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Viable but nonculturable bacteria: a survival strategy

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Abstract When bacteria are introduced into a new environment, environmental changes with which they are confronted may include temperature, nutrient concentration, salinity, osmotic pressure, and pH. Bacterial cells dynamically adapt to these shifts in their environment, employing a variety of genetic mechanisms. Bacteria, with the ability to utilize constitutive and inducible enzyme synthesis, can accommodate to growth-limiting nutrients and adjust or reroute metabolic pathways to avoid metabolic and/or structural disruption caused by specific nutrient limitations. Furthermore, they are able to coordinate their rates of synthesis to maintain their cellular structure and function. These adaptive capabilities provide bacterial cells with an extraordinary set of mechanisms by which they are able to respond to their surrounding environment and survive.

Key words Viable but nonculturable · Dormancy · Dormant · *V. cholerae* · Survival strategies

Introduction

Until recently, the ability to culture microorganisms on routine bacteriological media in the laboratory was considered sufficient proof of viability. However, depending on the efficiency, limitations, and/or selectivity of the medium employed, determination of viability of bacteria in environmental samples can be difficult.

In the past, cells observed to be present by microscopic examination but not able to be grown in the laboratory were called dead cells, vegetative cells, viable cells, nonviable cells, stressed cells, injured cells, or moribund cells. These terms were often applied quite ambiguously. Additional terms that have been employed to describe the metabolic and reproductive states of these bacteria can be found in the literature. Roszak and Colwell[1] coined the term "viable but nonculturable" for those bacterial cells with detectable metabolic function, but not culturable by available methods.

The viable but nonculturable phenomenon is not new for microbial ecologists. It has long been recognized that one of the major limitations to research in microbial ecology is the inability to isolate and grow in culture the vast majority of bacteria which occur in nature. Thus, the recognition of viable but nonculturable bacteria has revived and revitalized the interest of many investigators – particularly microbial ecologists – in the dormancy, survival, and persistence of microorganisms in the environment.

Bacterial cells that are "intact and alive," according to selected metabolic criteria, but do not undergo cell division in, or on, routinely employed bacteriological media, comprise a phenomenon that has been observed in soil and water bacteria, but not recognized widely, or accepted, by clinical microbiologists. Food microbiologists have addressed this phenomenon and called it "injury." They have published extensively on "resuscitation" of bacteria in foodstuffs.[2]

Materials and methods

Determining the metabolic state of organisms observed in direct counts, but not recoverable by plating or by the most probable number (MPN) method, was recognized as a problem in meat and other food as far back as the late 1800s. Knaysi[3] and Janinson[4] reported such observations, but did not propose specific terminology to define this phenomenon, which reflects the various mechanisms of bacte-

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rial survival. Individual strains within a species respond differently to environmental conditions, as well as to the length of exposure to those conditions. An extensive review of the viable but nonculturable phenomenon is provided by Colwell and Huq.[5]

Bacteria that have the greatest capacity to survive when in the starvation state have been shown to be small in size and to have lower metabolic rates. Dawes and Ribbons[6] have shown that spores share this characteristic, ie, a low metabolic rate, which allows them to survive, by shutting off almost all metabolic activity. The spores, thereby, remain viable. The "rounding-up" phenomenon, with concomitant reduction in cell volume, was reported for *Vibrio* spp. under low nutrient conditions, ie, in an environment relatively free of organic nutrient, at which time bacterial cells adjust to the substrate-limited, stress condition by adopting necessary physiological changes.[7] *Campylobacter jejuni* becomes typically coccoid-like, with intact membranes, when induced to the nonculturable state, still retaining viability.[8]

It has been suggested that, due to the morphological changes the cells undergo, "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. Anderson and Hoffman[9] were able to filter organisms from seawater that passed through a 0.45-μm filter membrane, but were retained on 0.22-μm membranes. These bacteria were later identified as *Spirillum*, *Leucothrix*, *Flavobacterium*, and *Vibrio* spp. The same range of size reduction has also been observed for viable but nonculturable *Vibrio cholerae*, since membrane filters with a pore size no larger than 0.22 μm must be used to collect the cells.[10]

For *V. cholerae*, the term "survival" had been viewed to reflect a high degree of host-adaptation, since cholera vibrios can be cultured for only very short periods of time when discharged into the environment. Now, evidence has accumulated showing *V. cholerae* to be an autochthonous inhabitant of brackish water and estuarine systems.[11] Thus, the very early studies of *V. cholerae*, prior to 1970, were aimed at identifying environmental conditions associated with unusual delay in the inevitable "death" of the "cholera vibrios," in order to establish the length of time after which an environment could be considered cholera-free, unless recontaminated with infected stool.

