

THE DETECTION, ISOLATION AND ENVIRONMENTAL APPLICATION OF UNCULTURABLE BACTERIA

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ABSTRACT

The modern industrialized world presents novel challenges to environmental science, requiring the constant development and deepening of knowledge. This is essential for characterizing new pollutants, better understanding bioremediation strategies, and identifying their limiting factors. The survival and activity of native microbial populations as well as their interactions with introduced microorganisms are critical to the effectiveness of bioremediation. Since the majority of natural microbiomes are still uncultivated, more research is required to understand their inherent roles in ecosystems. The identification, isolation, and environmental use of unculturable bacteria are the topics of this review.

Keywords: Detection, Isolation, Application, Unculturable bacteria

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INTRODUCTION

Since the eighteenth century, microbiologists have primarily relied on traditional plating techniques to obtain pure cultures. However, these culture-dependent methods, which

require the cultivation of microorganisms, cannot fully depict the microbial diversity in the biosphere. Despite Earth's impressively rich microbial diversity,

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over 99% of the potentially 10^{11} to 10^{12} microbial species remain undiscovered, with only a small fraction culturable by current techniques [1-4]. This limited microbial availability has created an urgent need to understand these missing species and their functions, as they hold significant potential for environmental sustainability in bioremediation and bio-waste industries. Metagenomics, a culture-independent technology, addresses this by harvesting the complete genetic material directly from an examined sample [5-7]. Large amounts of pollutants and xenobiotics are released into the environment by extensive industrial and agricultural operations, transportation mishaps, and inappropriate storage. One of the most important environmental problems of our time is the ensuing contamination of the air, water, and soil. The chemical structures and toxicological effects of contaminants, such as heavy metals, excess nutrients, and persistent organic pollutants, differ greatly. However, the majority endanger natural systems and present a significant risk to human health [8-13].

A variety of physicochemical treatments are available for removing environmental contaminants, but biological approaches remain among the most promising. Bioremediation, which utilizes the metabolic pathways of microorganisms or plants to neutralize pollutants, is a natural process that can be performed either ex situ or in situ. Contamination can be reduced by stimulating the indigenous

microflora with added nutrients (biostimulation) or by introducing microbial degraders (bioaugmentation) to the polluted site. When environmental pollutants are completely mineralized, the process generates carbon dioxide, water, and biomass as byproducts, making bioremediation a cost-effective and environmentally friendly approach. However, bioremediation can be inhibited by various abiotic factors (e.g., temperature, pH, moisture, electron acceptors or donors) and biotic factors (e.g., competition, predation). Often, even when environmental conditions appear optimal for biodegradation, efficiency falls short of expectations. Recent studies indicate that strains showing high pollutant biodegradation rates in controlled laboratory settings may be less effective and survive poorly in field-scale bioremediation. This observation further supports the existence of viable but nonculturable (VBNC) states in pollutant-degrading bacteria. Bacteria entering this state, where they are alive but have lost their culturability, could explain the discrepancies observed between laboratory and field performance [14-16].

NONCULTURABLE STATE

Until 1982, bacterial cells unable to grow on standard laboratory media were presumed dead. That year, however, the first description of the "viable but nonculturable" (VBNC) state emerged, marking the initial attempt to distinguish viability from culturability. Since then, numerous reports have revealed a spectrum of

bacterial physiological states, ranging from unstressed living cells to dead cells, with VBNC states falling within this range. VBNC cells retain viability but cannot proliferate on routinely used laboratory media [17, 18]. Similar to all living things, microbes react to shifting environmental conditions by triggering particular stress reactions and survival strategies when conditions fall short of ideal levels. By producing spores or going into a non-sporulating latent stage, bacteria may withstand harsh conditions. According to what is currently known, the VBNC state is an adaptation mechanism that allows bacteria to survive for a long time in harsh environments. Extreme temperatures, a lack of nutrients, elevated salinity, pH fluctuations, osmotic stress, reactive oxygen species, and exposure to food preservatives, heavy metals, organic pollutants, chlorination, UV light, or simply white light are some of these situations.

Although VBNC cells do not grow or form colonies on laboratory media, they are distinctly different from dead cells. VBNC cells maintain cellular integrity, possess uninjured membranes, and retain genomic or plasmid DNA. Unlike dead cells, VBNC cells exhibit metabolic and respiratory activities, have high ATP levels, and may perform transcription and gene expression. Entering this survival state triggers numerous physiological and molecular changes in VBNC cells compared to

normal viable and culturable cells [19-23]. Increased crosslinking in peptidoglycan cell walls, changes in outer membrane protein profiles, or changes in the fatty acid composition of cytoplasmic membranes are all consequences of environmental stimuli that cause VBNC states in bacteria. A lower surface-to-volume ratio may help reduce cellular energy requirements since many bacterial species show cell dwarfing or produce coccoid-shaped cells in a VBNC state. Nevertheless, morphological alterations by themselves cannot be regarded as conclusive characteristics for characterizing a VBNC condition. Given that most microorganisms inhabit nutrient-limited natural environments surrounded by biological competitors, it can be hypothesized that this environmental context accounts for the dominance of unculturable microbial populations on Earth, with a significant proportion likely existing in a VBNC state. A recent review documented VBNC states in over 100 bacterial species across almost 60 genera. Furthermore, some studies have revealed that eukaryotic cells, such as the yeast *Saccharomyces cerevisiae* and the vine spoilage yeast *Brettanomyces bruxellensis*, can also enter this survival state in response to sulfite stress [24-27].

