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REVIEW

Exploring the potential environmental functions of viable but non-culturable bacteria

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Abstract A conventional plate count is the most commonly employed method to estimate the number of living bacteria in environmental samples. In fact, judging the level of viable culture by plate count is limited, because it is often several orders of magnitude less than the number of living bacteria actually present. Most of the bacteria are in "viable but non-culturable" (VBNC) state, whose cells are intact and alive and can resuscitate when surrounding conditions are more favorable. The most exciting recent development in resuscitating VBNC bacteria is a bacterial cytokine, namely, the resuscitation-promoting factor (Rpf), secreted by *Micrococcus luteus,* which promotes the resuscitation and growth of high G+C Gram-positive organisms, including some species of the genus *Mycobacterium*. However, most of studies deal with VBNC bacteria only from the point of view of medicine and epidemiology. It is therefore of great significance to research whether these VBNC state bacteria also possess some useful environmental capabilities, such as degradation, flocculation, etc. Further studies are needed to elucidate the possible environmental role of the VBNC bacteria, rather than only considering their role as potential pathogens from the point view of epidemiology and public health. We have studied the resuscitation of these VBNC bacteria in polluted environments by adding culture supernatant containing Rpf from *M. luteus*, and it was found that, as a huge microbial resource, VBNC bacteria could provide important answers to dealing with existing problems of environmental pollution. This mini-review will provide new insight for considering the potentially environmental functions of VBNC bacteria.

Keywords VBNC · Rpf · Environmental functions · Microbial resources

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Introduction

Bacteria in the natural environment must be able to cope with a variety of stresses and adapt their genotypes and phenotypes to survive unfavorable oligotrophic conditions (Bakken and Olsen 1987; Ganzert et al. 2011). It is very common for bacteria to survive under extreme conditions by passing into a sporulating or non-sporulating dormancy, which means a reversible state of low to zero metabolic activity (Kaprelyants et al. 1993; Kaprelyants and Kell 1993; Chmielewski and Frank 1995). Since the original paper from the laboratory of Colwell in 1982 (Xu et al. 1982), over 400 papers have appeared describing various aspects of the phenomenon most commonly referred to as the "viable but non-culturable" (VBNC) state, in which bacterial cells are alive and capable of renewed metabolic activity but fail to grow on routinely employed bacteria media (Oliver 1995, 2005; Serpaggi et al. 2012). Despite their typically low levels of metabolic activity, VBNC state bacteria can become culturable upon resuscitation. Resuscitation promoting factor (Rpf) was discovered by Kaprelyants and co-workers studying non-sporulating gram-positive coccus *Micrococcus luteus* during prolonged

incubation in spent growth medium over a lengthy stationary phase (Kaprelyants and Kell 1993; Mukamolova et al. 1998). As a bacterial cytokine, Rpf can stimulate a number of gram-positive bacteria, including *Mycobacterium*, *Rhodococcus*, *Arthrobacter*, *Leifsonia, Bacillus*, *Nocardia*, *Kitasatospora* and *Streptomyces* (Biketov et al. 2000; Shleeva et al. 2002; Ding 2004; Nikitushkin et al. 2011). Ding and Yokota (2010) discovered that Rpf also stimulates the growth of several other Gram-negative organisms, such as *Curvibacter fontanus* sp. According to relevant databases, homologous genes are widely distributed throughout gram-positive bacteria such as *Mycobacterium, Streptomycetes* and *Corynebacteria* (Downing et al. 2004; Hartmann et al. 2004; Riano et al. 2012). Panutdaporn et al. (2006) reported that *Salmonella typhimurium* LT2 also has an rpf-like gene which shared 24.2 % sequence similarity with the *M. luteus* Rpf.

