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REVIEW ARTICLE

Thirty years of viable but nonculturable state research: Unsolved molecular mechanisms

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Abstract

Viable but nonculturable (VBNC) cells were recognized 30 years ago; and despite decades of research on the topic, most results are disperse and apparently incongruous. Since its description, a huge controversy arose regarding the ecological significance of this state: is it a degradation process without real significance for bacterial life cycles or is it an adaptive strategy of bacteria to cope with stressful conditions? In order to solve the molecular mechanisms of VBNC state induction and resuscitation, researchers in the field must be aware and overcome common issues delaying research progress. In this review, we discuss the intrinsic characteristic features of VBNC cells, the first clues on what is behind the VBNC state's induction, the models proposed for their resuscitation and the current methods to prove not only that cells are in VBNC state but also that they are able to resuscitate.

Keywords

Cell viability, gene expression, resuscitation, resuscitation promoting factors, viable but nonculturable state induction

History

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Viable but nonculturable and other nongrowing states

In 1982, Rita Colwell's laboratory described, for the first time, *Escherichia coli* and *Vibrio cholerae* cells that underwent a nonrecoverable stage of existence, but that remained viable (Xu et al., 1982). This was the first step for uncoupling the concepts of culturability and viability as, typically, cell viability is evaluated through culturability. Since then, many were the authors who tried to present a definition of viable but nonculturable (VBNC) cells. In an almost 10 years period, it was established that despite the fact that VBNC cells are not detectable by conventional culture techniques, they are able to produce new biomass and take up nutrients (Medema et al., 1992), maintain active metabolism (Rahman et al., 1994), respiration (Oliver et al., 1995), membrane integrity (Lloyd & Hayes, 1995) and gene transcription with specific messenger RNA (mRNA) production (Lleò et al., 2000). Along the years, the VBNC state started to be accepted as a survival strategy of cells exposed to adverse environmental circumstances.

In agreement with what is currently known about the VBNC state to correctly describe these cells, it should be stated that bacteria, when exposed to inauspicious environmental conditions, can go into a survival state in which they are no longer capable to form colonies on media formerly able to sustain their growth; they are intact cells that may have underwent changes in morphology (Takeda, 2011) and cell wall composition (Signoretto et al., 2002) and are still able to perform respiration (Oliver et al., 1995), gene transcription (Lleò et al., 2000) and protein synthesis (Rahman et al., 1994).

It is very common among microbiologists to perceive bacterial growth in three stages: lag phase, exponential phase and stationary phase. However, as ubiquitous life forms as bacteria are, we should be conscious that most of the time they are experiencing temperature or pH changes or even nutrient starvation. Since 1876, when Robert Koch and Ferdinand Cohn described bacterial spores (Higgins & Dworkin, 2012), many additional bacterial states in which bacteria are not growing were described. As general nongrowing states of bacteria are not the major goal of this review, only a brief description of additional bacterial nongrowing states is provided to stress out the differences and similarities between those and the VBNC state.

Sporulation

Sporulation was the first documented bacterial nongrowing state that can be observed in bacilli and clostridia. Spores are specialized cell forms, resistant to a multiplicity of harsh environmental assaults, which are formed through a heavily regulated genetic program that takes many hours and results in major changes in cellular morphology, biochemistry and physiology (Errington, 1993). The cell's decision to initiate sporulation is taken after integration of nutritional signals, population density and cell cycle stage (Errington, 1993). It is straightforward to point out the differences between sporulation and VBNC cell formation: while spores are metabolically inert forms with no detectable metabolic activity, VBNC cells are able to perform respiration (Oliver et al., 1995), transcription (Lleò et al., 2000) and protein synthesis (Rahman et al., 1994); spores are dehydrated and

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morphologically quite distinct from the growing cells (Higgins & Dworkin, 2012), whereas VBNC cells are mostly similar to growing cells with minor changes in cell wall composition (Signoretto et al., 2002) and that can experience stress-induced morphological changes (Takeda, 2011); even more, spores are much more resistant to environmental assaults than VBNC cells. Nevertheless, these processes are both bacterial adaptation strategies to environmental unfavorable conditions to growth and stay viable for several years (Amel & Amina, 2008; Higgins & Dworkin, 2012); all the more, both forms can revert from these states and become growing cells once again – the process by which spores turn into growing cells again is called germination (Higgins & Dworkin, 2012), while the process by which VBNC cells become growing cells again is called resuscitation (Nilsson et al., 1991).

Persistence

Almost 70 years after the first description of spores, Joseph Bigger described persister cells (Lewis, 2010), which are nongrowing variants of regular cells that form a small subpopulation on rapidly growing cultures and that are highly tolerant to antibiotics (Gerdes & Maisonneuve, 2012). Two major hypotheses, concerning the formation of persisters, co-exist: one states that unintended formation of various misfolded proteins causes stasis, producing persisters; alternatively, persisters' formation may be a programmed, epigenetic phenomenon with a genetic basis that has evolved to allow prokaryotic organisms to survive changing environments (Lewis, 2010). In favor of this last hypothesis is the observation that persisters can be formed in response to amino acid starvation, stationary phase and biofilm formation as well as the work on toxin–antitoxin modules, that suggests they might be involved in persisters' formation (Gerdes & Maisonneuve, 2012). Despite reports in several species [e.g. *E. coli*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus* and *Streptococcus pyogenes* (Gerdes & Maisonneuve, 2012; Lewis, 2010)] not much is known about their formation or the way they can switch back to rapid growth, i.e. resuscitate (Gerdes & Maisonneuve, 2012). The comparison between persistence and VBNC state is less obvious than the one with sporulation: for persister cells, morphological changes were never reported, and in the case of VBNC cells, these changes are known for a long time; evidences seem to accumulate about the involvement of toxin–antitoxin modules in persister cell formation what was never suggested for VBNC cell formation; moreover, persisters are formed, at low frequency, in parallel to growth, while VBNC cells are formed after longer periods of incubation in environmental conditions that do not sustain growth. The last is in fact an excellent argument for the conception of VBNC state as a survival strategy given that culturable cells will only commit to this state when environmental conditions are largely adverse. A similarity worth noticing is that both cell types can resuscitate, ultimately giving rise to a growing population. Even more, and contrary to sporulation, VBNC cell's formation and persister cell's formation are processes that were already described in a large taxonomical variety of bacteria (Gerdes & Maisonneuve, 2012; Lewis, 2010; Oliver, 2010).

Dormancy

Another bacterial nongrowing state that is frequently compared to VBNC state is dormancy. Dormant cells do not display significant metabolic activity, do not form colonies on agar plates or divide and can endure like this for extended periods (Kell & Young, 2000). This state is reversible, meaning that dormant cells can resuscitate (Kaprelyants et al., 1993). Two visions on how this state can be seen from the perspective of the cell are still currently under discussion: it can be seen as part of an essentially programmed developmental process, such as sporulation, or as a result of degradation due to loss of different cell functions (Kell & Young, 2000). Two opinions also co-exist concerning the differences between VBNC state and dormancy. Some authors (e.g. Oliver, 2005) consider that VBNC state and dormancy are different terms used to refer the same physiological state; in fact, dormant cells are viable (as extensively demonstrated through their ability to resuscitate) and nonculturable. On the other hand, authors working on actinobacterial dormancy (e.g. Mukamolova et al., 2003) do not use the term VBNC state mostly on the grounds of differences in metabolic activity; VBNC cells have measurable metabolic activity, while dormant cells' metabolic activity is below detection limits.

VBNC cells: they are everywhere

After description by Xu et al. (1982), VBNC cells were identified in a large number of bacterial species and a wide spectrum of environments. In 2005, Oliver reviewed the VBNC state in bacteria and presented a list of 52 species in which the VBNC state was described. Five years later, he reviewed the VBNC state in pathogenic bacteria and added four new species (Oliver, 2010). At present, 12 more can be added making a total of 68 species, enumerated in Table 1. The species are phylogenetically distributed: 40 species (17 genera) of γ-proteobacteria, 4 species (3 genera) of β-proteobacteria, 5 species (4 genera) of α-proteobacteria, 5 species (3 genera) of ε-proteobacteria, 1 species (1 genus) of bacteroidetes, 3 species (2 genera) of actinobacteria and 9 species (5 genera) of firmicutes. It is our opinion that this wide distribution of the VBNC state along the phylogenetic tree is in favor of the view of this state as a survival strategy.

Along a wide species distribution of VBNC state descriptions, also a broad environmental distribution can be observed. The first description of VBNC cells was in marine and estuarine water (Xu et al., 1982), and after that several authors detected VBNC cells in seawater (Maalej et al., 2004; Pommepuy et al., 1996), estuarine water (Oliver et al., 1995), tap water (Pawlowski et al., 2011), seabed sediments (Magarinos et al., 1994) or soil (Ben Abdallah et al., 2008; Reissbrodt et al., 2000). Moreover, several reports of VBNC cells in food came along the years in: drinking water (Defives et al., 1999; Liu et al., 2008), wine (Millet & Lonvaud-Funel, 2000), beer (Suzuki et al., 2006), grape fruit juice (Nicolo et al., 2011), mature apple fruit calyces (Ordax et al., 2009), lettuce (Dinu & Bach, 2011),

Table 1. Species in which the VBNC state was reported.

