Viable but Nonculturable Gastrointestinal Bacteria and Their Resuscitation

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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 acetotolerans*, *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_2O_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
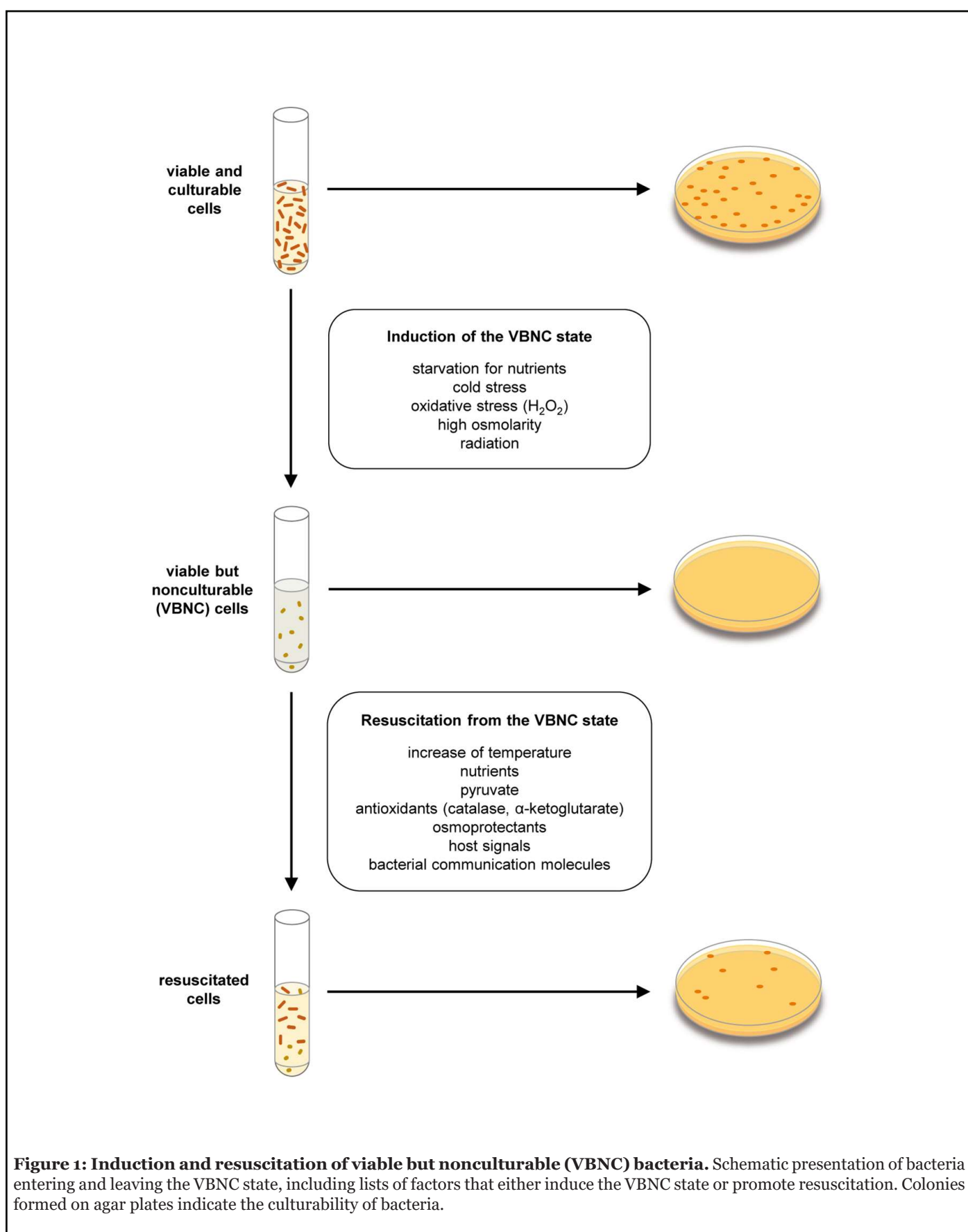
Figure 1: Induction and resuscitation of viable but nonculturable (VBNC) bacteria. Schematic presentation of bacteria entering and leaving the VBNC state, including lists of factors that either induce the VBNC state or promote resuscitation. Colonies formed on agar plates indicate the culturability of 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 (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].
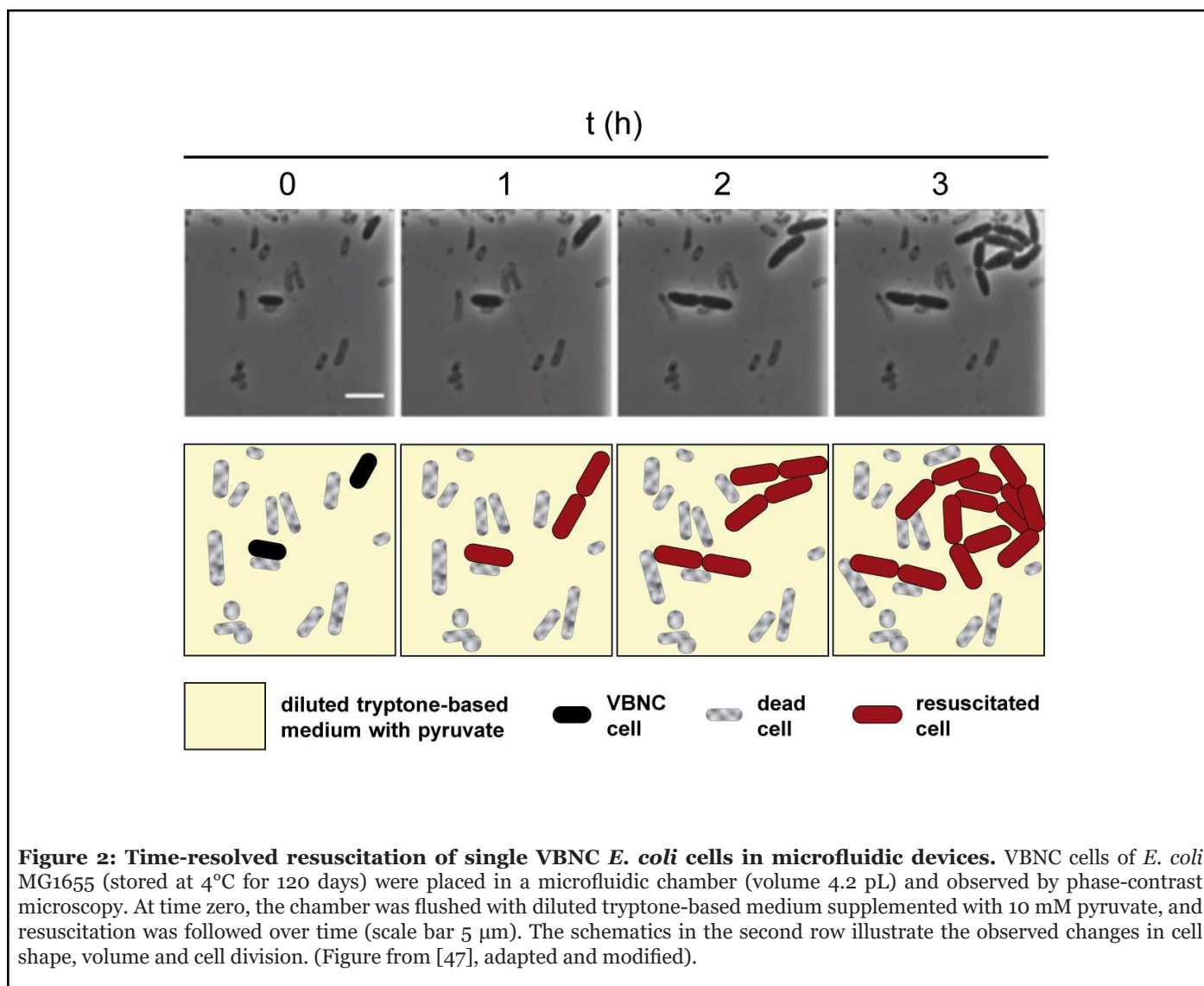
Figure 2: Time-resolved resuscitation of single VBNC *E. coli* cells in microfluidic devices. VBNC cells of *E. coli* MG1655 (stored at 4°C for 120 days) were placed in a microfluidic chamber (volume 4.2 pL) and observed by phase-contrast microscopy. At time zero, the chamber was flushed with diluted 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 *Acrobacter butzleri*, *Citrobacter freundii*, *E. coli*, *E. faecalis*, *P. aeruginosa* and *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 *Mycobacterium tuberculosis*, but also *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 *Salmonella* cells were shown to enable VBNC *Salmonella* to revive; similarly, *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 *C. jejuni*, pathogenic and non-pathogenic *E. coli*, *Edwardsiella tarda*, *Helicobacter pylori*, *L. monocytogenes*, *S. enterica*, *S. flexneri*, *V. cholerae* and *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 *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 *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 *E. coli* population [50], but is essential for the resuscitation of VBNC cells. VBNC *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 *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 *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 *V. cholerae*, *V. vulnificus*, *C. jejuni* or *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, *E. coli* O157:H7, *S. enterica*, *L. monocytogenes* and *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

