

VIABLE BUT NON-CULTURABLE BACTERIA IN THE MARINE ENVIRONMENT AND THE BIOTECHNOLOGICAL TOOLS TO DETECT THEM

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Contents

- 1. Introduction
- 2. History of the Viable but Non-culturable Phenomenon in Bacteria
- Glossary
- Bibliography
- Biographical Sketch

Summary

When bacteria move from one environment to another, the new environment with which they are confronted may include changes in temperature, nutrient concentration, salinity, osmotic pressure, pH, and other properties. Furthermore, such changes can occur daily within given environments such as estuaries and coastal systems. Bacterial cells adapt dynamically to such shifts in their environment by employing a variety of genotypic and phenotypic mechanisms. Constitutive and inducible enzyme synthesis enables bacteria to accommodate to growth-limiting nutrient concentrations and to adjust or reroute metabolic pathways to avoid potential metabolic and/or structural disruption. Furthermore, they are able to coordinate rates of synthesis to maintain cellular structure and function. These adaptive capabilities provide bacteria with mechanisms by which they are able to respond to their surrounding environment, not only to survive, but to thrive. Demonstration of these capabilities has been made possible through the newly developed tools of biotechnology.

1. Introduction

Until recently, the ability to culture microorganisms on artificial media in the laboratory constituted proof of their viability. Depending on the efficiency and/or selectivity of the media employed, interpretation of the viability of bacteria in environmental samples varied, with terms such as "live", "dead", "vegetative", "viable", "nonviable", "stressed", "injured", "moribund", and "static" being applied to such cells, often quite ambiguously. Additional terms used to describe metabolic and reproductive states of bacteria can be found in the literature. The term "viable but non-culturable" was first introduced in 1984

for bacteria that demonstrated detectable metabolic function, but were not culturable by any available bacteriological methods.

Thus, it has been recognized by microbial ecologists that one of the major limitations to research in microbial ecology is the inability not only to isolate and grow in culture the vast majority of the bacteria that occur in nature, but also to detect their presence. The "viable but non-culturable" phenomenon represents a state of dormancy, survival, and persistence in the environment. Bacterial cells that are "intact and alive", according to selected metabolic criteria, but are not recoverable by culture in or on routinely employed bacteriological media, have been observed in both terrestrial and aquatic environments and, most recently, in the clinical laboratory.

2. History of the Viable but Non-culturable Phenomenon in Bacteria

The problem of determining the metabolic state of organisms observed in direct counts, but not recoverable by plating or most-probable-number (MPN) counts, was recognized in the late 1800s. They did not propose specific terminology to define that stage of bacterial growth and survival. The phenomenon appears to reflect various mechanisms of bacterial survival, and individual strains within a species respond differently to environmental conditions as well as to the length of exposure to those conditions.

The term "viable" was proposed in 1954 to describe cells capable of multiplying and forming colonies, but the term "live" was suggested for respiring cells unable to divide under the same conditions. "Dead" bacteria were described, in 1962, as those that did not divide; however, it is considered that non-dividing bacteria may be in some sense "alive", because they retain their osmotic barrier even after "death". By this definition, cells are dead if they do not multiply.

Another term, "moribund", was suggested in 1967 for the transient state between viability and death that bacterial cells in or on bacteriological media enter when they are exposed to starvation and when they are incapable of multiplication, yet maintain other metabolic functions.

The word "dormancy", a term frequently employed among microbiologists, has been used to describe the state into which spore formers or cyst formers transform, but is not employed for organisms that do not produce spores or cysts. Spores and cysts produced by some soil microorganisms are mechanisms of survival. "Resting" forms are found in physiologically and genetically diverse groups of organisms, such as *Myxococcus*, *Azotobacter*, *Streptomyces*, *Bacillus*, and *Clostridium* species, notably when they are exposed to altered nutritional conditions. Both soil and aquatic bacteria are able to function at very low metabolic rates when environmental conditions are extreme, and spores develop when only a limited source of carbon, nitrogen, or phosphorous is available. The spore state is considered highly resistant to extreme environments, e.g., very high or low temperatures, desiccation, exposure to toxic chemicals, etc.

