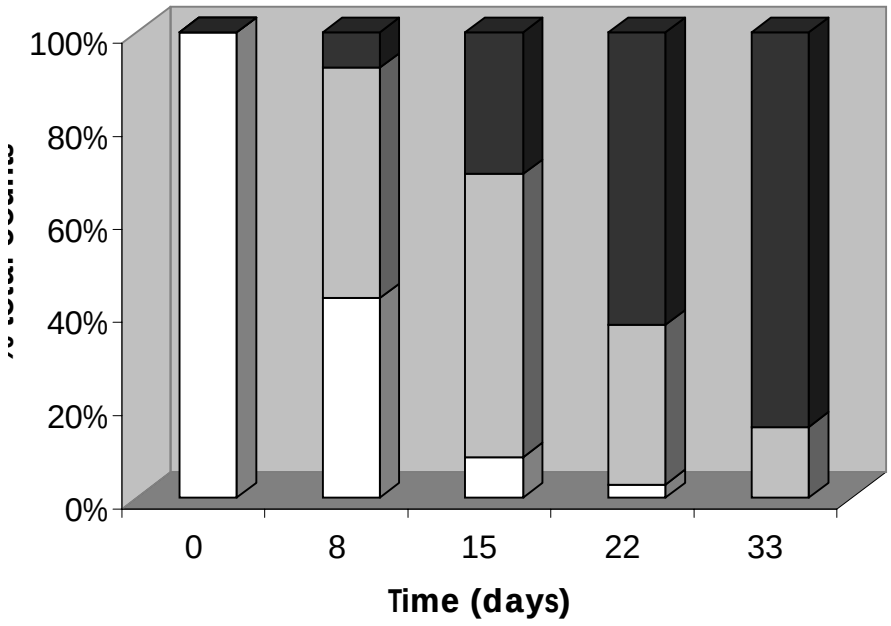

Fig. 1. Temporal evolution of the percentage of each cellular state in a *S. typhimurium* population during starvation in seawater. Each cellular state is expressed as a percentage of the initial total cell counts. White, active cells (CV3+); Grey, dead cells (CSE+); Black, non-active and non-dead cells (non-labelled by both dyes).

targets the activity of a cellular function with another that preferentially stains dead cells is of great interest. This approach has actually been developed in the BachLight live/dead staining kit from Molecular Probes Inc. (Eugene, Oregon) and the CV3/CSE labelling kit from Chemunex (Maisons-Alfort, France). As an example, the CV3/CSE kit has been applied to analysis of the physiological state of *S. typhimurium* cells starved in artificial seawater (Fig. 1). In this case, non-labelled cells may correspond to moribund cells, i.e., non-active with no detectable esterase activity (CV3-), and non-dead cells with intact membranes (CSE-).

Applications to Natural Communities

From an ecological point of view, at least three categories of cells could be distinguished within natural communities: active and non productive cells (those which play a potential role within communities); active and productive cells (those which contribute to the functional role and to the production of biomass at the time of sampling) and; dead cells (moribund and dead cells which represent particulate organic matter). The number of productive cells within natural communities, for instance, represents a more suitable parameter that could be used in models.

A major objective is to understand whether or not the cellular heterogeneity reported at the population level for selected organisms exposed to stressful conditions exists *in situ*. ANC cells of cultured bacteria may exist because bacteria in the natural environment are exposed to stressful conditions and are subjected to rapid changes, both spatial and temporal, in their environment. Therefore, when any environmental stress is applied, the initial physiological state of individual cells may vary depending on numerous factors, and may result in physiological heterogeneity within natural populations. One way to further understand the importance of this heterogeneity would be to analyse at a given time the diversity of species within each physiological state (productive, active and dead).

Over the past 20 years several methods have been developed to determine the fraction of active and productive bacteria. Although its ecological interpretation remains unclear, CTC labelling is often used to estimate the number of productive bacteria, and to calculate specific generation times within natural ecosystems ([19]. Conversely, the DVC method is commonly used to quantify the fraction of active cells. The DVC method has been useful in demonstrating that the fraction of active cells is sometimes much greater than that of culturable counts [6]. These cells are often called “viable cells” because of their apparent capacity to grow.

Assessment of microbial activity and productivity in complex systems, therefore requires an understanding of how the different members of the microbial community react in the assay. The ecological significance of all of these methods will remain unclear until we are able to relate active and productive cell counts to other parameters. The cell sorting capacity of flow cytometers is probably one of the most interesting techniques currently available to physically isolate bacterial cells in a given physiological or functional state for further characterisation by molecular techniques and/or parameters related to metabolic synthesis.

Analyses of active and culturable cells in natural marine communities suggests that the genetic diversity of active cells, as determined by a combination of the DVC and DGGE methods, is higher than that of culturable cells (Fig. 2). In this example, a seawater sample from a coastal area of the Mediterranean Sea was analyzed in March 1998. A total of 5.3 x 10^6 cells ml^{-1} was determined by FCM counting of SYBR-II stained cells. Active cell

counts (DVC+ = 22.2%) were higher than those of culturable counts (R2A, 8.3%; Zobell, 4.4%). The genetic diversity within each sorted fraction, as well as the genetic diversity of culturable cells, was analyzed by PCR amplification of 16S rDNA partial sequences, and by the DGGE technique (Fig. 2). The results suggests that; (i) the genetic diversity of active cells is high compared to that of culturable and productive cells and; (ii) the total genetic diversity determined by adding the patterns corresponding to each celular fraction (DVC+culturable cells) is larger than that reported from total DNA. Thus, the genetic and/or taxonomic diversity of cells as determined by cell sorting may be more representative of the fraction of active cells than that determined from the total community DNA.

