Viable but Nonculturable Gastrointestinal Bacteria and Their Resuscitation

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Received date: March 18, 2021, Accepted date: April 02, 2021

Abstract

The human gastrointestinal tract is colonized by a large diversity of health-associated bacteria, which comprise the gut microbiota. Sequence-based, culture-independent approaches have revolutionized our view of this microbial ecosystem. However, many of its members are nonculturable under laboratory conditions. Some bacteria can enter the viable but nonculturable (VBNC) state. VBNC bacteria do not form colonies in standard medium, although they exhibit – albeit very low – metabolic activity, and can even produce toxic proteins. The VBNC state can be regarded as strategy that permits bacteria to cope with stressful environments. In this commentary, we discuss factors that promote the resuscitation of VBNC bacteria, and highlight the role of extracellular pyruvate, based on our own work on the significance of pyruvate sensing and transport for the resuscitation of *Escherichia coli* cells from the VBNC state.

Viable but Nonculturable Gastrointestinal Bacteria

Viable but nonculturable (VBNC) bacteria are deeply dormant phenotypic variants that are characterized by a loss of culturability in conventional culture media, yet retain some viability markers [1]. Thus, low metabolic activity, nutrient uptake, membrane integrity, and respiration are all detectable in these dormant cells. In 1982, the VBNC state was first described for *Escherichia coli* and *Vibrio cholerae* [2]. Shortly afterwards, VBNC *Salmonella enteriditis* were found to regain culturability when placed under favourable conditions [3]. Since then, more than 100 bacterial species (including approximately 30 gastrointestinal bacteria) and some fungi have been reported to enter the VBNC state (see Dong et al. [4] and Li et al. [5] for excellent reviews). Among them are many food-borne, toxin-producing bacteria, such as *Aeromonas hydrophila*, *Bacillus cereus*, *Campylobacter jejuni*, *E. coli O157:H7*, *Lactobacillus acidophilus*, *Listeria monocytogenes*, *S. enterica*, *Shigella flexneri*, *V. cholerae*, but also probiotic bacteria, such as *Bifidobacterium animalis*, *Bf. longum* and *Bf. lactis* [6].

Characteristics of VBNC Bacteria

Bacteria enter the VBNC state in response to natural stresses. Stressful conditions that induce this form of dormancy in *E. coli* have been intensively studied, and include deprivation of essential nutrients, oxidative stress (H$_2$O$_2$), low temperature (4°C), high osmolarity and radiation (UV light, TiO$_2$-mediated photocatalysis) (summarized in Ding et al. [7]) (Figure 1). For most bacteria, nutrient limitation and cold stress are the frequently reported factors that trigger the entry of bacteria into the VBNC state. These environmental stresses could potentially kill whole populations, unless at least some cells enter this dormant state. Strikingly, nutrient limitation is also the major initiator of endospore formation, which is itself the outcome of a complex regulatory programme. Endospores are metabolically dormant and resistant to deleterious environmental conditions, including extremes of temperature, desiccation and ionizing radiation [8].

The VBNC state can be regarded as a survival strategy for non-spore-forming bacteria [9]. This implies that the mechanism that mediates the switch to the VBNC phenotype is genetically determined [10]. However, an alternative view of the process has also been proposed by Desnues et al. [11], who suggested that harsh environmental conditions result in oxidative damage to cells, which...
Figure 1: Induction and resuscitation of viable but nonculturable (VBNC) bacteria. Schematic presentation of bacteria entering and leaving the VBNC state, including lists of factors that either induce the VBNC state or promote resuscitation. Colonies formed on agar plates indicate the culturability of bacteria.
ultimately inhibits bacterial growth. In either case, bacteria must first sense changes in their environment, and then initiate a response which relies on an efficient regulatory network. Indeed, many genes and signalling pathways are known to be involved in activating the VBNC state in bacteria [4].

VBNC bacteria are viable, but they differ from culturable cells in several morphological, physiological and molecular features. They are usually smaller and rounded in shape, with a correspondingly increased surface-to-volume ratio, with a correspondingly increased surface-to-volume ratio (Figure 2) [12-14]. For example, in *Campylobacter* spp., the characteristic spiral shape in the exponential phase is transformed into a coccoid shape in the VBNC state [15]. These morphological changes are commonly found in VBNC cells; however, similar changes are also observed in non-VBNC cells that are exposed to stressful conditions, so changes in morphology alone cannot be used as the defining criterion of the VBNC state [16].