Acetobacter
A. aceti (Millet & Lonvaud-Funel, 2000)

Acinetobacter
A calcoaceticus (Lemke & Leff, 2006)

Aeromonas
A. hydrophila (Oliver, 2010)
A. salmonicida (Oliver, 2005)

Agrobacterium
A. tumefaciens (Oliver, 2005)

Alcaligenes
A. eutrophus (Oliver, 2005)

Arcobacter
A. butzleri (Fera et al., 2008)

Bifidobacterium
B. lactis (Lahtinen et al., 2008)
B. longum (Lahtinen et al., 2008)
B. animalis (Lahtinen et al., 2008)

Burkholderia
B. cepacia (Oliver, 2005)
B. pseudomallei (Oliver, 2005)

Campylobacter
C. coli (Oliver, 2005)
C. jejuni (Oliver, 2005)
C. lari (Oliver, 2005)

Citrobacter
C. freundii (Dhiaf et al., 2008)

Cytophaga
C. allerginae (Oliver, 2005)

Enterobacter
E. aerogenes (Oliver, 2005)
E. cloacae (Oliver, 2005)
E. agglomerans (Rowan, 2004)

Enterococcus
E. faecalis (Oliver, 2005)
E. hirae (Oliver, 2005)
E. faecium (Oliver, 2005)

Erwinia
E. amylovora (Oliver, 2010)

Escherichia
E. coli (Oliver, 2005)

Francisella
F. tularensis (Oliver, 2005)

Helicobacter
H. pylori (Oliver, 2005)

Klebsiella
K. aerogenes (Oliver, 2005)
K. pneumoniae (Oliver, 2005)
K. planticola (Oliver, 2005)

Lactobacillus
L. plantarum (Oliver, 2005)
L. lindneri (Suzuki et al., 2006)
L. paracollinoides (Suzuki et al., 2006)
L. lactis (Oliver, 2005)

Legionella
L. pneumophila (Oliver, 2005)

Listeria
L. monocytogenes (Oliver, 2005)

Oenococcus
O. oeni (Millet & Lonvaud-Funel, 2000)

Pasteurella
P. piscicida (Oliver, 2005)

Pseudomonas
P. aeruginosa (Oliver, 2005)
P. fluorescens (Oliver, 2005)
P. putida (Oliver, 2005)
P. syringae (Oliver, 2005)

Ralstonia
R. solanacearum (Oliver, 2005)

Rhizobium
R. leguminosarum (Oliver, 2005)
R. meliloti (Oliver, 2005)

Rhodococcus
R. rhodochrous (Oliver, 2005)

Salmonella
S. enteritidis (Oliver, 2005)
S. enterica (Oliver, 2005)

Serratia
S. marcescens (Oliver, 2005)

Shigella
S. dysenteriae (Oliver, 2005)
S. flexneri (Oliver, 2005)
S. sonnei (Oliver, 2005)

Sinorhizobium
S. meliloti (Oliver, 2005)

Vibrio
V. alginolyticus (Oliver, 2010)
V. anguillarum (Oliver, 2005)
V. campbellii (Oliver, 2005)
V. cholerae (Oliver, 2005)
V. fischeri (Oliver, 2005)
V. harveyi (Oliver, 2005)
V. mimicus (Oliver, 2005)
V. natriegens (Oliver, 2005)
V. parahaemolyticus (Oliver, 2005)
V. proteolytica (Oliver, 2005)
V. shiloi (Oliver, 2005)
V. vulnificus (Oliver, 2005)

Xanthomonas
X. axonopodis (Oliver, 2010)
X. campestris (Oliver, 2005)

Yersinia
Y. pestis (Pawlowski et al., 2011)

parsley leaves (Dreux et al., 2007) and salted salmon roe (Makino et al., 2000). In clinical settings and samples from human origin, only nongrowing *M. tuberculosis* were reported (Mukamolova et al., 2010).

The broad distribution of VBNC cells in bacterial species and environments reinforce that this state is a common mechanism to cope with unfavorable environmental conditions. Moreover, their existence cannot be overlooked and their real risk for human health should be properly evaluated.

VBNC cells: they are formed in mysterious ways

An extensive list of unfavorable environmental conditions was described to induce the VBNC state. Starvation is a general VBNC induction condition and besides that, VBNC induction conditions include temperatures outside those that are permissive to cell growth (Asakura et al., 2007; Besnard et al., 2000b, 2002; Coutard et al., 2007; Day & Oliver, 2004; Fera et al., 2008; Gonzalez-Escalona et al., 2006; Maalej et al., 2004; Nilsson et al., 1991; Pawlowski et al., 2011; Pinto et al., 2011), sunlight (Besnard et al., 2002; Pommepuy et al., 1996), low oxygen availability (Bovill & Mackey, 1997; Pinto et al., 2011), sulfur dioxide (Millet & Lonvaud-Funel, 2000), low redox potential (Mascher et al., 2000), high saline concentration (Besnard et al., 2000b, 2002; Mascher et al., 2000; Pinto et al., 2011), desiccation (Dinu & Bach, 2011; Dreux et al., 2007), heavy metal exposure (Aurass et al., 2011; del Campo et al., 2009; Ordax et al., 2006) and non optimal pH (Besnard et al., 2000b, 2002; Chaveerach et al., 2003; Pinto et al., 2011). To our knowledge, no large comparative study on the effect of diverse VBNC state inducing conditions exist. Such a study could be useful to determine which conditions would lead to faster VBNC state inductions. Nevertheless, it was suggested that as far as *E. coli* VBNC state inductions are concerned, the origin of the strains as well as the temperature are key factors for the speed of VBNC state inductions (Pinto et al., 2011).

Not much is known about the genetic program resulting in VBNC state induction. To our knowledge, no systematic large-scale screening of mutants for defects in VBNC state

induction was done. Nevertheless, three proteins were implicated into the VBNC state induction: RpoS (Boaretti et al., 2003; Kusumoto et al., 2012), polyphosphate kinase 1 (PPK1; Gangaiah et al., 2009) and EnvZ (Darcan et al., 2009). The effect of RpoS in VBNC state induction was studied in *E. coli* (Boaretti et al., 2003) and in *S. enterica* (Kusumoto et al., 2012). In both, RpoS depletion resulted in faster VBNC state induction. Moreover, in *E. coli*, *rpoS* deletion resulted in less ability of cells to stay in VBNC state for long periods (i.e. cells die faster) and to resuscitate after long periods in VBNC state. Polyphosphate kinases are involved in a wide variety of cell functions since they are implicated in polyphosphate formation. The effect of *ppk1* deletion in *Campylobacter jejuni* (Gangaiah et al., 2009) resulted in decreased ability of strains to form VBNC cells. But, since polyphosphate kinases are involved in a wide array of reactions in a cell, is it to wonder that it would also have some influence in VBNC state induction? Finally, the effect of EnvZ, an osmolarity sensor protein that is responsible for the regulation of *ompF* and *ompC* genes, was also evaluated in *E. coli*, and mutants in *envZ* were found not to induce the VBNC state. However, it is not clear if this phenotype is related to the VBNC induction condition (osmotic stress) or to the VBNC state phenomena itself.

VBNC cells: differences from culturable cells

As stated previously, VBNC cells present some changes from culturable cells besides the inability to growth, namely, in shape, cell wall composition, membrane composition and gene expression. Morphological changes in bacteria exposed to stressful conditions were already extensively reported – examples are *Campylobacter* spp. (Thomas et al., 2002), *Listeria monocytogenes* (Giotis et al., 2007), *Pasteurella piscicida* (Magarinos et al., 1994), *Salmonella bovismorbificans* (Ben Abdallah et al., 2008), *V. cholerae* (Krebs & Taylor, 2011), *Vibrio parahaemolyticus* (Chen et al., 2009) and *Vibrio vulnificus* (Nilsson et al., 1991). It was suggested that these morphological alterations may work as a coping mechanism of bacterial cells with stressful environmental conditions (Krebs & Taylor, 2011) – for example, starvation (Chen et al., 2009), low temperatures (Chen et al., 2009) or extreme pH (Giotis et al., 2007). In these unfavorable situations, decreasing size would enable the cells to have the greatest surface for nutrient uptake while maintaining the least amount of cell mass (Krebs & Taylor, 2011). If the environment becomes once again favorable, the cells change back and return to their normal shape (Chiu et al., 2008; Magarinos et al., 1994; Nilsson et al., 1991). This shape shift from bacilli to cocci was also described for cells in VBNC state (Colwell, 2000; Krebs & Taylor, 2011; Nilsson et al., 1991), which would return to the bacillary shape with resuscitation. Nevertheless, care should be taken when considering the shape shift of cells becoming VBNC since this may be a widespread mechanism to cope with stressful conditions and not a VBNC cell specific trait, i.e. VBNC cells might present changes in shape, but cells with changed shape are not necessarily in VBNC state.

