

“Viable But Non-Culturable Cells” are Dead

Sooyeon Song¹ and Thomas K. Wood^{2*}

¹Department of Animal Science, Jeonbuk National University, Jeonju-si, Jeollabuk-do, 54896 Korea

²Department of Chemical Engineering, Pennsylvania State University, University Park, PA, 16802-4400 U.S.A

*For correspondence. E-mail tuw14@psu.edu
Tel (+)1 814-863-4811; Fax (1) 814-865-7846

Running title: VBNCs are dead.

ABSTRACT

Most bacteria lead lives of quiet desperation, so they sleep. By sleeping, bacteria survive ubiquitous stress, such as antibiotics, and can resuscitate to reconstitute infections. As for other nearly-universal and highly-regulated processes such as biofilm formation, in persistence, a small population of cells have an elegantly-regulated pathway to become dormant. By inactivating their ribosomes, persister cells sleep through stress and resuscitate once (i) the stress is removed (ii) nutrients are presented, and (iii) ribosome content reaches a threshold. During stress, cells often become spheroid and die, becoming hollow, membrane-enclosed vessels. How cellular content is lost is unclear; but it is obvious that these “cell shells” are dead; i.e., “There’s no there there”. Critically, due to their intact membranes, the shells appear with membrane-impenetrant stains as “viable” particles. Unfortunately, the microbiology field of “viable but non-culturable cells” (VBNCs), though important for demonstrating the existence of dormant bacteria as a result of myriad stress states, has mistaken these non-viable shells as viable particles that mysteriously may be reborn, when an appropriate incantation is made. We argue here that if resuscitation occurs, it is the persister (always-viable) cell population that revives, rather than the cell husks, which are dead.

Persisters and VBNCs. These two resting phases share many common features, supporting the notion that the viable portion of both types of cells are identical (Kim et al., 2018a). For example, both persisters (Mulcahy et al., 2010) and VBNCs (Li et al., 2014) are involved in chronic infections, both are present in biofilms (Spoering and Lewis, 2001; Li et al., 2014), and both are produced by several stresses (e.g., nutrient, oxidative, acid (Hong et al., 2012; Li et al., 2014; Kim et al., 2018a)). For persister cell formation, the genetic basis of their formation, as discerned from single-cell experiments, is via inactivation of ribosomes in a subpopulation of cells by conserved

hibernating factors such as RaiA, RMF, and Hpf in *E. coli* (Song and Wood, 2020); these factors are induced by various stresses via the alarmone (p)ppGpp and are the basis of the (p)ppGpp ribosome dimerization model (Wood and Song, 2020). For resuscitation, persister cells, again based on single-cell experiments, detect nutrients, reduce (p)ppGpp and cAMP levels, and activate ribosomes (Kim et al., 2018b) via activation factors such as HflX (Yamasaki et al., 2020); these resuscitated cells then commence chemotaxis and foraging (Yamasaki et al., 2020). Unfortunately, the mechanism for VBNC formation is far less clear, and it has been argued that the addition of a quorum-sensing signal, enzymes such as catalase, antibiotics, temperature shift, or a special carbon source resuscitates these cells (Dong et al., 2020). However, as for persisters, elevating (p)ppGpp increases VBNCs (Boaretti et al., 2003). Rather than the proposed “dormancy continuum” model in which VBNCs are “more dormant” than persister cells and VBNCs do not resuscitate (Ayrapetyan et al., 2015), we found that the viable portion of what have been called VBNCs are persister cells (Kim et al., 2018a) and that the nonculturable cell fragments are dead (Kim et al., 2018a). Similarly, over two decades ago, Bogosian et al. showed by removing H₂O₂ stress that the nonculturable *Vibrio vulnificus* particles that are classified as VBNCs are dead (Bogosian et al., 2000), and one definition of dead is the inability of a bacterium to form a colony (Baquero and Levin, 2021). We extend this idea here through microscopy for many species shown to produce VBNCs.

TEM and lack of resuscitation indicates VBNCs are dead. Microscopy is the key for discriminating viable persister cells from non-viable VBNC particles.

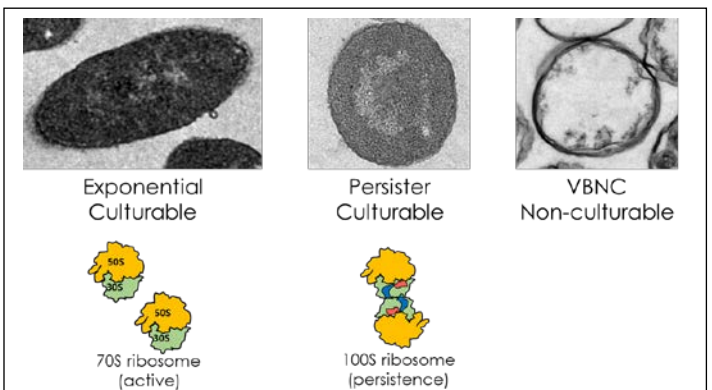


Fig. 1. TEM images of exponentially-growing, persister, and starved cells (VBNC). Exponentially-grown *E. coli* K-12 cells at a turbidity at 600 nm of 0.8, rifampicin-induced persister cells after 3 h ampicillin treatment, and VBNC cells 5 weeks of starvation in saline solution and ampicillin treatment for 3 h. Cartoons indicate the form of ribosomes, either as active 70S particles or hibernated 100S particles.