Other typical features include alterations in cell-wall and membrane composition. Importantly, VBNC bacteria are resistant to physical conditions and chemical agents that would be lethal to culturable bacteria, and are difficult to kill with antibiotics, partly due to their low levels of metabolism [4].

**Conditions and Factors that Promote Resuscitation**

VBNC cells can, nonetheless, resume cell division. The process of re-establishing culturability is termed resuscitation (Figure 1). Various factors that promote the restoration of culturability in gastrointestinal VBNC bacteria have been identified. For some species, simple reversion of the specific stress factors that induced the VBNC state triggers resuscitation. For example, an increase in temperature is sufficient for many species that enter the VBNC state on exposure to cold [17-19].

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**Figure 2: Time-resolved resuscitation of single VBNC *E. coli* cells in microfluidic devices.** VBNC cells of *E. coli* MG1655 (stored at 4°C for 120 days) were placed in a microfluidic chamber (volume 4.2 pL) and observed by phase-contrast microscopy. At time zero, the chamber was flushed with diluted tryptone-based medium supplemented with 10 mM pyruvate, and resuscitation was followed over time (scale bar 5 μm). The schematics in the second row illustrate the observed changes in cell shape, volume and cell division. (Figure from [47], adapted and modified).
The addition of chelators and osmoprotectants can also reverse VBNC states induced by toxic levels of metals and high osmolarity, respectively [20,21]. Supplementation with complex nutrients, such as soluble extracts of plant or animal tissues, following starvation has been shown to restore culturability of various enteric VBNC bacteria, such as \textit{Acrobacter butzleri}, \textit{Citrobacter freundii}, \textit{E. coli}, \textit{E. faecalis}, \textit{P. aeruginosa} and \textit{S. enterica} [22–27]. However, the metabolite(s) directly responsible for initiating the process of resuscitation remain unknown in many cases.

Other gastrointestinal VBNC cells require specific biological stimuli to escape from the dormant state – for instance, signals emitted by the host or other bacteria. Resuscitation-promoting-factors (RPFs) are a family of proteins secreted by actively growing Actinobacteria such as \textit{Mycobacterium tuberculosis}, but also \textit{S. enterica} serovar Oranienburg [28,29]. RPFs play a distinctive role in the resuscitation of these species. Interestingly, these proteins show striking structural similarity to cell-wall-hydrolyzing enzymes. Molecules involved in cell-to-cell communication, designated as autoinducers (AIs), have also been found to resuscitate some intestinal VBNC bacteria [30,31]. For example, addition of AIs from supernatants of culturable \textit{Salmonella} cells were shown to enable VBNC \textit{Salmonella} to revive; similarly, \textit{E. coli} AIs can make VBNC cells of the same species culturable again [22,28]. Even an inter-species resuscitating effect of AIs on VBNC bacteria has been observed [32]. Several studies have demonstrated that host signals can confer culturability on some VBNC gastrointestinal bacteria. Thus, species such as \textit{C. jejuni}, pathogenic and non-pathogenic \textit{E. coli}, \textit{Edwardsiella tarda}, \textit{Helicobacter pylori}, \textit{L. monocytogenes}, \textit{S. enterica}, \textit{S. flexneri}, \textit{V. cholerae} and \textit{V. parahaemolyticus} have been restored to the culturable state with the help of host signals [13,30,33–40]. Resuscitation was achieved either by the addition of eukaryotic cell extracts, co-culture with eukaryotic cells, incubation in fertilized eggs or passage through the host.

Furthermore, antioxidants such as catalase, superoxide dismutase or α-ketoglutarate can promote resuscitation by scavenging reactive oxygen species [22,41,42]. Moreover, several studies have described pyruvate as crucial for resuscitation [5,27,41,42]. Pyruvate is known to scavenge hydrogen peroxide [43] and the hydroxyl radical [44], and prevents lipid peroxidation [45]. Pyruvate and other α-ketoacids scavenge oxygen radicals by a non-enzymatic oxidative decarboxylation mechanism [41,46].