Although a large amount of work has been done characterizing the formation and resuscitation of the VBNC state in pathogenic bacteria (Signoretto and Canepari 2008; Oliver 2010), at present there is hardly any information concerning the environmental functions underlying the VBNC state. It is worth pointing out that VBNC state bacteria can be of great significance in environmental rehabilitation, since VBNC bacteria might represent a potent microbial resource. Here, combined with our researches (Ding 2004; Ding et al. 2007, 2009, 2011, 2012; Ding and Yokota 2010; Su et al. 2011a, b, 2012), more recent findings on the resuscitation and application of VBNC state bacteria are summarized in Fig. 1. The primary aims of this short review are critical to reconsidering the potential environmental function of VBNC state bacteria, and to developing a novel efficient method for excavating and obtaining highly desirable pollutant-degrading microorganisms.

Formation of VBNC bacteria

The existence of a VBNC state has long been recognized. We found that *M. luteus* had only slight changes in total cell count after 1 year of storage, while the viable count decreased by more than five orders of magnitude from the initial values, meanwhile, a rapid decrease in the mean cell diameter (from 1.4 to 0.3 μm) was observed. Using the fluorescent dye rhodamine 123, we also observed several morphological and physiological changes (Ding 2004; Ding et al. 2011). Indeed, bacteria in the VBNC state can undergo dramatic variations in their morphology and physiology. A common phenomenon is their inability to form colonies on routine bacteriological media, even though the cells are alive and capable of renewed metabolic activity (Oliver 2005, 2010).

A variety of chemical and environmental factors, including oligotrophic conditions (Lothigius et al. 2010; Rezaeinejad and Ivanov 2011; Bukh et al. 2012), extreme temperature (González and Hänninen 2012; Trevors et al. 2012), oxidative stress (Atack and Kelly 2009; Reuter et al. 2010), low salinity stress (Pinto et al. 2011), UV light shock (Hamblin et al. 2005; Chen et al. 2012), osmotic pressure (Asakura et al. 2008; Chong et al. 2008), organic pollutants and heavy metals (Divol et al. 2012; Luna et al. 2012; Zhang et al. 2012) have been reported to induce formation of the VBNC state. More than 60 species to date (the number increases all the time) have been reported to enter VBNC state (Oliver 2005), including a number of food-borne human pathogens posing potential health risks (Dinu and Bach 2011; Dwivedi and Jaykus 2011; Siegumfeldt and Arneborg 2011). Although the formation of the VBNC state has been well investigated, little is known about the precise nature and genetic pathways underlying this state. Furthermore, a large number of papers have shown that VBNC state cells retain their potential for virulence and can recover culturability, but only a few researchers have focused on the potential safety hazards of VBNC state in food and agricultural systems (Artz et al. 2006; Dinu and Bach 2011). Above all, as the vast majority of unknown microorganisms, VBNC state bacteria need to be studied in order to identify new microbial resources and microbial environmental functions.

Resuscitation of VBNC bacteria

Since the original paper from Kaprelyants (Kaprelyants et al. 1993; Kaprelyants and Kell 1993) in 1993, the role of a group of extracellular bacterial proteins has been concerned with resuscitation. A bacterial cytokine was isolated, named as the resuscitation-promoting factor (Rpf), secreted by *M. luteus,* which promotes the resuscitation and growth of VBNC state organisms when added in picomolar concentrations to minimal media (Mukamolova et al. 1998). The resuscitation of pathogenic bacteria, such as *Mycobacterium tuberculosis, Campylobacter* spp., *Escherichia coli, Pseudomonas aeruginosa*, *Helicobacter pylori*, *Legionella pneumophlia* and numerous pathogenic *Vibrio* spp. from the VBNC state has been widely studied (Oliver 1995, 2005, 2010; Biketov et al. 2000; Downing et al. 2004; Panutdaporn et al. 2006; Hett et al. 2007; Asakura et al. 2008; Signoretto and Canepari 2008; Nikitushkin et al. 2011; Rezaeinejad and Ivanov 2011; Bukh et al. 2012; González and Hänninen 2012; Riano et al. 2012). However, there are no researches concerning the Rpf function for the VBNC bacteria from the point of view of microbial resources and microbial environmental functions.