The remarkable discoveries of the past decade have revealed the existence of the dormant or somnambulant, ie, viable but nonculturable, state into which *V. cholerae* 01 and *V. cholerae* non-01 enter, in response to nutrient deprivation and other environmental conditions.[12] This phenomenon provides a new perspective and imparts a dynamic meaning to the term "survival." The evidence shows that *V. cholerae* cells do not die when discharged into aquatic environments, but instead remain viable, and are capable of transforming into the culturable state if environmental conditions again become favorable.[13]

The implications of dormancy of *V. cholerae* 01 are both significant and relevant. That is, dormant, ie, viable but nonculturable cells, are not recoverable on conventional bacteriological media that are routinely used to examine water for public health safety. However, nonculturable forms of *V. cholerae* 01, when inoculated into rabbit ileal loops, or drunk by human volunteers, caused large amounts of fluid accumulation and diarrhea, respectively.[12,14]

The evidence, primarily based on physiological studies of *V. cholerae* in aquatic environments, is that the dormant or viable but nonculturable state transcends mere survival and raises the more fundamental question of the adaptation and response of microorganisms to environmental conditions.[1]

Studies employing microcosms simulating saline, estuarine, brackish, and freshwater environments have provided new and important information on *V. cholerae* physiology and the effects of temperature and salinity, adherence, and colonization of chitinaceous and mucilaginous macrobiota. Studies prior to 1970 were based on methods for the isolation and characterization of *V. cholerae* originally developed for the clinical diagnosis of cholera in hospital laboratories.[15] The many difficulties associated with the isolation of *V. cholerae* 01 from the aquatic environment can be related to one simple fact: methods for isolating *V. cholerae* developed for clinical specimens containing large numbers of actively growing cells are not suitable for environmental samples. Clinical samples are likely to contain fewer cells adapted to a variety of environmental conditions, including (most commonly) low nutrient concentration, pH in the range of 7–8 (seawater), fluctuating temperatures and pH, variations in oxygen tension, and exposure to UV via sunlight.[16]

The findings of Colwell et al.[12] that *V. cholerae* 01 in environmental samples may not grow on laboratory media routinely used for isolation was a pivotal point in the debate concerning the ecology of *V. cholerae*. Viable but "nonculturable" cells go undetected unless appropriate methods for detection, eg, molecular, immunological, or direct microscopy, are employed. By combining modern molecular biological techniques with immunological direct detection methods, it can be convincingly demonstrated that viable but nonculturable cells of *V. cholerae* 01 occur even in clinical specimens from patients with the disease.[12,14,17,18]

Because the standard plate count cannot be used to enumerate viable but nonculturable organisms, alternative methods were developed to enumerate these bacteria, based on direct counting, the most common of which is epifluorescence microscopy and acridine orange staining.[19,20] The acridine orange direct count (AODC) was taken as a presumptive count of viable organisms, since it was assumed that intact, double-stranded DNA bound monomers of the dye which fluoresce with a green color, but broken, single-stranded DNA bound as dimers which fluoresce red-orange. At the very least, the AODC permitted enumeration of unlysed cells containing intact DNA. Subsequent studies, unfortunately, showed that the color shift cannot be used to distinguish viable from non-viable cells.

Therefore, a more persuasive assay for testing viability is the direct viable count (DVC) developed by Kogure et al.[21] Active cells are identified by growth, without multiplication, after incubation for 6 h at 25°C, in response to the

addition of yeast extract in the presence of nalidixic acid. Under these conditions, active cells carry out protein synthesis in the absence of DNA replication or cell division and produce elongated cells that are easily identified by their size. In general, the AODC count is higher than DVC enumeration for a given bacterial suspension, suggesting that nonsubstrate responsive cells may not be viable. However, this observation requires further study, because the substrate employed for the DVC can influence the end result.