Cell envelope

The characterization of VBNC cell's wall of the Gram-positive *Enterococcus faecalis* (Signoretto et al., 2000) and the Gram-negative *E. coli* (Signoretto et al., 2002) was reported by Signoretto et al. in the beginning of the 2000s decade. One of the parameters evaluated was the mechanical resistance of the cell wall, and while for *Ent. faecalis*, the VBNC cells were twice as resistant as growing cells (Signoretto et al., 2000); for *E. coli*, this was not observed (Signoretto et al., 2002). Another assessed factor was the chemical composition of the peptidoglycan evaluated by the extent of oligomerization of muropeptides. In both cases, an increase in peptidoglycan cross-linking was reported (Signoretto et al., 2000, 2002), even though in the Gram-positive it was most likely due to an increase in oligomers with higher order than dimers (Signoretto et al., 2000) and in the Gram-negative it reflects a preferential increase in total dimers but not higher order oligomers (Signoretto et al., 2002). Ultimately, the relative amount of enzymes involved in peptidoglycan metabolism was evaluated. Noteworthy is the increase, in *Ent. faecalis*, of the penicillin-binding proteins (PBPs) important for cell growth under suboptimal environmental conditions (Signoretto et al., 2000) and the decrease, in *E. coli*, of the PBPs responsible for peptidoglycan assembly and growth (Signoretto et al., 2002). These observations are in agreement with the vision of VBNC cell formation as a regulated phenomenon.

Another relevant peptidoglycan modification described in VBNC cells of *Ent. faecalis* was O-acetylation of muramoyl residues. VBNC cells of several strains of *Ent. faecalis* showed an increase between 44 and 72% of O-acetylation when compared to exponentially growing cells (Lleò et al., 2000). This peptidoglycan modification, as well as the presence of teichoic acids, inhibits the activity of lytic transglycosylases as these enzymes require a free C-6-hydroxyl group on the muramoyl residues to act (Moynihan & Clarke, 2011). This increase in O-acetylation may be a mechanism by which the cells regulate their sensitivity to hydrolytic enzymes produced by other cells (resistance mechanism) or regulate the action of their own lytic transglycosylases.

Cytoplasmic membranes are of outmost importance for a cell since they separate the cell contents from the external environment, regulate movement of substances going in or out of the cell and allow for energy transduction (Denich et al., 2003). Moreover, an array of proteins essential for cell metabolism is located in the membrane. Several environmental factors can change the physical properties of the biological membranes, e.g. temperature, chemical pollutants, ions, pressure, nutrients and the growth phase of the microbial culture (Denich et al., 2003). In 2004, Day et al. published the results of a study of the fatty acid composition of cell membranes in *V. vulnificus* entering VBNC state and reported a decrease in hexadecanoic acid (16:0) from 50% of the total fatty acid composition prior to temperature downshift (VBNC cell formation inducing stimulus) to 5% after 24 days of incubation, when cells were already in VBNC state. Moreover, the ability to enter VBNC state was lost when the synthesis of fatty acids was chemically prevented, i.e. the cells died instead. It seems reasonable to hypothesize that membrane composition is altered when cells enter VBNC state, but from this report, it was not clear if these changes were due to the VBNC state inducing stimuli [starvation in combination with low temperature, conditions known to alter

the membranes (Denich et al., 2003)] or if those were in fact characteristic of a cell in VBNC state. A similar comparative study using different VBNC state inducing stimuli could probably answer this question.

In 2008, Muela et al. studied the changes in membrane protein composition in conditions known to induce the VBNC state in *E. coli*. Authors used cells incubated into three different media and observed that, among all conditions tested, 18 proteins showed variations in expression level along the studied time. Nevertheless, only four proteins showed changes in their expression regardless of the condition tested. Those were elongation factor Tu, enolase, D-3 phosphoglycerate dehydrogenase and threonine synthase. All these proteins are somehow related to nutrient stress and may reflect a common feature between all the tested conditions, i.e. starvation. In a similar way to the study on the changes in lipidic composition of the membrane, it is not clear if the modifications are characteristic of the VBNC state or of the VBNC state induction conditions.

In the same year, Asakura et al. (2008) studied the proteome of *E. coli* exposed to oxidative stress conditions. The authors stated that outer membrane protein W (OmpW) expression was induced in VBNC state and that deletion of *ompW* resulted in an increased cell recovery while its overexpression decreased it. Even though the results could point for a role of OmpW in stress adaptation, it is our belief that the authors were working with injured cells and not VBNC cells; two different physiological states as described below.

The studies published so far concerning modifications of the membrane, lipidic (Day & Oliver, 2004) or proteic (Asakura et al., 2008; Muela et al., 2008) fractions, can give some insights on how cells cope with stressful conditions, but the experimental strategy needs to be highly improved to be sure that these modifications are in fact characteristic of VBNC cells, e.g. cells in VBNC state induced by several distinct factors should be characterized.

Gene expression

The controversy around the formation of VBNC cells, similar to what happened with other bacterial nongrowing states, was in discussion for a long time: are they formed by a degradative process leading to cell death or by a programmed process by which cells survive through adaptation to unfavorable environmental condition (Maalej et al., 2004)? One of the best ways to show that the later hypothesis is the correct one is to show that VBNC cells do perform gene transcription and protein synthesis in a way that would be distinct from culturable cells and consequently several were the authors that underwent studies on VBNC cells' gene expression. Two years ago, gene expression data collected from analysis of planktonic and biofilm VBNC cells of pathogenic bacteria were reviewed (Trevors, 2011), nevertheless, without a focus on mechanism elucidation.

In 2002, Sabina Heim et al. studied the proteome of VBNC *Ent. faecalis* cells. When comparing exponentially growing cells and VBNC cells, six proteins were found to be present at lower levels (the chaperonin GroEL – that prevents misfolding and promotes the refolding of polypeptides generated under stress conditions; the chaperonin DnaK – known to participate in the response to hyperosmotic shock; enolase – an essential enzyme in glycolysis; adenosine triphosphate (ATP)-synthase β-chain – for ATP production; EF-Tu – involved in protein biosynthesis; and enoyl-acyl carrier protein – involved in fatty acid biosynthesis), while three proteins were found in higher amounts (elongation factor Ts (EF-Ts) – that mediates the regeneration of elongation factor Tu (EF-Tu); fructose-bisphosphate aldolase – enzyme involved in gluconeogenesis and glycolysis; and catabolite regulator protein – involved in regulation of carbon metabolism). Also approaching the problematic from a proteomic point of view, Lai et al. (2009) compared *V. parahaemolyticus* protein profiles of VBNC cells to those of exponentially growing cells. More than a dozen proteins were enhanced in VBNC state, among which, it is possible to find proteins related to transcription (i.e. RNA polymerase), translation (i.e. EF-Tu), synthesis of ATP, gluconeogenesis-related metabolism (i.e. glyceraldehyde 3-phosphate dehydrogenase) and antioxidants. As can be noted, reports disagree in the behavior of the EF-Tu protein in VBNC cells.

Concerning the assessment of transcribed genes, the broader study belongs to Asakura et al. (2007) that reported microarray analysis of *V. cholerae* VBNC cells. From the 58 genes reported to be induced at least 5-fold in VBNC state, more than 50% belong to four categories: regulatory functions, transport and binding, cellular processes and energy metabolism. The eight genes reported to be repressed in the VBNC state belonged to four categories: central intermediary metabolism, transport and binding proteins, cellular processes and cell envelope. In 2006, González-Escalona et al. had already evaluated the expression of a small set of genes involved in protein translation (*tuf*) or stress response (*relA* and *rpoS*) of *V. cholerae*. These authors reported a reduction in all four RNAs when comparing exponentially growing to VBNC cells. Interestingly, comparing starved cells with VBNC revealed an increased amount of *relA* mRNA and a reduction in 16S ribosomal RNA.