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

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 transplantations (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

References

1. Oliver JD. The viable but nonculturable state in bacteria. Journal of Microbiology. 2005;43(1):93-100.

2. Xu HS, Roberts N, Singleton FL, Attwell RW, Grimes DJ, Colwell RR. Survival and viability of nonculturable *Escherichia coli* and *Vibrio cholerae* in the estuarine and marine environment. Microbial Ecology. 1982 Dec 1;8(4):313-23.

3. Roszak DB, Grimes DJ, Colwell RR. Viable but nonrecoverable stage of *Salmonella enteritidis* in aquatic systems. Canadian Journal of Microbiology. 1984 Mar 1; 30 (3): 334-8.

4. Dong K, Pan H, Yang D, Rao L, Zhao L, Wang Y, et al. Induction, detection, formation, and resuscitation of viable but non-culturable state microorganisms. Comprehensive Reviews in Food Science and Food Safety. 2020 Jan;19(1):149-83.

5. Li L, Mendis N, Trigui H, Oliver JD, Faucher SP. The importance of the viable but non-culturable state in human bacterial pathogens. Frontiers in Microbiology. 2014 Jun 2;5:258.

6. Millet V, Lonvaud-Funel A. The viable but non-culturable state of wine micro-organisms during storage. Letters in Applied Microbiology. 2000 Feb;30(2):136-41.

7. Ding T, Suo Y, Xiang Q, Zhao X, Chen S, Ye X, et al. Significance of viable but nonculturable *Escherichia coli*: induction, detection, and control. Journal of Microbiology and Biotechnology. 2017;27(3):417-28.

8. Higgins D, Dworkin J. Recent progress in *Bacillus subtilis* sporulation. FEMS Microbiology Reviews. 2012 Jan 1;36(1):131-48.

9. Colwell RR. Viable but nonculturable bacteria: a survival strategy. Journal of Infection and Chemotherapy. 2000 Jul 1;6(2):121-5.

10. Roszak DB, Colwell RR. Survival strategies of bacteria in the natural environment. Microbiological Reviews. 1987 Sep;51(3):365-79.

11. Desnues B, Cuny C, Grégori G, Dukan S, Aguilaniu H, Nyström T. Differential oxidative damage and expression of stress defence regulons in culturable and non-culturable *Escherichia coli* cells. EMBO Reports. 2003 Apr;4(4):400-4.

12. Rahman I, Shahamat M, Kirchman PA, Russek-Cohen E, Colwell RR. Methionine uptake and cytopathogenicity of viable but nonculturable *Shigella dysenteriae* type 1. Applied and Environmental Microbiology. 1994 Oct 1;60(10):3573-8.

13. Du M, Chen J, Zhang X, Li A, Li Y, Wang Y. Retention of virulence in a viable but nonculturable *Edwardsiella tarda* isolate. Applied and Environmental Microbiology. 2007 Feb 15;73(4):1349-54.

14. Adams BL, Bates TC, Oliver JD. Survival of *Helicobacter pylori* in a natural freshwater environment. Applied and Environmental Microbiology. 2003 Dec 1;69(12):7462-6.