In 1983, cells were described as "nonviable" once the cells lost the ability to form a colony. It was also acknowledged that the number of "nonviable" respiring cells was 10-fold greater than the number of those that were capable of multiplying.

As stated above, after comparing various available methods for isolation and detection of cells, a team of researchers proposed in 1984 the term "viable but non-culturable" to describe *Salmonella enteritidis* in the stage when it is detectable by direct viable count (DVC) but is not culturable. They were able to "resuscitate" cells assumed by other investigators to have "died off" in seawater, defining the non-culturable cells as viable but non-culturable. It was subsequently proposed, in 1987, that a resting or "somicell" stage for Gram-negative bacteria, analogous to spore formation in Gram-positive bacteria. Two years later in 1989, in an attempt to show the genetic basis of nonculturability, an interesting level of homology was observed between described fragments of *Vibrio* genome and *spo* genes when probes prepared from cloned *spo* genes from *Bacillus* spp. were used. The presence of a few *spo* genes and a number of sigma factors in the genome of *V. cholerae* was confirmed in 2000.

Bacteria that have the greatest capacity to survive starvation are small and have lower metabolic rates than other bacteria. It was shown in 1965 that this characteristic low metabolic rate is shared with spores, which exhibit a capacity for survival by shutting off almost all metabolic activity, yet remaining viable. The "rounding up" phenomenon, with concomitant reduction in cell volume, was reported for *Vibrio* species under low nutrient conditions, i.e., in an environment relatively free of organic nutrients, at which time the bacterial cells are observed to adjust to the substrate-limited stress condition by adopting necessary physiologic changes. *Campylobacter jejuni* also becomes a typical coccoid-like cell with intact cell membranes and retains its viability after entering the non-culturable state.

When morphologic changes take place, the rounding up and reduction in size of the cells may be as much as 15- to 300-fold, depending on the level of nutritional stress. It is possible to filter organisms from seawater that pass through a 0.45- μ -pore-size filter membrane, but which are retained on 0.22- μ m-pore-size membranes. The bacteria with this capability are *Spirillum*, *Leucothrix*, *Flavobacterium*, and *Vibrio* species. This size reduction was also observed for viable but non-culturable *V. cholerae*. It is concluded that filter membranes with pores no larger than 0.22 or 0.10 μ m must be used to collect cells from the natural environment.

The three O-antigenic forms or subtypes of *Vibrio cholerae* 01 (Ogawa, Inaba, and Hikojima) are based on structure and response to various tests that determine an organism's role in causing disease. For purposes of discussion, epidemic strains of *V. cholerae* 01, i.e., the "cholera vibrio", are defined as possessing the 01 antigen, being capable of producing cholera toxin (CT⁺), and, therefore, causing cholera in susceptible hosts. Both classical and El Tor biovars are included in *V. cholerae* 01. Nontoxicogenic *V. cholerae* 01 strains comprise those isolates that agglutinate in 01 antiserum, but do not possess the cholera toxin gene. Nevertheless, these strains may produce diarrheal disease and are designated nonepidemic *V. cholerae* 01. A non-01 *V. cholerae* strain, *V. cholerae* 0139, which does not agglutinate with 01 antiserum, has caused epidemics in India, Bangladesh, and elsewhere.

Environmental and clinical isolates are identical by 5S rRNA and DNA fingerprinting. Interestingly, both the 5S rRNA and the 16S rRNA data indicate a deep branching of *V. cholerae* from other vibrios. Little difference is found in the major outer membrane

proteins of clinical and environmental isolates of *V. cholerae* 01 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A band of 22-kDa protein was observed for all the environmental strains, but not for the clinical strains. A significant decrease in the concentration of several of the protein bands, including the 22-kDa band, was detected for environmental strains passaged through rabbit ileal loops, demonstrating a possible environmentally-induced change.