Virulence

Since pathogenicity of VBNC cells is a question of outmost importance in assessing the real danger these cells can pose to human health, in 2005, Vora et al. reported the results of a 95 oligonucleotide microarray study showing that human pathogenic *Vibrio* spp., while in VBNC state, continued to express known toxin (*ctxAB*, *rtxA*, *hlyA*, *tl*, *tdh* and *vvhA*) and virulence (*tcpA* and *TTSS*) genes. In 2006, Smith & Oliver reported the results of a small expression study where they evaluated *in situ* the expression of six genes throughout VBNC entry, staying and subsequent resuscitation of *V. vulnificus*. Genes were *vvhA*, coding for *V. vulnificus* cytolysin, *rpoS*, for the RNA polymerase sigma factor, *tufA*, for the elongation factor Tu, *wza* and *wzb*, which gene products are involved in capsule synthesis and *katG*, for periplasmic catalase. Results showed a down-regulation of *katG* and *vvhA* mRNAs during VBNC state. One year later, Coutard et al. (2007) recovered this issue and studied the transcription profile of a few virulence genes in *V. parahaemolyticus* resuscitation. Expression of gene *tdh2*, coding for the thermostable direct hemolysin, and genes *escU*, *spa24* and *vopP* coding, respectively, for the cytosolic, inner

membrane and effector proteins of the enterotoxic system (type III secretion system) was evaluated, and no transcriptional induction was observed. Lothigius et al. (2010) also assessed the expression of virulence genes in VBNC cells of *E. coli*. The presence of mRNA of *estA* (coding for heat-stable toxin), *eltB* (for heat labile enterotoxin), *cfaB* (for colonization factor antigen I fimbrial subunit B), *csvA* (for fimbriae major subunit) and *cvbB* (for fimbriae periplasmic chaperone) was evaluated as well as the production and secretion of the toxins. Surprisingly, no production or secretion of the toxins was observed, but genes encoding toxins and colonization factors (*cfaB*, *csvA* and *cvbB*) were expressed.

Globally, the results, here summarized, allow us to defend that VBNC state is, in fact, a genetically programmed state, that genes involved in response to stress are consistently up-regulated, which is in agreement with unfavorable conditions leading to VBNC state entry. Moreover, genes coding for the toxins are down-regulated during permanence into VBNC state suggesting that VBNC cells are not pathogenic unless they resuscitate. It is plausible to suggest that VBNC state entry after exposure to unfavorable conditions may be a very different genetic program than the one leading to virulence; in this case, not only the cells are exposed to a completely different set of environmental stimuli but also the beneficial outcomes of each situation are opposite.

Descriptions of VBNC state induction by pathogenic species raised a concerned about the retention of virulence by cells in this state and several studies concerning the virulence of VBNC cells were done over the years and a comprehensive review on the VBNC state of pathogenic bacteria and the virulence of VBNC cells was presented a few years ago (Oliver, 2010). Not only was the virulence of VBNC cells studied through gene expression, as reviewed above, but also by means of *in vivo* assays. *V. vulnificus* was found to resuscitate *in vivo* and invade a mouse model (Oliver & Bockian, 1995), *V. cholerae* resuscitate in the human intestine (Colwell et al., 1996) and *Vibrio* spp. were found to resuscitate in mice (Baffone et al., 2003). *C. jejuni* was found to resuscitate in both yolk sacs of embryonated eggs (Cappelier et al., 1999) and mouse intestine (Baffone et al., 2006). *Aeromonas hydrophila* was reported as pathogenic after resuscitation (Maalej et al., 2004). *E. coli* was found to produce enterotoxins while in VBNC state (Pommepuy et al., 1996) and *L. monocytogenes* despite the ability to resuscitate *in vivo* was not virulent while in VBNC state (Cappelier et al., 2005). In addition, adhesion of VBNC cells to eukaryotic cells was evaluated in *Shigella dysenteriae* (Rahman et al., 1996), *Ent. faecalis* (Pruzzo et al., 2002) and *V. cholerae* (Pruzzo et al., 2003); and in all cases, it was observed that these cells were able to adhere but at a reduced rate.

Globally, these studies seem to show that cells from pathogenic species while in VBNC state are not virulent but if or when resuscitate are once again virulent. Moreover, the ability of VBNC cells to resuscitate *in vivo* seems like a common trait of these cells.

VBNC cells: how to identify them

The ability to identify cells in VBNC state is crucial not only in research but also for applied purposes. In order to state that a cell is in VBNC state, two major cell attributes, inability to form colonies on agar plates and cell viability, should be verified. Evaluate the ability of cells to form colonies on agar plates is a standard procedure in any microbiology laboratory but, to be confident of the inability to form colonies much care must be taken. First of all, one needs to think about the concept of VBNC cells: these cells were culturable at some point and after that they lost the ability to grow. This implies that culturability should be evaluated in media that can support the growth of that particular strain. It is quite common in clinical and food microbiology laboratories to use selective or differential media with antibiotics or stressful conditions. For the purpose of identifying VBNC cells, we do not advise the use of such conditions since stressed cells, not in VBNC state, might not grow exclusively because they are too stressed to cope with the selective conditions; culturability should be evaluated in media as rich and as non stressful as possible.

In 2001 Bogosian & Bourneuf shared their concerns about the confusion between VBNC cells and injured cells. The latter were then defined as having an increased sensitivity to growth media components that were not normally inhibitory. The recognition of the existence of cells in this injured state is the main reason why media used to evaluate the inability of cells to form colonies should be as non stressful as possible. Bloomfield et al. (1998) proposed an explanation for the formation of injured cells: imbalance between catabolism and anabolism. Cells in rich media grow at maximal rate implying metabolic maximal rate, and catabolism and anabolism are balanced. When cells reach stationary phase, they shut down the main anabolic processes but the catabolic processes are not reduced by the same extent. In these conditions, free radicals are intracellularly produced, and if not rapidly reverted, this can lead to cell injury. Bogosian et al. (2000) reported that cold-shocked cells of *V. vulnificus* were peroxide sensitive; and Kong et al. (2004) studied this in detail and suggested that hydrogen peroxide sensitivity might contribute to nonculturability but not fully account for VBNC state induction.

To be sure that these injured cells are not mistaken for VBNC cells, the supplementation of the media, used to evaluate culturability, with sodium pyruvate was suggested since this compound is used in microbiological media to degrade hydrogen peroxide (Bogosian et al., 2000; Bogosian & Bourneuf, 2001). As extensively seen (Arana et al., 2007; Asakura et al., 2007, 2008; Boaretti et al., 2003; Gupte et al., 2003; Heim et al., 2002; Lleò et al., 2001; Pawlowski et al., 2011; Pinto et al., 2011; Rahman et al., 1994; Reissbrodt et al., 2000), even when no colonies appear on rich media, several colonies are formed on rich media supplemented with sodium pyruvate or catalase, both hydrogen peroxide-degrading compounds. Injured cells are not considered VBNC cells since they are formed by means of cell degradation and are dying instead of adapting to unfavorable environmental conditions. Moreover, injured cells can be recovered by media supplementation with hydrogen peroxide-degrading compounds, whereas VBNC cells cannot.

Along the years, several methods for evaluating bacterial viability were used: membrane integrity (Arana et al., 2007; Asakura et al., 2007; Aurass et al., 2011; Basaglia et al., 2007;

Boaretti et al., 2003; Defives et al., 1999; Dinu & Bach, 2011; Dreux et al., 2007; Fera et al., 2008; Gonzalez-Escalona et al., 2006; Heim et al., 2002; Kusumoto et al., 2012; Lai et al., 2009; Lleò et al., 2001, 2007; Muela et al., 2008; Nicolo et al., 2011; Ordax et al., 2006; Panutdaporn et al., 2006; Pawlowski et al., 2011; Reissbrodt et al., 2000; Senoh et al., 2010; Smith & Oliver, 2006), membrane potential (Kaprelyants & Kell, 1992), enzymatic activity (Dinu & Bach, 2011), respiration (Amel & Amina, 2008; Baffone et al., 2003; Basaglia et al., 2007; Besnard et al., 2000b, 2002; Boaretti et al., 2003; Bovill & Mackey, 1997; Cappelier et al., 1999; Gangaiah et al., 2009; Gupte et al., 2003; Muela et al., 2008; Nilsson et al., 1991; Oliver & Bockian, 1995; Rodriguez et al., 1992; Thomas et al., 2002), responsiveness to nutritional stimuli (Barcina et al., 1995; Besnard et al., 2000a, b; Besnard et al., 2002; Boaretti et al., 2003; Darcan et al., 2009; Day & Oliver, 2004; Kogure et al., 1979; Maalej et al., 2004; Oliver & Bockian, 1995; Oliver et al., 1995; Signoretto et al., 2002; Xu et al., 1982) and mRNA detection (Lleò et al., 2000, 2001) among others.

Evaluation of cytoplasmic membrane integrity is extensively used to conclude about cell viability and uses a combination of two distinct fluorescent stains with different cell permeability (Boulos et al., 1999): SYTO®9 and propidium iodide. The first is a green fluorescent nucleic acid stain that labels all bacteria in a population (those with intact membranes and those with damaged membranes) and the second is a red fluorescent nucleic acid stain that penetrates only bacteria with damaged membranes causing a reduction in SYTO®9 fluorescence. In this way, cells with intact membranes will stain fluorescent green and cells with damaged membranes fluorescent red.