Further investigations of this kind may contribute to a better understanding of the significance and diversity of ANC and productive cells in the marine environment, as well as of the relationships that exist between taxonomic, physiological and genetic diversity. Although new methods are available under development, the dyes used for activity assessment should be improved and more related to cellular energy metabolism. The recent development of new instruments such as solid-phase cytometry, which can detect rare

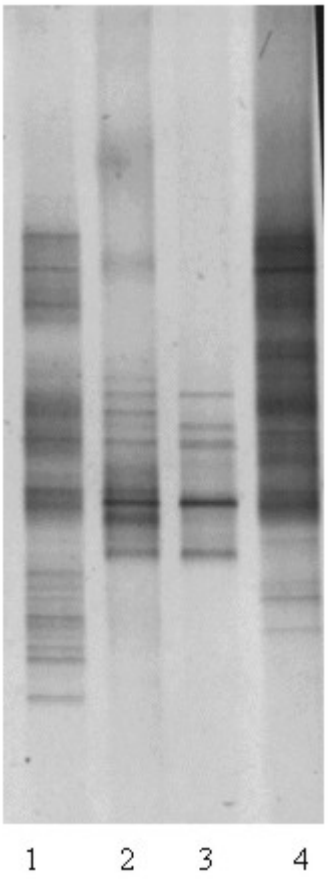

Fig. 2. Analysis of a seawater sample from the Mediterranean coast (Banyuls-sur-Mer, France). The genetic diversity within total, active and culturable cells was determined by PCR amplification of partial 16S rDNA sequences and DGGE analysis. Banding patterns from each cellular fraction are reported. Total cells (pattern 4) were stained with the SYBR-II dye and counted by flow cytometry (FCM). Active cells were determined by the DVC method and elongated cells were sorted by FCM (pattern 1). Culturable cells were determined on R2A (pattern 2) and Zobell (pattern 3) media.

events in the presence of a background of non-targeted cells, will contribute to our understanding of the importance of ANC cells within natural communities. This instrument has great potential for the rapid detection and quantification of species (pathogens and others) within natural bacterial communities.

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References

1. Barer MR, Gribbon LT, Harwood CR, Nwoguh CE (1993) The viable but non-culturable hypothesis and medical bacteriology. Rev Med Microbiol 4:183-191
2. Bianchi A, Giuliano L (1996) Enumeration of viable bacteria in the marine pelagic environment. Appl Environ Microbiol 62:174-177
3. Button DK, Schut F, Quang P, Martin R, Robertson BR (1993) Viability and isolation of marine bacteria by dilution culture: theory, procedures, and initial results. Appl Environ Microbiol 59:881-891
4. DelGiorgio PA, Gasol JM, Vaqué D, Mura P, Agusti S, Duarté C (1996) Bacterioplankton community structure: protist control net production and the proportion of active bacteria in a coastal marine community. Limnol Oceanogr 41:1169-1179
5. Joux F, Lebaron P, Troussellier M (1997a) Changes in cellular states of the marine bacteria *Deleya aquamarina* under starvation conditions at two temperatures. Appl Environ Microbiol 63:2686-2694
6. Joux F, Lebaron P, Troussellier P (1997b) Succession of cellular states in a *Salmonella typhimurium* population submitted to starvation in artificial seawater microcosms. FEMS Microbiol Ecol 22: 65-76
7. Joux F, Lebaron P (1997) Improved direct viable count method for aquatic bacteria: ecological implications. Appl Environ Microbiol 63:3643-3647
8. Karner M, Fuhrman JE (1997) Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. Appl Environ Microbiol 63:1208-1213
9. Kell BD, Kapreylants AS, Weichart DH, Harwood CR, Barer MR (1998) Viability and activity in readily culturable bacteria: a review and discussion of the practical issues. Antonie van Leeuwenhoek 73:169-187
10. Kjelleberg S (1993) Starvation in bacteria. Plenum Press, New York.
11. Lebaron P, Joux F (1994) Flow cytometric analysis of the cellular DNA content of *Salmonella typhimurium* and *Alteromonas haloplanktis* during starvation and recovery in seawater. Appl Environ Microbiol 60:4345-4350
12. Lebaron P, Catala P, Pathuisot N (1998) Effectiveness of SYTOX Green stain for bacterial viability assessment. Appl Environ Microbiol 64:2697-2700
13. Lloyd D, Hayes A (1995) Vigour, vitality and viability of microorganisms. FEMS Microbiol Lett 133:1-7
14. McDougald D, Rice SA, Weichart D, Kjelleberg S (1998) Nonculturability: adaptation or debilitation? FEMS Microbiol Ecol. 25:1-9

15. Morita RY (1997) Bacteria in oligotrophic environments. Chapman and Hall, New York.
16. Oliver JD (1993) Formation of viable but non-culturable cells. In: Kjelleberg, S (ed) Starvation in bacteria. Plenum, New York. pp 239-272
17. Oliver, JD (1995) The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol Lett 133:203-208
18. Postgate JR (1976) Death in macrobes and microbes. Symp Soc Gen Microbiol 26:1-18
19. Sommaruga R, Conde D (1997) Seasonal variability of metabolically active bacterioplankton in the euphotic zone of a hypertrophic lake. Aquat Microb Ecol 13:241-248
20. Xu HS, Roberts N, Singleton FL, Atwell RW, Grimes DJ, Colwell RR (1982) Survival and viability of non-culturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microb Ecol 8:313-323