The anecdotal information that persisted for nearly 100 years from the turn of the century maintained that the only reservoir of toxigenic *V. cholerae* 01, the epidemic strain of cholera, was the human intestinal tract and that this strain was taxonomically separate from *V. cholerae* non-01, also known as "nonagglutinable vibrios", found in aquatic, predominantly estuarine environments. An important advance was made when it was shown that, using DNA-DNA hybridization, 01 and non-01 *V. cholerae* constitute a single species. The distinction between "cholera vibrios" and other *V. cholerae* was vigorously championed at that time, but a mounting volume of evidence, including results of numerical taxonomy, DNA-DNA hybridization, and nucleic acid sequencing, provided solid underpinning for this conclusion. The epidemic of cholera occurring in India and Bangladesh, in which *V. cholerae* 0139 was isolated and concluded to be responsible for the epidemic, suggested the need to finally put to rest the notion of cholera vibrios and *V. cholerae* non-01 strains as separate species.

The evidence that has been accumulated for cholera is similarly increasing in volume for *Legionella*, *Campylobacter*, and *Helicobacter*. The evidence is now compelling for environmental sources of *Campylobacter jejuni*, and *Legionella pneumophila* and the long-term survival of *Helicobacter pylori* in the environment, and possibly autochthonous in nature.

Physiological investigations, done in parallel with the genetic analysis of control of the VBNC state, focused on two aspects of the VBNC response. Metabolic activity of *V. cholerae* and *E. coli* cells at the point of entry into the VBNC state was investigated in substrate uptake experiments, in which uptake of ³H-labeled thymidine and ¹⁴C-labeled glucose and acetate were measured. Uptake of substrate decreased as cells entered the VBNC state and dramatically increased with a temperature upshift from 4°C to 30°C, with recovery of culturable cells of *V. cholerae*. The increased uptake was not observed with *E. coli*, which did not recover culturability. Transition from the VBNC state, after a temperature upshift from 4°C to 30°C, was elucidated, showing that the mechanism(s) of genetic control of VBNC state must be known before this phenomenon can be fully understood.

Temperature is an important inducer of the VBNC state. Experimental evidence suggests that increased temperature of *Campylobacter jejuni* cultivation results in decreased recoverability on culture plates. The ability of bacterial cells to respond to shifts in environmental parameters involves a variety of phenotypic and genotypic mechanisms. To remain viable, bacteria must utilize both constitutive and inducible enzyme synthesis, accommodate to growth-limiting nutrients and adjust or reroute metabolic pathways to escape metabolic or structural disruption caused by specific nutrient limitations. The adaptability of bacteria in this regard is extraordinary and has been documented by many investigators over the past several decades. In one study, the

uptake of ^3H -labeled thymidine and ^{14}C -labeled glucose with acetate as the substrate decreased as cells of *V. cholerae* and *E. coli* entered the VBNC state and dramatically increased upon an upshift of temperature from 4°C to 30°C . In addition, the ability of the microbial cells to coordinate rates of synthesis to maintain cellular structure and carry out metabolic functions provides bacterial cells with a significant advantage in responding to the surrounding environment.

Clinical and environmental pathogens representing more than 13 genera including the families *Enterobacteriaceae*, *Vibrionaceae* and *Aeromonadaceae* have been reported to occur in the viable but non-culturable state. Although it is believed that growth-limiting factors have a major influence on the survival of microorganisms in the viable but non-culturable state, the effect of temperature appears to be extremely important. Morphological changes have been demonstrated in that the cells become coccoid and smaller in size with the central region compressed and surrounded by denser cytoplasm, because temperature change has induced the viable but non-culturable state. Organisms not capable of growing on routine bacteriological culture media are considered to be non-culturable, whereas demonstration of viability by direct methods (i.e., measuring the uptake of substrates) will allow determination of viability.

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