In 1992, Rodriguez et al. reported the use of the compound 5-cyano-2,3-di-(p-tolyl)tetrazolium chloride (CTC) to detect activity of the respiratory chain. CTC competes with oxygen as a final electron acceptor of the respiratory chain and when reduced forms an insoluble fluorescent red compound inside the cells. In this case, live cells (cells with a working respiratory chain) will be stained in red and dead cells (the others) will not be stained. Three years later, Ullrich et al. (1996) drew our attention to the toxic effects of CTC to the growth of bacteria. Nevertheless, when this compound is applied to viable cell enumeration, the cells are not usually subsequently plated (Besnard et al., 2000a; Rodriguez et al., 1992).

In 1979, Kogure et al. described a microscopic methodology that allowed the direct enumeration of living bacteria. Cells would be incubated in rich media in the presence of antibiotics, conditions in which cells will not divide but instead elongate. Live cells will look elongated, while dead cells will not suffer changes in their cell length. Several years later, Barcina et al. (1995) reported some changes to the method, namely changing the antibiotic from nalidixic acid to ciprofloxacin, to allow its application to both Gram-positive and negative. In 2000a, Besnard et al. described a similar procedure optimized for *L. monocytogenes* VBNC cell detection. Despite the simplicity and subsequent widespread use of this method, several were the critiques arising: the mechanism underlying this behavior was never explained, the uptake of nutrients may occur without elongation (Rice et al., 2000), the bacteria response to the antibiotic may not be uniform (Rice et al., 2000); and if cells elongate in response to nutrients and one needs antibiotics to prevent their division, then why are they not able to form colonies on media plates? Nevertheless, a strong correlation between this direct viable count and other methods was described (Villarino et al., 2000).

It is a widespread opinion that the ultimate proof that cells are, in fact, viable despite their inability to grow is their ability to revert from this state and became culturable again, i.e. to resuscitate.

Resuscitation of VBNC cells: the burden of the proof

Two years after the first description, in 1982, of the VBNC state in *E. coli* and *V. cholerae*, it was also Rita Colwell's laboratory that first described resuscitation in *Salmonella enteritidis* (Roszak et al., 1984). Care should be taken when evaluating resuscitation because several concepts are sometimes mistaken with resuscitation, most due to the confusion between the VBNC and the injured states. Resuscitation is defined as the reversal of metabolic and physiological processes, which characterize the nonculturable state (Baffone et al., 2006), meaning that the VBNC cells become culturable again. On the other hand, revival defines the transition of injured cells to an active state after release from stress, while recovery is the transition of injured cells to an active state by media supplementation with hydrogen peroxide-degrading compounds (Imazaki & Nakaho, 2009). In 2009, Sachidanandham & Gin defined a new term, revitalization, that combined resuscitation followed by cell division of the resuscitated cells; but to our knowledge, this is not a common term in the literature.

In all resuscitation experiments, it is crucial to prove that what is seen as growth is in fact result of resuscitation and not revival or regrowth, i.e. growth of culturable cells. In a mixed population composed of cells in different physiological states (culturable, injured, VBNC and dead cells) exposed to a resuscitation stimulus, the number of culturable cells can increase. Three hypotheses must be considered: regrowth of the culturable cells, revival of the injured cells or resuscitation of the VBNC cells.

Methods to prove resuscitation

Along the years, several were the experimental approaches aiming to prove resuscitation and from our perspective proving resuscitation gets straightforward if some cell populations could be excluded. A culturable cell population exposed to a VBNC state induction stimulus will reduce the number of culturable cells and that of the other populations (injured, VBNC and dead) will increase. Eventually, culturable cells will fall below detection limit, and only three types of cells can remain in the population: injured, VBNC or dead. If the environmental VBNC-inducing conditions would not change, injured cells will, similarly, decrease in number until it drops below the detection limit. At this point, only two types of cells can remain in the population (Figure 1). This is, in our opinion, the optimal population to perform resuscitation experiments with; nonetheless, it does not exclude the requirement for quantification of the number of cells that

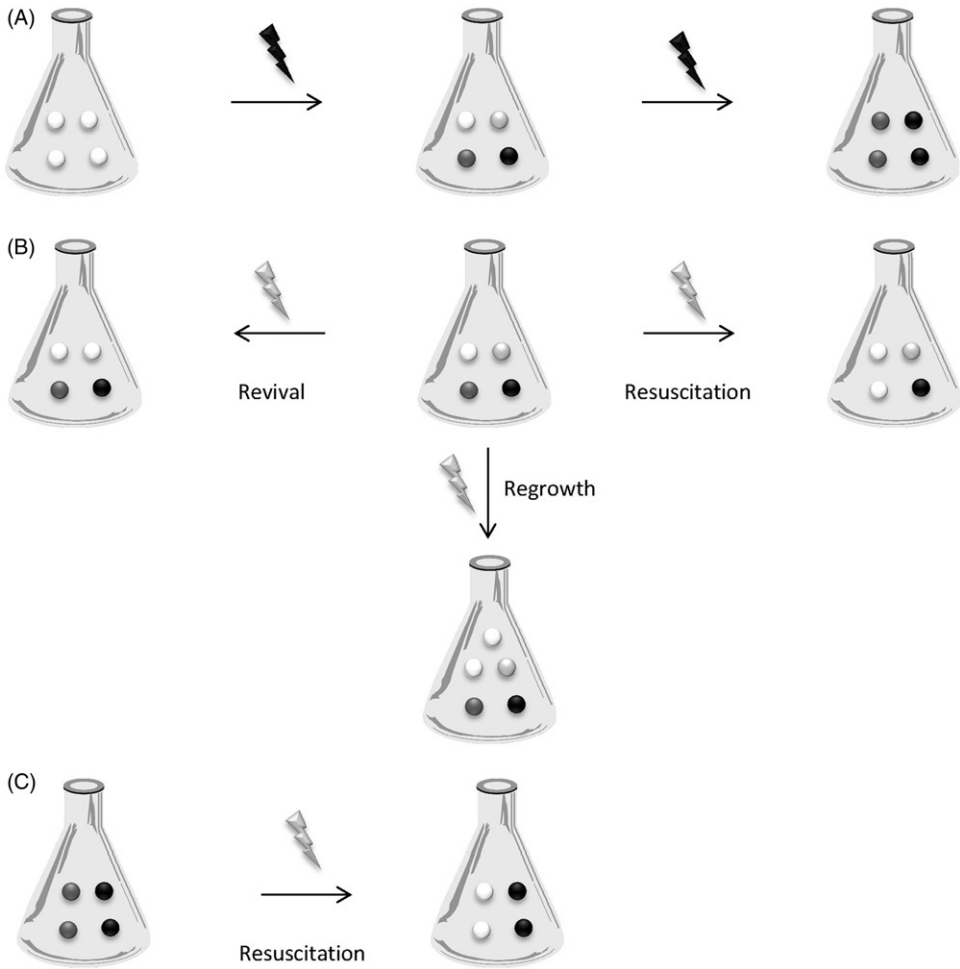

Figure 1. How to distinguish resuscitation from revival and regrowth. (A) A suspension of culturable cell (white circles) is subjected to a stress condition (black lightning bolt). As the time goes by, culturable cells will die (black circles), become injured (light gray circles) or VBNC (dark gray circles). Eventually, all cells of the initial culturable cells suspension would become either dead or VBNC. At any given time, culturable cells (c) might be quantified through plating in rich media, while culturable and injured cells ($c+i$) might be quantified through plating in media supplemented with H_2O_2 degrading compounds. Total cells (t) and viable cells (v) might be quantified microscopically. The number of injured cells can be derived from $(c+i)-c$, the number of VBNC cells from $v-(c+i)$ and the number of dead cells from t–v. (B) If a suspension in which culturable, injured, VBNC and dead cells are present is subject to a given resuscitation stimulus (gray lightning bolt) and the number of culturable cells (c) rises, three hypothesis must be considered: (i) revival of injured cells, $c_{final}=(c+i)_{initial}$; (ii) regrowth of the culturable cells, $c_{final}>(c+i)_{initial}$; (iii) or resuscitation of the VBNC cells, $c_{final}>(c+i)_{initial}$ also applies. A more complex case in which regrowth, revival and resuscitation all happens must not be excluded. (C) If a suspension composed only by dead and VBNC cells is subject to a resuscitation stimulus and growth appears, only resuscitation must be considered since c and $c+i$ equals ''0''. Most times, this ''0'' corresponds in fact to a detection limit, and the number of cells that have resuscitated must be above that threshold.

resuscitated. If that number, commonly quantified through a most probable number approach, is above the culturable and injured cells' detection limits, resuscitation can be stated, otherwise regrowth or revival cannot be excluded (Figure 1). With a similar approach, it was possible to state resuscitation of *E. coli* (Pinto et al., 2011).

In 1997, Whitesides and Oliver proved resuscitation of *V. vulnificus* by diluting cells, to levels in which the presence of growing cells was very unlikely, excluded regrowth from their resuscitation experiments; nevertheless, these authors did not account for the presence of injured cells and therefore for their revival.