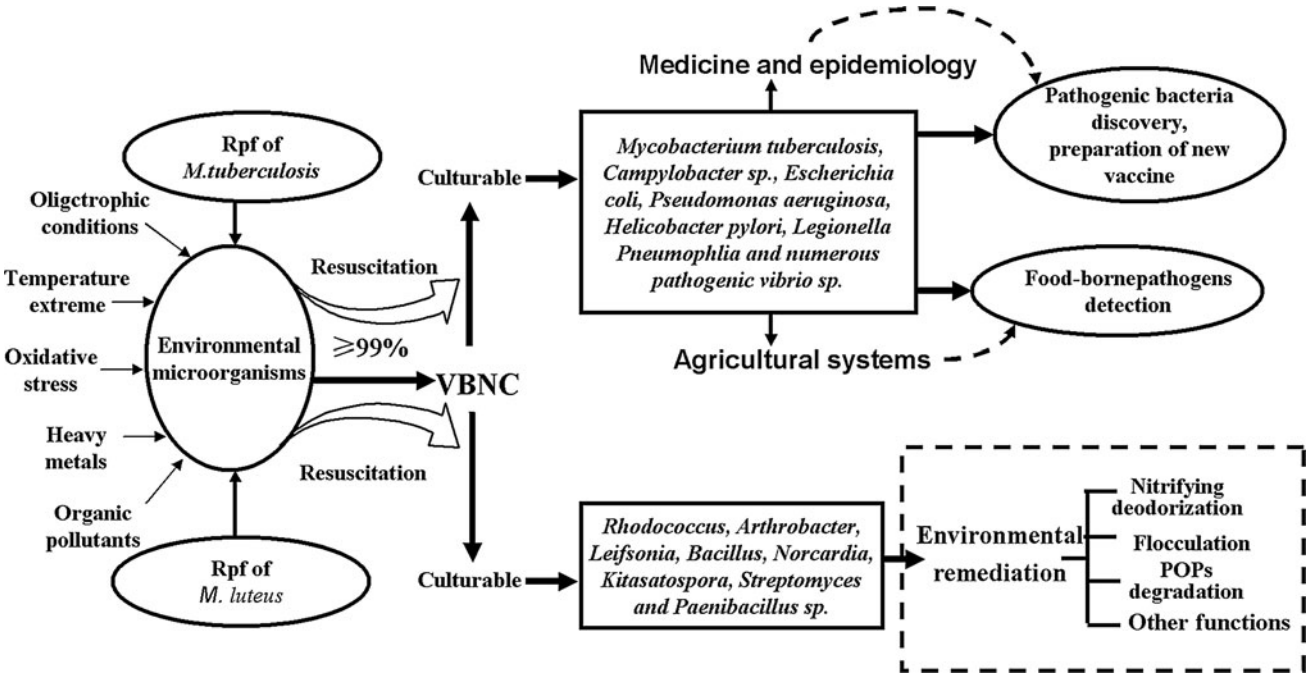

Fig. 1 Schematic summary of the resuscitation and application of VBNC state bacteria

We have obtained the Rpf protein encoded by the rpf gene from *M. luteus*. The gene was isolated, sequenced, and expressed in *E. coli* (Ding 2004; Ding et al. 2012). The effect of this Rpf on VBNC state bacteria was evaluated by the method of most probable number (MPN) and denaturing gradient gel electrophoresis (DGGE). The results suggested that Rpf increased the viable cell count of VBNC state *M. luteus* cultures at least 100-fold and stimulated their growth. Meanwhile, we found 28 kinds of soil out of 68 studied samples that had an obvious response to Rpf, and 40 VBNC state strains belonging to *Rhodococcus*, *Arthrobacter*, *Leifsonia*, *Bacillus*, *Nocardia*, *Kitasatospora*, *Streptomyces* and *Paenibacillus* species were isolated (Table 1). It is worth noting that Rpf can resuscitate not only the high G+C Gram-positive organisms, but also the low G+C Gram-positive organisms such as *Bacillus* and *Paenibacillus* (Ding 2004; Ding et al. 2007, 2009, 2011, 2012; Ding and Yokota 2010). Furthermore, Rpf also stimulates the growth of several other Gram-negative organisms, such as *Curvibacter fontana* sp. (Ding and Yokota 2010). In a word, Rpf enables culturing difficult-to-culture bacteria and is highly worthy of application to phylogenetic analysis for the separation of accidentally cultured bacteria.