15. Thomas C, Hill D, Mabey M. Culturability, injury and morphological dynamics of thermophilic *Campylobacter* spp. within a laboratory-based aquatic model system. Journal of Applied Microbiology. 2002 Mar;92(3):433-42.

16. Pinto D, Santos MA, Chambel L. Thirty years of viable but nonculturable state research: unsolved molecular mechanisms. Critical Reviews in Microbiology. 2015 Jan 2;41(1):61-76.

17. Maalej S, Gdoura R, Dukan S, Hammami A, Bouain A. Maintenance of pathogenicity during entry into and resuscitation from viable but nonculturable state in *Aeromonas hydrophila* exposed to natural seawater at low temperature. Journal of Applied Microbiology. 2004 Sep;97(3):557-65.

18. Whitesides MD, Oliver JD. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Applied and Environmental Microbiology. 1997 Mar 1;63(3):1002-5.

19. Nilsson L, Oliver JD, Kjelleberg S. Resuscitation of *Vibrio vulnificus* from the viable but nonculturable state. Journal of Bacteriology. 1991 Aug 1;173(16):5054-9.

20. Dwidjosiswojo Z, Richard J, Moritz MM, Dopp E, Flemming HC, Wingender J. Influence of copper ions on the viability and cytotoxicity of *Pseudomonas aeruginosa* under conditions relevant to drinking water environments. International Journal of Hygiene and Environmental Health. 2011 Nov 1;214(6):485-92.

21. Aurass P, Prager R, Flieger A. EHEC/EAEC O104: H4 strain linked with the 2011 German outbreak of haemolytic uremic syndrome enters into the viable but non-culturable state in response to various stresses and resuscitates upon stress relief. Environmental Microbiology. 2011 Dec;13(12):3139-48.

22. Pinto D, Almeida V, Almeida Santos M, Chambel L. Resuscitation of *Escherichia coli* VBNC cells depends on a variety of environmental or chemical stimuli. Journal of Applied Microbiology. 2011 Jun;110(6):1601-11.

23. Dhiaf A, Bakhrouf A, Witzel KP. Resuscitation of eleven-year VBNC *Citrobacter*. Journal of Water and Health. 2008 Dec;6(4):565-8.

24. Fera MT, Maugeri TL, Gugliandolo C, La Camera E, Lentini V, Favaloro A, et al. Induction and resuscitation of viable nonculturable *Arcobacter butzleri* cells. Applied and Environmental Microbiology. 2008 May 15;74(10):3266-8.

25. Lleo MM, Bonato B, Tafi MC, Signoretto C, Boaretti M, Canepari P. Resuscitation rate in different enterococcal species in the viable but non-culturable state. Journal of Applied Microbiology. 2001 Dec;91(6):1095-102.

26. Zhang S, Ye C, Lin H, Lv L, Yu X. UV disinfection induces a VBNC state in *Escherichia coli* and *Pseudomonas aeruginosa*. Environmental Science & Technology. 2015 Feb 3;49(3):1721-8.

27. Liao H, Jiang L, Zhang R. Induction of a viable but non-culturable state in *Salmonella Typhimurium* by thermosonication and factors affecting resuscitation. FEMS Microbiology Letters. 2018 Jan;365(2):fnx249.

28. Panutdaporn N, Kawamoto K, Asakura H, Makino SI. Resuscitation of the viable but non-culturable state of *Salmonella enterica* serovar Oranienburg by recombinant resuscitation-promoting factor derived from *Salmonella Typhimurium* strain LT2. International Journal of Food Microbiology. 2006 Feb 15;106(3):241-7.

29. S Kaprelyants A, V Mukamolova G, Ruggiero A, A Makarov V, R Demina G, O Shleeva M, et al. Resuscitation-promoting factors (Rpf): in search of inhibitors. Protein and Peptide Letters. 2012 Oct 1;19(10):1026-34.