Viable but nonculturable cells will not form colonies on agar media or grow in broth culture, as shown for *Escherichia coli*,[10] *C. jejuni*,[8] *Salmonella enteritidis*,[1] and other Gram-negative and Gram-positive bacteria.[22]

The polymerase chain reaction (PCR) has been used to detect *V. cholerae* in food and stool specimens by various investigators.[23,24] PCR is especially useful for environmental specimens in which the concentration of bacterial cells is usually very low. Koch et al.[23] claim to be able to detect as few as one *V. cholerae* cell per 10 g of food, with amplification reaction from crude bacterial lysates. Earlier, Shirai et al.[25] reported the use of PCR for detection of the cholera enterotoxin operon of *V. cholerae* 01 in stool specimens. They concluded that the cells of *V. cholerae* were "dead" when they did not grow on plates, a premature conclusion in the absence of corroborating evidence of cell death. More recently, we have been able to demonstrate the presence of viable but nonculturable cells of *V. cholerae* 01, using PCR in laboratory microorganisms and culture-negative stool samples,[26] combined with the DVC, using a fluorescent-labeled monoclonal antibody.

A modified fluorescent antibody method, originally described by Brayton and Colwell,[27] has been optimized to kit form, reducing the time required to complete the test to less than 10 min.[28,29] A test optimized for clinical specimens, the immunoassay CholeraSMART (New Horizons Diagnostics, Columbia, MD, USA) reported by Hasan et al.,[26] has proven promising also. All three of these test kits require no culture, making the tests valuable for the detection of *V. cholerae*, even in the nonculturable stage.

In other studies, Pearson et al.[30] reported that *Legionella pneumophila* which did not grow on standard laboratory media were detected in hospital water and ventilation systems, using immune epifluorescence microscopy. Quantification by this technique and comparison with DVC yielded excellent agreement when the *Legionella* were culturable. No evidence of cross-reactivity was detected with six groups of microorganisms isolated from the samples in which *Legionella* were detected. Recovery of viable cells not culturable on laboratory media has been reported by passage through fertilized egg yolks. The techniques for the detection of viable but nonculturable *Legionella* provide a new approach and rationale for the detection and quantification of *Legionella* in the environment. Previous work on the survival and growth of *Legionella* can now be interpreted on the basis of this new information, thereby extending our understanding of the epidemiology and pathogenicity of human infections associated with *Legionella*.

Discussion

The survival strategies of the cholera vibrios shape epidemic patterns. For example, we have been able to show a correlation of the epidemic pattern of cholera in recent years to climatological events, including El Niño. Predictive capacity for this infectious disease is being developed, utilizing knowledge of the viable but nonculturable state into which *V. cholerae* is now known to enter, as the following illustrates.

Cholera is a disease that can strike very suddenly – within 24–48 h, and victims may die when epidemics are severe. The understanding of cholera epidemics is traceable to the basic understanding of epidemiology founded by John Snow in the nineteenth century. In epidemiological studies, Snow plotted cases of cholera in central London and showed that the distribution of cases was associated with one of the wells in the center of London. In developing countries, cholera is common where only multi-use water supplies are available. When cooking utensils are washed, a child takes water to drink, clothes are laundered, and cattle are walked and watered and washed – all in the same area of a body of water – transmission of infectious disease is nearly inevitable.

Cholera, being a diarrheal disease, is a severe dehydrating illness with profuse watery diarrhea and vomiting, but only 25% of cases of cholera may be manifestly apparent. Three-quarters of cholera cases are mild, with the disease transmitted most commonly via the water supply. Thus, the numbers of cases actually reported may represent only the tip of the iceberg.

Very simply stated, the physical effects of cholera are caused by the bacteria attaching to the wall of the intestine and interfering with sodium and potassium transport. Loss of fluid via diarrhea results. Hospital treatment entails replacing the volume of fluid lost through diarrhea and vomiting, either by oral rehydration or by intravenous methods. Intravenous rehydration poses a difficult problem because the amount of fluid required for patient with a very severe case can be quite large. If there are 100 000 patients, and even if only a few thousand are severe, the demand for intravenous fluid replacement can put a severe burden on the capacity of a developing country.

The current global epidemics of cholera are part of the seventh pandemic, which began in the 1960s. Understanding the riverine and estuarine sources of the cholera vibrio is very important, especially since it is associated with plankton. In our early studies, we found the cholera bacterium to be associated with plankton in tidal rivers and coastal waters. In Chesapeake Bay in the eastern United States, we can and still are isolating and tracking the cholera vibrio. *Vibrio cholerae* can be detected in Chesapeake Bay in areas where the salinity levels are approximately 15 parts per 1000. However, the water treatment systems are effective, and the population surrounding the Bay is free of cholera.

In nature, cholera bacteria attach to the egg cases of microscopic animals, the zooplankton, notably the copep-

ods. The eggs are dispersed into the water, with the bacteria heavily and readily attaching to the egg cases of gravid females. The role vibrios play in nature is critical. For example, *V. cholerae* is a chitin digester. It breaks down the exoskeletal shell that is common to crabs and shrimp and copepods.