In 2007, Arana et al. described a complex protocol to prove resuscitation. First of all, the mixed cultures used to resuscitate could be composed of injured cells, VBNC and dead cells since only the presence of culturable cells was excluded. This initial suspension was diluted in flasks of resuscitation media supplemented with antibiotics, that could be bactericidal (Garrod, 1948; Zeiler, 1985), and aliquots were used to evaluate culturability in rich media. Simultaneously, the initial suspension was diluted in most probable number tubes with the resuscitation media. Accordingly, to the authors' interpretation, resuscitation could only be stated if growth was observed in resuscitation tubes and also from samples from flasks with antibiotics. In our opinion, resuscitation could be stated, in the absence of antibiotics, every time that the most probable number of cells to originate the visible growth is above the one determined for culturable and injured cells.

The use of benzylpenicillin to kill all culturable cells in a mixed suspension, and in this way exclude regrowth, was reported by Lleò et al. (1998). We do not support the use of antibiotics in resuscitation experiments since if one wants to study a process of which almost nothing is known from the molecular point of view, inhibit DNA metabolism, protein synthesis or cell wall metabolism could lead to false

negative results. For example, it was actually shown already that protein synthesis happens in VBNC cells (Heim et al., 2002; Lai et al., 2009; Rahman et al., 1994).

Resuscitation window

Another important aspect to have in mind while pursuing resuscitation is to perform the resuscitation experiments inside the resuscitation window, that is defined as the period of time in which VBNC cells can resuscitate in response to the stimulus under study. This period was described for *Micrococcus luteus* resuscitation by supernatants of growing cells (Mukamolova et al., 1998b), for *V. cholerae* resuscitation by co-culture with eukaryotic cells (Senoh et al., 2010) and for *E. coli* resuscitation by amino acids (Pinto et al., 2011). The length of this window can go between 3 months (Pinto et al., 2011; Senoh et al., 2010) and 10 days (Mukamolova et al., 1998b) and it was suggested to relate with the age of the VBNC cells (Mukamolova et al., 1998b).

In VBNC state induction, the number of the culturable cells is decreasing, while the number of VBNC cells is increasing. This implies that at any given time, VBNC cells that can be found in the cell suspension are not all the same age. If all these cells have resuscitation windows of the same length, the number of cells able to resuscitate will decrease with time (Figure 2), it could explain some lower resuscitation numbers.

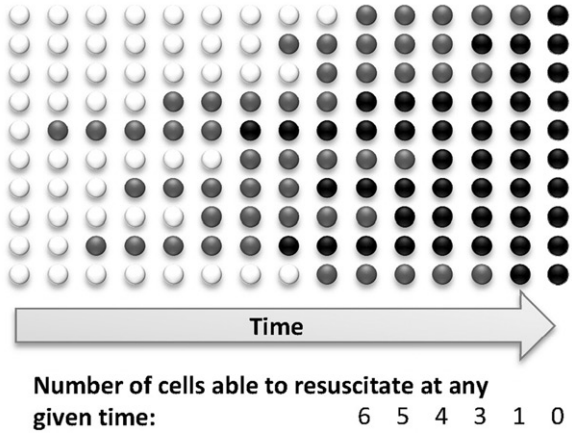

Figure 2. Resuscitation window. At start, a population composed exclusively by culturable cells (white circles) is exposed to a VBNC inducing stimulus and VBNC cells are progressively formed. If each VBNC cell is able to resuscitate (dark gray circles) in response to a specific stimulus for a limited time period (e.g. 5 arbitrary time units) after which they lose the ability to resuscitate (black circles), at any given time, the number of cells able to resuscitate will change. If resuscitation experiments would only be performed after all culturable cells enter VBNC state, the number of cells able to resuscitate could only decrease in number as the time goes by. For simplification purposes, the possibility of formation of injured or dead cells was overlooked.

Resuscitation reports

In the same way, as the species distribution of VBNC state reports also resuscitation was described in a few species and by different stimuli (Table 2). Nevertheless, resuscitation was not obtained for *Sinorhizobium meliloti* VBNC cells induced in soil (Basaglia et al., 2007), *L. monocytogenes* VBNC cells induced in parsley leaves (Dreux et al., 2007), and for *E. coli* VBNC cells induced at room temperature (Arana et al., 2007), but this number of unsuccessful resuscitation attempts might be underestimated since this type of reports are not commonly published. It is curious to notice that the taxonomical distribution of VBNC state reports (68 species of 34 genera) is much wider than that of resuscitation (10 species of 8 genera) what could reflect how challenging resuscitate VBNC cells can be, mostly due to our lack of knowledge on the process.

Resuscitation of VBNC cells: models in Gram-negatives

E. coli has been for many years considered the model organism for the study of molecular biology in Gram-negative bacteria. As far as the VBNC state is considered, Gram-negative bacteria most studied are *Vibrio* spp. and *E. coli*. However, from a molecular point of view, it is about the last that much is known not only because the VBNC state of *E. coli* was among the ones that were firstly described (Xu et al., 1982) but also because changes in cell wall composition (Signoretto et al., 2002), membrane (Asakura et al., 2008; Muela et al., 2008), gene expression (Muela et al., 2008; Pommepuy et al., 1996) and even the influence of RpoS in VBNC state induction (Boaretti et al., 2003) were already studied. Despite one report on the inability to resuscitate *E. coli* (Arana et al., 2007), resuscitation was already demonstrated to be mediated by the heat-stable autoinducer (Reissbrodt et al., 2000), rich culture media (Ozkanca et al., 2009), temperature upshift (Pinto et al., 2011), supernatants of growing cells (Pinto et al., 2011) or amino acids (Pinto et al., 2011).

Resuscitation mediated by supernatants

In 1999, Freestone et al. reported that Gram-negative bacteria were able to produce, in response to the hormone norepinephrine, a compound able to stimulate growth of bacteria by reducing the apparent lag phase. Two years later, Weichart & Kell (2001) published the biochemical characterization of this compound. They determined that it was dialyzable, heat stable, acid- and alkali-stable as well as protease-resistant and suggested it to be a small, nonproteinaceous, non-ionic molecule. Even though this heat-stable autoinducer has been first described to be produced in response to norepinephrine, it was also identified in Luria Bertani medium, tryptone soy broth and even M9 minimal medium (Freestone et al., 2001). Reissbrodt et al. (2000) were the first to report VBNC state resuscitation mediated by this compound, while resuscitation mediated by supernatants produced in tryptone soy broth was reporter 9 years later (Pinto et al., 2011).

The scout hypothesis was proposed in 2009 by Epstein. The author proposed that microorganisms rather than awake from nongrowing states in response to environmental stimulus may do it randomly. If by exposure to environmental unfavorable conditions, cells became nonculturable; from time to time (randomly), a cell spontaneously became culturable again. If the environmental conditions are still unfavorable at that time, the scout would die; if environmental conditions are favorable, then the scout starts to divide. Adding this hypothesis to what is known about the

Table 2. Reports of VBNC cells' resuscitation.

Resuscitation stimuli	Species in which it was observed
Amino acids	*E. coli* (Pinto et al., 2011)
Gas mixture	*C. jejuni* (Bovill & Mackey, 1997)
Heat shock in rich culture media	*S. enterica* (Gupte et al., 2003)
Heat-stable autoinducer	*E. coli* (Reissbrodt et al., 2000)
	S. enterica (Reissbrodt et al., 2000)
In amoeba	*Legionella pneumophila* (Steinert et al., 1997)
In animal models	*C. jejuni* (Baffone et al., 2006; Cappelier et al., 1999)
	V. vulnificus (Oliver & Bockian, 1995)
Resuscitation promoting factor	*S. enterica* (Panutdaporn et al., 2006)
Rich culture media	*Arcobacter butzleri* (Fera et al., 2008)
	Ent. faecalis (Lleò et al., 1998, 2001)
	Ent. hirae (Lleò et al., 2001)
	E. coli (Ozkanca et al., 2009; Pinto et al., 2011)
Supernatants of growing cells	*E. coli* (Pinto et al., 2011)
Temperature upshift	*A. hydrophila* (Maalej et al., 2004)
	E. coli (Pinto et al., 2011)
	V. parahaemolyticus (Wong et al., 2004)
	V. vulnificus (Oliver et al., 1995)

supernatants' active compound, the following model can be proposed: an initial population of VBNC cells exists, and from time to time a scout resuscitates. If environmental conditions are not permissive for growth, the scout would die (Figure 3A); if, on the contrary, conditions are already favorable, the scout starts to grow (Figure 3B). While growing, it produces and secretes the heat-stable autoinducer (stimulated or not by norepinephrine) that would accumulate in the extracellular media and enter other bacteria through a TonB-dependent receptor (Freestone et al., 2001) triggering resuscitation of the remaining cells in the population.

Resuscitation mediated by amino acids

As stated before, sporulation and VBNC state share some common features, and therefore it can be reasonable to hypothesize the existence of some common features between spore germination and VBNC state resuscitation, mainly since VBNC state resuscitation mediated by amino acids was already reported in *E. coli* (Pinto et al., 2011).