30. Chaisowwong W, Kusumoto A, Hashimoto M, Harada T, Maklon K, Kawamoto K. Phsysiological characterization of *Campylobacter jejuni* under cold stresses conditions: Its potential for public threat. Journal of Veterinary Medical Science. 2012;74(1):43-50-

31. Ayrapetyan M, Williams TC, Oliver JD. Interspecific quorum sensing mediates the resuscitation of viable but nonculturable vibrios. Applied and Environmental Microbiology. 2014 Apr 15;80(8):2478-83.

32. Reissbrodt R, Rienaecker I, Romanova JM, Freestone PP, Haigh RD, Lyte M, et al. Resuscitation of *Salmonella enterica* serovar Typhimurium and enterohemorrhagic *Escherichia coli* from the viable but nonculturable state by heat-stable enterobacterial autoinducer. Applied and Environmental Microbiology. 2002 Oct 1;68(10):4788-94.

33. Baffone W, Casaroli A, Citterio B, Pierfelici L, Campana R, Vittoria E, et al. *Campylobacter jejuni* loss of culturability in aqueous microcosms and ability to resuscitate in a mouse model. International Journal of Food Microbiology. 2006 Mar 1;107(1):83-91.

34. Cappelier JM, Minet J, Magras C, Colwell RR, Federighi M. Recovery in embryonated eggs of viable but nonculturable *Campylobacter jejuni* cells and maintenance of ability to adhere to HeLa cells after resuscitation. Applied and Environmental Microbiology. 1999 Nov 1;65(11):5154-7.

35. Colwell RR, Brayton P, Herrington D, Tall B, Huq A,

Levine MM. Viable but non-culturable *Vibrio cholerae* O1 revert to a cultivable state in the human intestine. World Journal of Microbiology and Biotechnology. 1996 Jan;12(1):28-31.

36. Boehnke KF, Eaton KA, Fontaine C, Brewster R, Wu J, Eisenberg JN, et al. Reduced infectivity of waterborne viable but nonculturable *Helicobacter pylori* strain SS 1 in mice. Helicobacter. 2017 Aug;22(4):e12391.

37. Cappelier JM, Besnard V, Roche SM, Velge P, Federighi M. Avirulent viable but non culturable cells of *Listeria monocytogenes* need the presence of an embryo to be recovered in egg yolk and regain virulence after recovery. Veterinary Research. 2007 Jul 1;38(4):573-83.

38. Imamura D, Mizuno T, Miyoshi SI, Shinoda S. Stepwise changes in viable but nonculturable *Vibrio cholerae* cells. Microbiology and Immunology. 2015 May;59(5):305-10.

39. Chaveerach P, Ter Huurne AA, Lipman LJ, Van Knapen F. Survival and resuscitation of ten strains of *Campylobacter jejuni* and *Campylobacter coli* under acid conditions. Applied and Environmental Microbiology. 2003 Jan 1;69(1):711-4.

40. Senoh M, Ghosh-Banerjee J, Ramamurthy T, Colwell RR, Miyoshi SI, Nair GB, et al. Conversion of viable but nonculturable enteric bacteria to culturable by co-culture with eukaryotic cells. Microbiology and Immunology. 2012 May;56(5):342-5.

41. Mizunoe Y, Wai SN, Takade A, Yoshida SI. Restoration of culturability of starvation-stressed and low-temperature-stressed *Escherichia coli* O157 cells by using H2O2-degrading compounds. Archives of Microbiology. 1999 Jun;172(1):63-7.

42. Morishige Y, Fujimori K, Amano F. Differential resuscitative effect of pyruvate and its analogues on VBNC (viable but non-culturable) *Salmonella*. Microbes and Environments. 2013;28(2):180-6.

43. O'Donnell-Tormey J, Nathan CF, Lanks K, DeBoer CJ, De La Harpe J. Secretion of pyruvate. An antioxidant defense of mammalian cells. The Journal of Experimental Medicine. 1987 Feb 1;165(2):500-14.

44. Woo YJ, Taylor MD, Cohen JE, Jayasankar V, Bish LT, Burdick J, et al. Ethyl pyruvate preserves cardiac function and attenuates oxidative injury after prolonged myocardial ischemia. The Journal of Thoracic and Cardiovascular Surgery. 2004 May 1;127(5):1262-9.