Genes similar to Rpf are widespread among high G+C Gram-positive bacteria, such as *Mycobacterium smegmatis* (four genes) and *M. tuberculosis* (five genes) (Downing et al. 2004; Hartmann et al. 2004; Nikitushkin et al. 2011; Riano et al. 2012). Thus, Rpf-homologous proteins have the same stimulatory effect on bacterial resuscitation and these Rpf-like compounds have been reported in several genera (Hett et al. 2007; Nikitushkin et al. 2011). Some studies indicated that the mechanism by which Rpf resuscitate VBNC bacteria is the Rpfs are peptidoglycan hydrolases, which are involved in the complex process of cell wall digestion in order to allow cell division to occur (Bakken and Olsen 1987; Hett et al. 2007, 2008; Hett and Rubin 2008; Kana and Mizrahi 2009; Wyckoff et al. 2012). Studies by Reissbrodt et al. (2002) indicated that Rpf was a heat-stable "autoinducer of growth" that was secreted by a variety of gram-positive and gram-negative bacterial species when incubated in media with the human catecholamine hormone norepinephrine (Sperandio et al. 2003; Freestone et al. 2006; Senoh et al. 2012). Mukamolova et al. (2005) demonstrated that Rpf possessed muralytic activity that was probably responsible for its observed action. If correct, these reports would have major implications for the resuscitation of enteropathogens from the VBNC state. However, the mechanism of Rpf activity, both in resuscitating VBNC bacteria and in stimulating growth when bacteria are in a state of low activity under adverse environmental conditions, remains to be uncovered. Further studies are needed to elucidate the mechanism of Rpf resuscitation of VBNC bacteria.

Exploring the potential environmental function of VBNC bacteria

Most bacteria in nature are in the VBNC state, little work has been done to explore the potential environmental

Table 1 Summary of isolated strains of VBNC state bacteria after adding Rpf from *M. luteus* (Ding 2004)

Allied genera	Isolated strains	Resuscitation ratio (fold)	16S rDNA sequence similarity (%)
Rhodococcus	11	7–490	96–100
Arthrobacter	1	100	99
Streptomyces	6	5–10	98–100
Leifsonia	2	49–73	97–98
Nocardia	1	14	97
Kitasatospora	4	5–14	97–98
Paenibacillus	3	7–10	98–99
Bacillus	12	5–490	98–100