THE DETECTION OF VBNC BACTERIA

While there are no direct methods for detecting VBNC (viable but non-

culturable) bacteria, a combination of techniques can reveal their viability. VBNC cells, though alive, cannot grow on standard liquid or semi-solid bacteriological media. Therefore, simple plating methods can confirm that living cells are present despite a lack of colony formation. Kogure's procedure allows for the microscopic detection of normal-shaped VBNC or dormant cells. This method involves staining elongated living cells with acridine orange after exposure to nalidixic acid, a cell division inhibitor. Once unculturability is confirmed, a variety of fluorescent stains are available for fluorescent microscopy. These stains mainly identify the cells' enzymatic activity or membrane integrity. For example, total and viable bacteria can be concurrently detected by counterstaining germs with 5-cyano-2,3-ditolyl tetrazolium chloride and 4,6-diamino-2-phenyl indole (DAPI). Two nucleic acid stains are combined in the LIVE/DEAD® BacLight™ bacteria viability kit: red-fluorescent propidium iodide, which only penetrates the damaged membranes of dead cells, and green fluorescent SYTO® 9 for living bacteria. The LIVE/DEAD® BacLight™ kit is widely used in fluorescence microscopy, fluorescent microplate readers, flow cytometers, and fluorimeters because of these qualities [28-33].

The direct viable count method, which uses the DNA-gyrase inhibitor novobiocin, combined with fluorescent in situ hybridization, has made it possible to detect and enumerate VBNC *Helicobacter pylori*

cells. Molecular methods that exclusively target DNA, such as normal PCR or qPCR, cannot differentiate between viable and dead cells. However, combining qPCR with the microbial dye propidium monoazide allows for the detection of nucleic acids exclusively in living cells. Only damaged membranes are penetrated by PMA, which binds to extracellular or dead cell DNA to prevent PCR amplification. Since bacterial mRNA only exists in metabolically active cells and has a short half-life, new research suggests using reverse transcriptase PCR to monitor stress-related genes in order to determine cell viability. Based on these factors, molecular techniques can identify VBNC bacteria following a failed culturability test in addition to being appropriate for differentiating between viable and dead cells [34-37].

INTERCELLULAR METABOLITES

Dormancy is a widespread natural phenomenon that enables survival in hostile environmental conditions. Bacteria, including both spore-forming and non-sporulating species, can enter a zero- or low-activity dormant state, which they reverse when conditions become optimal. This reversal can be triggered by external factors or random awakening events. Within dormant microbial populations, "scout" cells periodically and stochastically emerge. A scout cell is a newly reactivated cell that has exited dormancy due to infrequent and essentially random internal events. Scouts are not genetic

variants and are indistinguishable from the typical growing cells of the population. They explore available resources and, if growth conditions are suitable, initiate the transition from dormancy in the rest of the population by secreting intercellular signaling metabolites (e.g., autoinducers, resuscitation promoting factors, siderophores, catalases). If conditions are not suitable, the scout dies, and a new scout will randomly arise from the same dormant population in due course [38, 39].

Uncultured and viable but non-culturable (VBNC) microorganisms, which constitute the vast majority of microbial populations, also randomly form scouts. These scouts explore resources and can trigger resuscitation (transition from a zero- or low-activity state) in the rest of the population by secreting signaling molecules. The artificial resuscitation of previously unculturable microorganisms and the utilization of their potential abilities could open novel avenues for environmental rehabilitation. The stochastic appearance of microbial scouts in dormant bacterial communities may explain not only the random pattern of latent infections but also why a few cultivable bacteria exist in all VBNC populations [40-42]. Gene expression stochasticity, a crucial component of bacterial epigenetics, may affect transitions between states like dormancy and scout production independently of environmental stimuli, according to recent research

that has shown evidence of spontaneous awakening. The use of intercellular signaling molecules to artificially awaken dormant cells into scouts may prove useful in the future for both environmental biotechnology and human health sciences.

Bacterial Resuscitation

The entire process is reversible because, in contrast to dead cells, VBNC cells have visible metabolic activity, which enables them to regain culturability. Since some environmental changes cause the VBNC condition, removing or neutralizing these stressors can aid in the restoration of culturability. VBNC cell recovery may be aided by favorable growing conditions, such as ideal temperature, more energy sources, appropriate nutritional concentrations, chemical agents, or co-culturing with other species. However, in most cases, bacterial resuscitation is more complex than simply removing adverse factors, differing between bacteria and being inducible by various stimuli [43, 44].