45. Varma SD, Hegde K, Henein M. Oxidative damage to mouse lens in culture. Protective effect of pyruvate. Biochimica et Biophysica Acta (BBA)-General Subjects. 2003 Jun 11;1621(3):246-52.

46. Constantopoulos G, Barranger JA. Nonenzymatic decarboxylation of pyruvate. Analytical biochemistry. 1984 Jun 1;139(2):353-8.

47. Vilhena C, Kaganovitch E, Grünberger A, Motz M, Forné I, Kohlheyer D, et al. Importance of pyruvate sensing and transport for the resuscitation of viable but nonculturable *Escherichia coli* K-12. Journal of Bacteriology. 2019 Feb 1;201(3).

48. Behr S, Brameyer S, Witting M, Schmitt-Kopplin P, Jung K. Comparative analysis of LytS/LytTR-type histidine kinase/response regulator systems in γ-proteobacteria. PLoS One. 2017 Aug 10;12(8):e0182993.

49. Kristoficova I, Vilhena C, Behr S, Jung K. BtsT, a novel and specific pyruvate/H+ symporter in *Escherichia coli*. Journal of Bacteriology. 2018 Jan 15;200(2).

50. Vilhena C, Kaganovitch E, Shin JY, Grünberger A, Behr S, Kristoficova I, et al. A single-cell view of the BtsSR/YpdAB pyruvate sensing network in *Escherichia coli* and its biological relevance. Journal of Bacteriology. 2018 Jan 1;200(1).

51. Fakruddin M, Bin Mannan KS, Andrews S. Viable but nonculturable bacteria: food safety and public health perspective. ISRN Microbiol 2013: 703813.

52. Ramamurthy T, Ghosh A, Pazhani GP, Shinoda S. Current perspectives on viable but non-culturable (VBNC) pathogenic bacteria. Frontiers in Public Health. 2014 Jul 31;2:103.

53. Oliver JD, Bockian R. In vivo resuscitation, and virulence towards mice, of viable but nonculturable cells of *Vibrio vulnificus*. Applied and Environmental Microbiology. 1995 Jul 1;61(7):2620-3.

54. Pruzzo C, Tarsi R, del Mar Lleo M, Signoretto C, Zampini M, Colwell RR, et al. In vitro adhesion to human cells by viable but nonculturable *Enterococcus faecalis*. Current Microbiology. 2002 Aug;45(2):105-10.

55. Jones DM, Sutcliffe EM, Curry A. Recovery of viable but non-culturable *Campylobacter jejuni*. Microbiology. 1991 Oct 1;137(10):2477-82.

56. Dinu LD, Bach S. Induction of viable but nonculturable *Escherichia coli* O157: H7 in the phyllosphere of lettuce: a food safety risk factor. Applied and Environmental Microbiology. 2011 Dec 1;77(23):8295-302.

57. Nicolo MS, Gioffre A, Carnazza S, Platania G, Silvestro ID, Guglielmino SP. Viable but nonculturable state of foodborne pathogens in grapefruit juice: a study of laboratory. Foodborne Pathogens and Disease. 2011 Jan 1;8(1):11-7.

58. Cunningham E, O'Byrne C, Oliver JD. Effect of weak acids on *Listeria monocytogenes* survival: evidence for a viable but nonculturable state in response to low pH. Food Control. 2009 Dec 1;20(12):1141-4.

59. Oliver JD. Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiology Reviews. 2010 Jul 1;34(4):415-25.

60. Rivers B, Steck TR. Viable but nonculturable uropathogenic bacteria are present in the mouse urinary tract following urinary tract infection and antibiotic therapy. Urological Research. 2001 Feb 1;29(1):60-6.

61. Ayrapetyan M, Williams T, Oliver JD. Relationship between the viable but nonculturable state and antibiotic persister cells. Journal of Bacteriology. 2018 Oct 15;200(20).

62. Yin C, He D, Chen S, Tan X, Sang N. Exogenous pyruvate facilitates cancer cell adaptation to hypoxia by serving as an oxygen surrogate. Oncotarget. 2016 Jul 26;7(30):47494-510.