**Resuscitation of VBNC \textit{E. coli} cells can be activated by pyruvate uptake**

In a recent study [47], we demonstrated that pyruvate is not only an antioxidant, but is avidly taken up by starved and cold-stressed \textit{E. coli} VBNC cells, and promotes their return to a culturable state. Uptake of pyruvate under these conditions is mediated by the high-affinity transporter BtsT, whose expression is under the control of the pyruvate-sensing network BtsSR/YpdAB [48,49]. This pyruvate-sensing network is not only important for the homogenization of the physiological states within an \textit{E. coli} population [50], but is essential for the resuscitation of VBNC cells. VBNC \textit{E. coli} cells that lack this network are essentially unable to resuscitate in the presence of pyruvate, as confirmed by their inability to resume DNA replication and protein biosynthesis. The resuscitation of VBNC \textit{E. coli} was monitored in a time-resolved manner in microfluidic devices (Figure 2). Remarkably, resuscitation of cells was accompanied by visible changes in cell volume within minutes after exposure to pyruvate – a phenomenon that needs to be explored in more detail. The accompanying proteomic study revealed that VBNC \textit{E. coli} cells are characterized by a significantly increased copy number of the high-affinity pyruvate/H⁺ symporter BtsT. Consequently, wild-type VBNC cells were able to take up pyruvate within seconds of its provision. Several enzymes involved in pyruvate metabolism were also found to be strongly upregulated in the proteome of the VBNC cells, and we have suggested that pyruvate becomes the preferred carbon source in starving cells because – unlike glucose – it does not need to be activated by phosphorylation prior to uptake.

**The Relevance of VBNC cells in the Gastrointestinal Tract**

Entry into the VBNC state enables enteric pathogens and non-pathogens to survive in adverse environments, but poses health risks in clinical settings, for the food industry and for water supplies, once such cells become culturable again [51,52].

Many pathogens, such as \textit{V. cholerae}, \textit{V. vulnificus}, \textit{C. jejuni} or \textit{E. faecalis}, not only enter the VBNC state and return to culturability, but retain or regain their pathogenicity once resuscitated [35,53–55]. In light of the fact that host signals can in principle promote resuscitation of enteric pathogens, the possibility must be considered that these bacteria can regain their pathogenicity in the human intestinal tract after having survived in a dormant – and effectively undetectable – VBNC state, and can thus represent an underestimated risk factor. For example, \textit{E. coli} O157:H7, \textit{S. enterica}, \textit{L. monocytogenes} and \textit{P. aeruginosa} can all survive standard disinfection treatments in the VBNC state in food or drinks [26,56–58]. Once they access to the host, these pathogens could be resuscitated, initiate their virulence programs and cause infection. Furthermore, due to their low metabolic activity, VBNC cells are effectively resistant to antibiotics...
On the other hand, the non-culturability of some gastrointestinal bacteria is certainly related to lack of knowledge of the specific molecular triggers that initiate the resuscitation process [61]. This is an important consideration in the context of fecal transplantsations (also known as bacteriotherapy), i.e., the transfer of stool from a healthy donor into the gastrointestinal tract of patients suffering from - for example - *Clostridioides difficile*-induced colitis. The transfer of the bacteria requires a period during which the cells are outside of a host. Exposure to lower temperature and/or oxygen might be sufficient to induce the VBNC state in some species, and it is unclear whether they resume growth in the new host.

## Conclusion

In a recent study, we have shown that pyruvate is crucial for VBNC *E. coli* cells to return to the culturable state. Pyruvate is one of the main factors involved in the resuscitation of VBNC bacteria. It should be emphasised here that many bacteria secrete pyruvate under conditions of overflow metabolism [48]. In addition, mouse and human cells also secrete pyruvate as an antioxidant to neutralise reactive oxygen species [43], and cancer cells in particular release pyruvate to adapt to hypoxia [62]. Thus, the secretion of pyruvate may be of more general significance for the gut microbiota, and thus for human health, than previously thought. In summary, it is of great importance to gain a broader and more systematic understanding of the induction and resuscitation of VBNC bacteria in the gastrointestinal tract.

## Acknowledgment

This research was funded by the Deutsche Forschungsgemeinschaft (JU270/19-1 and Project No. 395357507 – SFB 1371 to K.J.). We thank Cláudia Vilhena, Eugen Kaganovitch, Alexander Grünberger, Magdalena Motz, Ignasi Forné and Dietrich Kohlheyer for excellent work on this project.

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[59]. Hence, VBNC cells might, for instance, account for reinfections with *H. pylori* or recurring urinary tract infections by uropathogenic *E. coli* [1,60].

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