Germination receptors detect small molecules that trigger the emergence of spores from dormancy. In *Bacillus subtilis* 15 germination proteins were described which group in three to compose a specific receptor: GerA receptor (composed by GerAA, GerAB and GerAC) triggers spore germination in response to L-alanine; GerB receptor (composed by GerBA, GerBB and GerBC) triggers germination in response to a combination of L-asparagine, glucose, fructose and potassium ions; GerK receptor (composed by GerKA, GerKB and GerKC) is involved in germination triggered by L-alanine also with glucose, fructose and potassium ions; two other germination receptor operons were identified (*ykfQRT* and *yndDEF*), but their germinants are yet unknown (Ross & Abel-Santos, 2010). After germination receptors' activation ions are released from the spore, lytic enzymes are activated and catalyze the spore cortex hydrolysis. Interestingly, there is some degree of similarity between *B. subtilis* GerKB and *E. coli* L-proline/proton and L-serine/proton symporters (ProY and SdaC, respectively).

Given this, a model in which amino acid-mediated resuscitation is independent on the scout hypothesis may be proposed (Figure 3C). An initial population of VBNC state cells enters in contact with amino acids, which will bind cell surface receptors that can be similar to those involved in germination of spores. Two scenarios can be idealized: either these proteins act exclusively as receptors able to trigger resuscitation without the need for the amino acid to enter the cell; or they would act as transporters that would mediate the entry of the amino acid into the cell where it would bind other receptor and trigger resuscitation.

It seems reasonable to think that despite the difference in the stimuli leading to resuscitation of *E. coli* VBNC, supernatants or amino acids, they would just represent two branches of the same final pathway. However, despite the existence of experimental results that support some of the hypothesis described here, much work is needed to elucidate the molecular mechanism underlying *E. coli* resuscitation.

Resuscitation of VBNC cells: resuscitation promoting factors in action

In 1998a Mukamolova et al. reported the identification and characterization of a small protein of *Mic. luteus* that, in picomolar concentrations, promoted resuscitation of dormant cells. Besides this activity, the resuscitation-promoting factor (Rpf) was also able to stimulate growth (Mukamolova et al., 1998a, 1999) through lag-phase reduction (Mukamolova et al., 1999). It was proposed that Rpf proteins could act as signaling molecules (cytokines) that when secreted to the medium by growing cells would bind surface receptors of dormant cells and trigger resuscitation (Figure 4A).

Unlike *Mic. luteus, M. tuberculosis* possesses five Rpf genes (A to E) (Mukamolova et al., 2002). Growth, survival and resuscitation *in vitro* (Downing et al., 2005; Tufariello et al., 2004) as well as virulence *in vivo* (Biketov et al., 2007; Downing et al., 2005; Kana et al., 2008) was evaluated in multiple mutants of this microorganism. Results suggested that Rpfs are partially, but not totally, redundant with a hierarchy in function or potency. Another set of evidences against functional redundancy came from expression analysis of *rpf* genes of *M. tuberculosis* (Gupta et al., 2010). *rpfC* was shown to be consistently expressed in all growth phases and

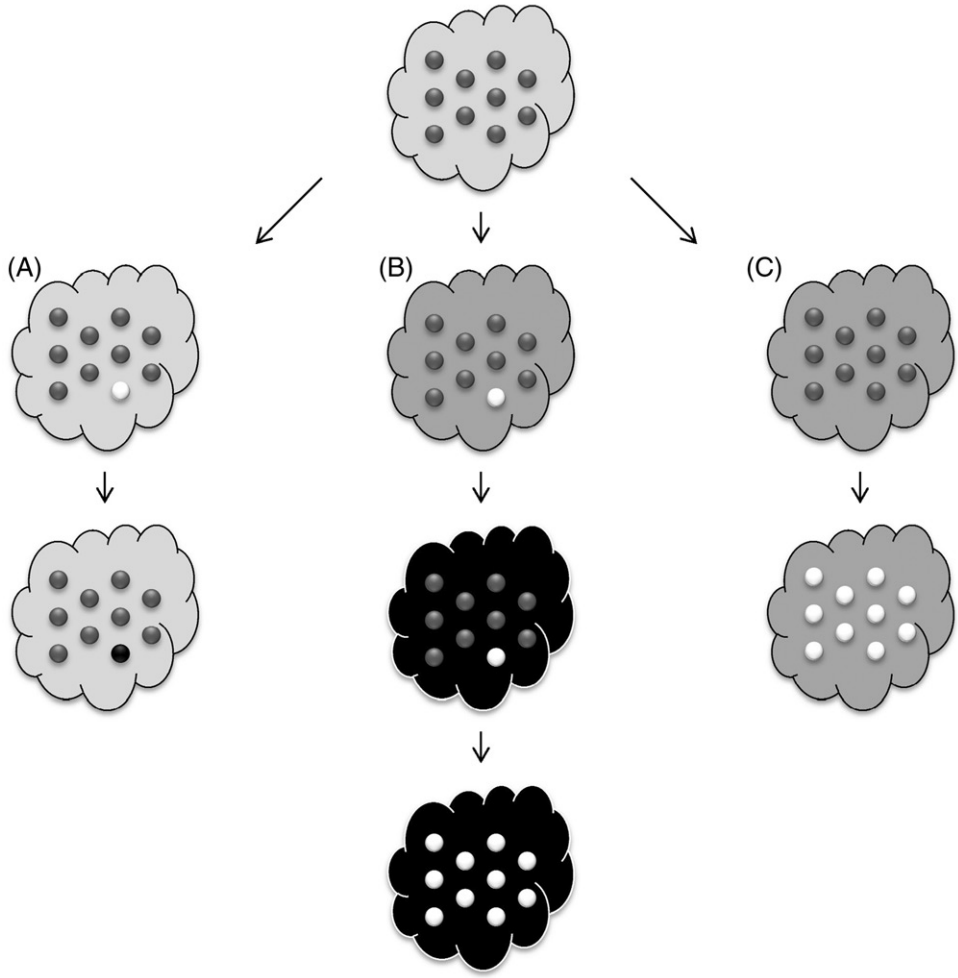

Figure 3. Resuscitation models. (A and B) In a population composed by VBNC cells (dark gray circles) from time to time, a cell becomes culturable (white circles) and is said to be a scout. (A) If the environmental conditions are not yet favorable for growth (light gray cloud), e.g. VBNC state-inducing conditions are still present, the scout dies (black circle). (B) If the environmental conditions are favorable for growth (dark gray cloud), e.g. nutrients are present or temperature approached optimal, the scouts starts growing, producing and secreting signaling molecules (black cloud), e.g. heat-stable autoinducer or resuscitation promoting factors, that are sensed by the other cells triggering resuscitation. (C) An alternative model, independent of scout's existence, hypothesizes that VBNC cells are exposed to the resuscitation stimulus, e.g. amino acids or other nutrients, which directly trigger resuscitation.

physiological stresses tested, *rpfA* and *rpfD* to have maximum expression ratios in early resuscitation and *rpfD* and *rpfE* to have higher expressions under stress. Expression of *rpf* genes in human tissues infected with *M. tuberculosis* was also detected (Davies et al., 2008).

The first insights on the molecular mechanism of Rpfs were obtained through bioinformatics identification of a transglycosylase-like domain with a lysozyme-like fold (Cohen-Gonsaud et al., 2004). In agreement with this, *Mic. luteus* Rpf was shown to lyse crude preparations of cell wall as well as degrade a muralytic enzyme synthetic substrate in addition to a weak proteolytic activity (Telkov et al., 2006). Mutation of the putative catalytic glutamate reduced lytic activity as well as resuscitation ability suggesting that the lytic activity is responsible for resuscitation promoting activity (Telkov et al., 2006).

RpfB was shown to interact with a putative endopeptidase designated Rpf-interacting protein A (RipA), which is secreted but remains cell-associated and acts like a peptidoglycan hydrolase (Hett et al., 2007). This interaction and their co-localization at the septum (Hett et al., 2007) suggest that these proteins might be involved in the final stages of cell division, e.g. the separation of the daughter cells.

These observations are conflicting with the proposed role of Rpf as a cytokine (Figure 4A): would a protein intended to be a cytokine have lytic activity? On the light of the new set of available data, a second model in which Rpfs would degrade or remodel the dormant cells' characteristic peptidoglycan (Figure 4B) was proposed. Supporting this hypothesis is also the characterization of changes in the peptidoglycan chemistry in VBNC state – more resistant, increased cross-link and O-acetylation (Signoretto et al., 2000, 2002); maybe the cleavage of a specific cell state peptidoglycan needs specific enzymes with those particular specificities.