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Transmission electron microscopy (TEM) (**Fig. 1**) shows *E. coli* persister cells resemble exponentially-growing cells in that they have a dense cytoplasm, but persisters usually change their morphology from the rod shape of exponential growth to spheroid (Kim et al., 2018a); these cells rapidly resuscitate in 0 to 6 h (Kim et al., 2018a) based on their ribosome content (Kim et al., 2018b). In contrast, TEM shows 96% of *E. coli* VBNC particles formed from starvation after five weeks have intact membranes but lack normal cytosolic material (Kim et al., 2018a); critically, these “cell shells” cannot resuscitate due to their lack of normal cell components (Kim et al., 2018a). Similarly, EHEC heated to 50°C for 2 h forms cells with over 50% empty cytosol whereas growing cells have dense cytosol (Fu et al., 2020). Also, the plant pathogen, *Ralstonia solanacearum* forms cell shells under VBNC-inducing conditions based on copper treatment (Um et al., 2013). Therefore, various stress conditions give rise to intact membranes which lack normal cytosolic material (cell shells) that have been called VBNCs; hence, these cells are dead.

Errors in “viability” based on membrane staining.

Although there are inherent difficulties in interpreting the data of others, it appears there are frequent mistakes in the VBNC literature that are propagated based on naïve interpretations of membrane stains. For example, Fu et al. (Fu et al., 2020) heat-treated *Escherichia coli* O157:H7 (EHEC) and found after 2 h at 50°C, all cells were no longer culturable, as expected, by anyone familiar with the autoclave. Unfortunately, the authors relied on propidium iodide (PMA)-quantitative polymerase chain reaction (qPCR) to determine the number of VBNC particles rather than TEM; PMA enters cells with compromised membranes, binds DNA, and inhibits qPCR (Dong et al., 2020). Fu et al. concluded these particles that were not stained with PMA were VBNCs and were viable (Fu et al., 2020). In contradiction to this conclusion, their data showed that after more than 2 h at 50°C, no cells could be resuscitated even though the VBNC count based on PMA-qPCR was 6.5×10^6 particles/mL; the only time VBNC cells could resuscitate is when heating was for 2 h or less and culturable cells were present (Fu et al., 2020); i.e., it seems clear the cells that could form colonies were their culturable fraction rather than the much larger VBNC population (Fu et al., 2020). In addition, their transmission TEM images show that after 2 h of heat treatment, many cells become spheroidal and are clearly dead due to protein aggregation and empty cytosol. However, since these cell shells have intact membranes and are not affected by PMA, the particles were considered by the authors as viable VBNCs. In our own lab, we have shown that the viable fraction of EHEC VBNCs that are formed from starvation are persister cells (Kim et al., 2018a). Critically, we showed via TEM that the non-viable particles are dead cells, since, they lack cytosol, as well as showed that DNA-staining dyes like propidium

iodide that cannot penetrate membranes are not a suitable tool for studying VBNCs as they fail to indicate as dead those cells that lack cytosol (and possibly DNA) but have intact membranes (Kim et al., 2018a).

Aggregation indicates VBNCs are dead. Fu et al. (Fu et al., 2020) showed their VBNC cells contained as many as 1,028 aggregated proteins and then claimed there was “reversible aggregation” that resuscitated the cell shells. Similarly, Pu et al. also used propidium iodide staining to indicate VBNC particles and found nearly 1,000 different aggregated proteins (Pu et al., 2019). We feel it is highly unlikely large numbers of denatured proteins can be reconstituted, as there is no known biological process for restoring life to particles containing so many different denatured proteins in aggregates. In fact, large numbers of protein aggregates are a clear indication of death (Baquero and Levin, 2021). The claims of “reversible aggregation” and “protein aggresome” by some VBNC authors (Pu et al., 2019; Fu et al., 2020) and even those in the persister field (Dewachter et al., 2021; Huemer et al., 2021) is necessitated due to the failure to realize these former cell particles are dead as shown by their TEM (Fu et al., 2020) and protein aggregation data (Pu et al., 2019; Fu et al., 2020; Huemer et al., 2021).

Perspectives (or Where Angels Fear to Tread). Since antibiotic tolerance is a very serious problem, with projected global deaths of 100M/yr by 2050 (Huemer et al., 2020), it behooves us to understand why cells survive stress and current antibiotics; hence, discriminating between dormant persister cells and empty cell shells (VBNCs) is critical. We argue that those cells that can rejuvenate and reconstitute infections are not part of a dormancy continuum, but instead, only persister cells are dormant and capable of re-growth whereas VBNCs are dead and result from the death of persisters and non-persisters. Critically, for advances to be made in this field, TEM should be used (**Fig. 1**) along with colony counts to determine if cells are viable or dead, rather than on utilizing indirect methods like DNA stains which incorrectly view cell shells as viable.

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