Autoinducers

Many microorganisms excrete secondary metabolites or pheromones involved in regulating cellular differentiation, maintaining virulence, or communicating with conspecifics. Autoinducers (AIs) are small, heat-stable, hormone-like signaling molecules, primarily produced by Gram-negative bacteria. These gene expression regulators play a crucial role in cell-to-cell interspecies communication,

known as quorum sensing. AIs were first discovered in *Escherichia coli* cultures grown in a norepinephrine-containing medium, where they stimulated growth rate and reduced the lag phase.

Siderophores

Iron is a key component of redox enzymes and is necessary for basic biological functions like electron transport and DNA and amino acid synthesis. Soluble ferrous iron (Fe^{2+}) has a very limited biological availability despite its critical significance. When iron is scarce, microorganisms create siderophores, which are tiny ferric ion (Fe^{3+}) chelator molecules that scavenge insoluble iron and carry it into cells. Additionally, siderophores encourage cell proliferation. For example, the siderophore schizokinen can function as a growth factor by shortening *Bacillus megaterium*'s lag phase. Even in nutrient-rich conditions, bacterial growth can be restricted by a shortage of iron or siderophores, indicating their critical involvement in the resuscitation of unculturable bacteria.

TRENDS IN ISOLATING UNCULTURABLE BACTERIA

Uncultivated bacteria cannot grow in standard laboratory media because of their slow growth rates or tendency to enter dormancy. Consequently, standard cultivation techniques can lead to fast-growing species overshadowing those that are slow-growing or rare. Given these challenges, initial efforts to isolate previously uncultured bacteria

involved mimicking natural conditions. This included using lower nutrient concentrations, smaller inoculum sizes, and longer incubation times. Beyond modifying isolation media (substrates and nutrients) and easily controllable laboratory factors (e.g., pH, temperature, and oxygen availability), developing new cultivation methods should also consider biotic factors. These include synergistic interactions, dependence on secondary metabolites, and interspecies competition [45-47]. In the last decade, isolation techniques that replicate natural environments in the laboratory and through in situ cultivation have gained interest, as natural milieus typically contain all necessary nutrients for bacterial growth. However, the primary focus has been on resuscitating viable but non-culturable (VBNC) bacteria by adding recombinant Rpf or extracellular organic matter from *M. luteus*.

SIGNIFICANCE AND ENVIRONMENTAL APPLICATION OF UNCULTURABLE BACTERIA

Most microorganisms in natural environments live under suboptimal conditions, facing challenges such as limited energy and resources, unfavorable temperatures, pH fluctuations, and the presence of competitors and predators. These stressors can inhibit their ability to divide or even survive. Consequently, entering a dormant or low-activity state serves as a crucial

survival strategy, preserving both genetic and phenotypic diversity. These diverse microbial "seedbanks"—large reservoirs of inactive individuals—warrant significant attention. Resuscitating these microorganisms could unlock their promising potential for various ecological processes. While this phenomenon has been extensively studied in pathogenic bacteria, particularly in relation to latent infectious diseases, food safety, and new pharmacological products, much less is known about viable but non-culturable (VBNC) bacteria in polluted environments and their role in bioremediation. Given that most bacteria naturally exist in a VBNC state, these uncultured species represent a vast, unexplored pool of microbial resources (e.g., metabolic enzymes, biosurfactants) for environmental rehabilitation.

Recent studies increasingly support the idea that uncultured bacteria are vital for biodegrading environmental pollutants. Metagenomic analysis of chronically polluted coastal sediments, for example, revealed a high abundance of aromatic ring-hydroxylating oxygenase genes in uncultured microorganisms, potentially responsible for polycyclic aromatic hydrocarbon biodegradation. Exploiting metagenomic data has also proven beneficial for characterizing enzymes from uncultured microorganisms in polluted soils and marine environments, enhancing our understanding of the catabolic versatility of these microbes. For

instance, Salam and colleagues identified uncultured genera involved in the natural attenuation of hydrocarbons in contaminated tropical African soil. Similarly, Chandra and Kumar detected uncultured bacterial members in post-methanated distillery sludge that likely play a significant role in neutralizing recalcitrant pollutants and reducing phyto- and genotoxicity [48-51]. Beyond molecular approaches, the targeted isolation and resuscitation of previously uncultured bacteria using growth-promoting factors have also gained considerable interest over the past decade.

CONCLUSION

Finding the hidden microbial diversity and overcoming barriers to transferring pollutant-degrading microorganisms from labs to field locations are the main difficulties facing bioremediation today. Even in field cleanup methods, increasing their capacity for survival and biodegradation is necessary to boost bioconversion rates. A deeper understanding of the metabolic pathways involved in the microbial degradation of recalcitrant contaminants requires more knowledge about the unculturable majority of bacteria, their relationships with coexisting microbial community members, and how they can be integrated as key players in environmental rehabilitation.

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