Work done by many investigators over the past 20 years shows that, in the spring, when phytoplankton blooms occur, zooplankton graze on the phytoplankton. The bacteria become abundant, being "amplified," as the numbers of zooplankton increase. The ability of the organism to survive in a kind of spore-like or dormant stage, as described above, is very important.

With human volunteers – volunteers in a study carried out at the University of Maryland Medical School at the Center for Vaccine Development – we were able to show that within 48 h of the ingestion of viable but nonculturable cells, we could culture bacterium in the stool of the volunteers. Thus, the bacterium, when in the nonculturable stage, can cause disease, a phenomenon common to bacteria in the environment, ie, pathogens that are waterborne or environmentally transmitted, eg, *Salmonella*, *Shigella*, *Legionella*, and *E. coli*; and this is an important concept for water microbiologists and clinicians.

The association between cholera vibrios and copepod adults and juveniles, especially the nauplii, is critical. The pattern that has been observed is that the number of copepods, hence the number of vibrios, increases just before epidemics break out in the spring and in the fall. The epidemics in Bangladesh are bimodal – in some years they are very severe and in other years they are not so severe – but twice a year there are epidemics. This bimodality can be correlated with the spring and fall peaks in plankton abundance.

Using remote sensing, we have been able to measure sea surface temperature and sea surface height. This has allowed us to make a discovery, quite fortuitously, that there is a bimodality of sea surface temperature and sea surface height, like the bimodality of the epidemics. When we plotted the data for the epidemics (hospitalization rates of cholera cases from 1966 to the present), we were able to show a correlation of the epidemics with sea surface temperature, the latter being related to plankton blooms.[31]

During the epidemic of cholera in 1990 and 1991, there were about 300 000 cases of cholera in Peru, and 100 000 victims were hospitalized, with about 3000 deaths in that 1 year alone. In 1997–1998, the results were very interesting, since the 1997–1998 El Niño had been predicted and, therefore, we were able to mobilize public health teams in Latin America to look for *V. cholerae* in the environment, starting in August 1997 when the El Niño was just beginning.

By satellite imagery obtained from August 1997 to June 1998, we observed that, along the coast of Peru, sea surface temperatures increased. In September, there were a few dozen cases of cholera in Peru; by October, about 200; by December, about 655; and by January, more than 1000; and, in total, by June 1998, there were more than 30 000 cases, approximately. We were able to detect the cholera vibrio in environmental samples early in August 1997, with numbers of vibrios detected in the environmental samples increasing from January to February 1998. Thus, real-time correlation was achieved. We are currently doing retrospective analysis of plankton data to determine whether there is a specific association of a certain species of copepod with *V. cholerae*.

Recent interannual changes in the strength and seasonal evolution of the surface level southwest monsoon winds have been related to variations in the summer phytoplankton blooms in the Bay of Bengal. Correlation of satellite remote sensing data with hydrographic and meteorological data sets and cholera case data for Bangladesh provided strong evidence that cholera cases occurred when there was a rise in ocean surface temperatures.

From all of this evidence, it is now possible to utilize remote sensing and computer processing to integrate ecological, epidemiological, and remotely sensed spatial data for the purpose of developing predictive models of cholera outbreaks. The ability to predict conditions conducive to pandemics of cholera will allow public health measures to be taken prospectively, rather than retrospectively.

It is worth highlighting one other aspect of our work. For the Bangladeshi population, for whom the filtration and chlorination of drinking water on a massive scale is not yet possible, the question becomes, what constructive measures can be taken? We chose to test whether simple filtration might reduce the severity of the epidemics. Experiments were done which showed that filtration of water, using cloth, retained the copepods and other particulate matter. When water collected from a Bangladesh pond was poured through inexpensive sari cloth, folded four to eight times, 99% of the vibrios were removed from the water.[32] In January 1998, a sociological study was done to determine whether it would be culturally acceptable for people to filter water through sari cloth before drinking it. Indeed, it proved to be so. We are now in the process of determining whether, with this very simple technology, we can reduce the incidence of cholera.

In summary, the exploration of *V. cholerae* in the environment has led to the discovery of the viable but nonculturable phenomenon and to the origin of epidemic cholera. With this knowledge, simple technology can now be employed, with the potential of preventing the massive epidemics of cholera that occur with regularity in the developing world.

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