Studies on *Mic. luteus* Rpfs of environmental isolates showed that some are released to the growth media, while others, with longer linkers between the LysM domain and the lysozyme-like domain, are bound to the cell wall (Koltunov et al., 2010). Moreover, it was observed that peptidoglycan fragments originated either from Rpf-mediated cleavage or from ultrasonication, had a stimulatory effect on resuscitation (Nikitushkin et al., 2012).

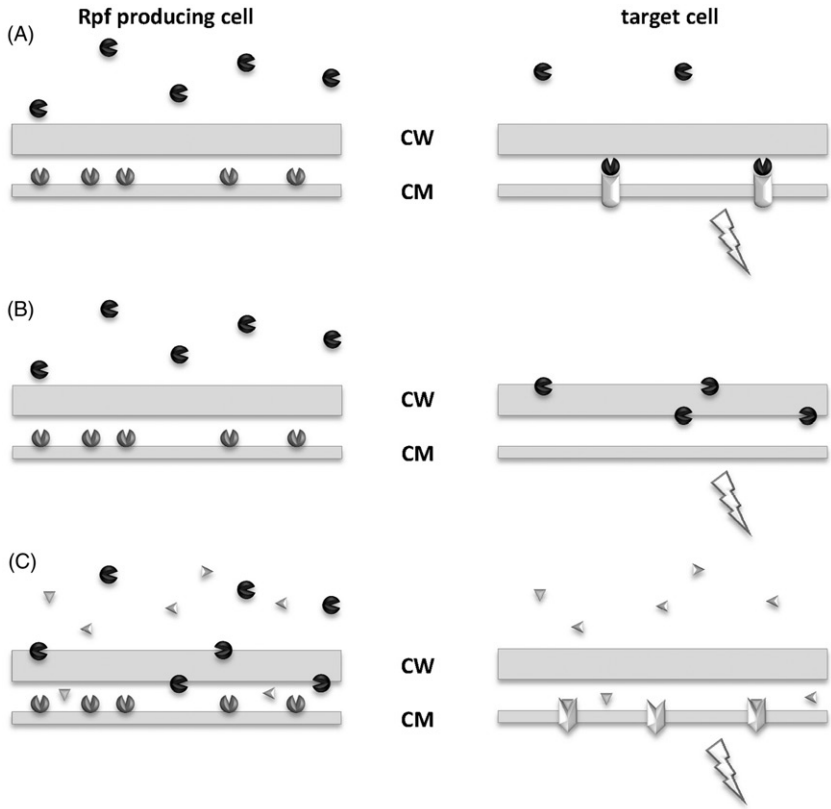

Figure 4. Models for Rpf-mediated resuscitation. Rpf proteins may be secreted by the producing cell (black pies) or remain bound to the producer cell's surface (dark gray pies). To date, three models for its resuscitation promoting activity were proposed. (A) Rpf proteins may act as cytokines; after its secretion by the producing cell they bind to specific receptors (light gray rectangles) on the target cell's surface, triggering resuscitation (white lightning bold). (B) Rpf proteins may act on the cell wall (CW) of the target cell, leading to its remodeling and triggering resuscitation (white lightning bold). (C) Rpf proteins may act on the producing cell's cell wall, partially cleaving it and releasing small peptidoglycan fragments (light gray triangles) that would bind PknB-like receptors (light gray chevrons) on the target cell's surface, triggering resuscitation (white lightning bolt). CM refers to the cytoplasmic membrane.

This new set of observations is not in agreement with either of the models proposed so far. If the Rpf would act as a cytokine (Figure 4A), then the ultimate active molecule would be the protein. Therefore, why are peptidoglycan fragments active in stimulation of resuscitation? Furthermore, if to act, the protein needs to bind receptors of other cells, then how to look to the cell bound Rpfs described so far? If, on the other hand, it is the Rpf-mediated peptidoglycan remodeling of dormant cells that triggers resuscitation (Figure 4B), how to include in the model that Rpfs are known to be active *in vitro* in the absence of RipA, that dormant cells were never reported to be arrested in the final stages of cell division or how to explain why are muropeptides active (Nikitushkin et al., 2012)?

A new model was proposed that states that the action of Rpf proteins on the peptidoglycan releases low molecular weight muropeptides that once in the extracellular media could be sensed by other cells through serine/threonine-protein kinase PknB. PknB-like receptors that would then trigger resuscitation (Figure 4C). This model integrates the lytic activity proposed for Rpfs (Telkov et al., 2006), and the resuscitation-promoting activity of the Rpf generated muropeptides (Nikitushkin et al., 2012). Moreover, it does not conflict with the existence of cell-bound Rpfs (Koltunov et al., 2010), since these would degrade the producing cells' peptidoglycan leading to the release of the muropeptides, or with its interaction with RipA (Hett et al., 2007).

This last model relies in the assumption that the muropeptides produced through Rpf peptidoglycan degradation will be sensed by the dormant cells (Figure 4C). It was suggested that PknB, an eukaryotic-like Ser/Thr membrane kinase, may respond to those muropeptides (Shah et al., 2008). In *M. tuberculosis*, PknB has an extracellular domain capable of binding specific peptidoglycan fragments (Mir et al., 2011). A moderate effect of resuscitation stimulation of a peptide with high affinity to PknB (Mir et al., 2011) was observed. Despite this indirect evidence of the involvement of PknB on resuscitation, its essential nature for growth of *M. tuberculosis* (Fernandez et al., 2006) turns challenging its definitive confirmation. Nonetheless, studies on its homolog, *B. subtilis* PrkC, proved it acts as a germination receptor able to bind peptidoglycan fragments (Shah et al., 2008). The proposed mechanism of action states that PkrC indicates, in this way, the presence of growing bacteria and thus serve as a signal for dormant cells that growth-enabling conditions exists (Shah et al., 2008).

L. monocytogenes' homologs

We propose *L. monocytogenes* as a good model organism to study resuscitation in Gram-positive bacteria. Despite the ability of *L. monocytogenes* to enter VBNC state been known since 2002 (Besnard et al.), to our knowledge, resuscitation from VBNC state was never reported. Then, why do we believe it to be a good model to study resuscitation? First of all, because in 2005, Ravagnani et al. suggested that Rpfs homologs might be present on the genome of several firmicutes. Not only was *L. monocytogenes* among those firmicutes as is one of those with more available molecular tools.

Concerning the other proteins involved in the actinobacterial Rpf action models, when RipA was described in *M. tuberculosis*, the similarity between its catalytic domain and that of p60 protein, a cell wall endopeptidase of

L. monocytogenes, was noted (Hett et al., 2007). To our knowledge, no study done so far evaluated the ability of Lmo0186 or Lmo2522 to interact with p60. It may be interesting to evaluate the role of p60 in resuscitation even though RipA was never definitely implicated on resuscitation. Moreover, Ser/Thr kinases are widespread in bacteria (Pereira et al., 2011), and in *L. monocytogenes*, two Ser/Thr kinases were identified: Lmo1820 and Lmo0618. The first one, designated PrkA, shows high homology with the catalytic domains of other well-studied Ser/Thr kinases such as *B. subtilis* PrkC (68% identity) and *M. tuberculosis* PknB (46% identity), involved in germination or resuscitation, respectively (Lima et al., 2011). In addition, *L. monocytogenes* PrkA and *M. tuberculosis* PknB share the structural organization (Lima et al., 2011). Given this observations, the role of PrkA in *L. monocytogenes* resuscitation should be investigated.

Conclusion

The VBNC state is recognized for 30 years; but for most of this time, the existence of VBNC cells was controversial. It altered the mode how bacterial cell viability is perceived and prompted the development of diverse techniques to assess life essential traits of bacteria but, to date, no gold standard for viability confirmation exists.

Regardless of three decades of reports of VBNC bacteria, understanding of the way VBNC state induction and resuscitation occur is extremely limited. This can express the delayed acknowledgment of the VBNC state as a stress adaptive strategy as well as the improper judgment of injured cells as VBNC cells. Along the years, numerous authors recognized this problematic and become cautious regarding the confidence of VBNC cells' identification, but unfortunately this was not the case for all authors. We recommend that this issue should be accounted for at all times, and each author's clarification of criteria used for VBNC cells' identification be instigated by journal reviewers.

The acknowledgement of the existence of VBNC cells will be responsible for a change in the manner how bacteria are detected in food and clinical settings since conventional methods do not detect them causing large underestimation of bacterial load. Comprehensive knowledge of how bacterial cells enter and leave VBNC state will ultimately allow an enormous improve of microorganisms' detection.

In spite of a number of evidences and proposed models thus far, not much is in fact known about the molecular mechanisms underlying VBNC state induction and resuscitation. Combined and focalized efforts should be made to understand these processes namely agreement over model organisms to study these processes. Advances in this topic will have crucial importance in all fields of microbiology.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article. (Authors are grateful to Fundação para a Ciência e a Tecnologia (Portugal) for financial support through Doctoral Fellowship SFRH/BD/46111/2008 and Project Grant PTDC/AGRALI/098020/2008.)

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