function of VBNC bacteria. In our previous studies, several bacteria in the VBNC state were isolated from soil and sewage treatment systems by adding culture supernatant from *M. luteus* containing the Rpf protein to culture media (Ding 2004; Ding et al. 2012). Adopting Kaolin suspension as the active evaluation system, four strains (M3, M7, M8 and M11) with high flocculating activity were screened out (Ding et al. 2011; Su et al. 2011b;), and belonged to *Arthrobacter*, *Chryseobacterium* and *Rhodococcus*. The flocculating efficiency in Kaolin suspension was 82.24, 78.29, 72.54 and 66.83 %, respectively. According to the principle of effective biotechnology (EM), multiple colonies of M3 (*Arthrobacter*) and M7 (*Chryseobacterium*) with higher flocculation efficiency was selected, and the flocculation efficiency reached 87.13 %. Under optimal conditions of medium composition, culture conditions and flocculating activity, the flocculation efficiency could reach as high as 98.23 % (Su et al. 2011b). Qualitative and quantitative analysis showed that the main components of this bioflocculant, namely, MAC37 were polysaccharide substances containing a small amount of protein. The application of the MAC37 bioflocculants in adhesive wastewater treatment showed that the removal rate of turbidity, color and COD_{cr} from the adhesive wastewater were 92.57, 94.73 and 92.12 %, respectively (Su et al. 2012). In addition, we used *M. luteus* culture supernatant to isolate VBNC bacteria from a micro-aerobic printing and dyeing wastewater treatment system. Based on physiological and biochemical characteristics, and a BLAST search on the basis of 16S rDNA sequences, the isolated VBNC bacteria belonged to the genera *Cupriavidus* and *Gordonia.* Many environmental functions, such as the preferential degradation of aromatics (Hassanshahian et al. 2011; Bacosa et al. 2012), promotion of metal pollution bioremediation (Biondo et al. 2012; Hajdu and Slaveykova 2012), and biodegradation of di-n-butyl phthalate (Jin et al. 2012) have been reported about these two genera. Therefore, we will select additional environmental samples and study their response to Rpf, intending to identify more VBNC bacteria with desirable environmental functions.

It is common knowledge that artificial mixed cultures consisting of purified cultivable isolates from enrichment cultures are less efficient in polychlorinated biphenyl (PCB) and biphenyl degradation than mixed-culture (Abraham et al. 2002). The reason would be that there are abundant of VBNC bacteria in mixed-culture that have potential capability to degrade PCBs and biphenyl. Furthermore, DeBruyn et al. (2009) found that *Mycobacterium* populations have potential biodegradation in nature attenuation of high molecular weight polycyclic aromatic hydrocarbons (PAHs).

Most recently, we used *M. luteus* culture supernatant to explore the potential biphenyl-degrading capability of the VBNC microbial community. It was indicated that the enrichment culture produced by the addition of Rpf enhanced the efficiency of degradation of biphenyl, cell growth (OD_{600}) and microbial community diversity in the mixed-culture significantly. When the concentration of biphenyl was 2,000 mg/L, the biphenyl degradation efficiency of the control group (with inactivated Rpf) and the treatment group (with Rpf) was 17.7 and 81.2 %, respectively. Meanwhile, the OD_{600} increased by 1.91 with added Rpf. Furthermore, a concentration of 1,500 mg/L biphenyl could be almost completely degraded in 24 h using Rpf at a dosage of 15 % (v/v). In addition, based on DGGE patterns of 16S rRNA gene sequences analysis, it was observed that the Shannon–Weaver diversity index (H) increased by 0.35 with added Rpf. And colonies that were unique in the treatment group, with no counterpart in the control group, were insolated. Six strains (GenBank under accession numbers KC577542-KC577547) from species of *Arthrobacter*, *Rhodococcus*, *Chryseobacterium*, *Achromobacter* and *Alcaligenes* were obtained.

As a huge microbial resource, VBNC bacteria can provide important answers to the existing problems of environmental pollution, especially for microbial degradation of persistent organic pollutants (POPs). Therefore, the resuscitation and stimulation function of Rpf will open up a new avenue for exploring the potential of VBNC or uncultured microorganisms for environmental bioremediation.

Conclusion

There is now a large amount of substantial evidence demonstrating that numerous bacteria, both pathogenic and non-pathogenic are capable of entering the VBNC state. Although little is known about the precise nature and genetic pathway underlying the VBNC state, it is clear that Rpf proteins can promote the resuscitation and growth of VBNC state organisms, both gram-positive and negative.

Recovering culturable VBNC state bacteria not only expands the biodiversity cognition, but also gives greater opportunities to excavate and obtain microorganisms with desirable environmental functions. Undeniably, much attention should be paid to the function of VBNC bacteria after resuscitation culture, and further studies are required to change this situation.

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