Low Temperature Induces Infectious Nonresuscitatable VBNC Cells in Ralstonia Pseudosolanacearum

Neha Faridi
DIBER: DRDO Defence Institute of Bio-Energy Research

Merwyn Packia raj Samuel (✉ merwyn@diber.drdo.in)
DIBER: DRDO Defence Institute of Bio-Energy Research

Shalini Bhatt
DIBER: DRDO Defence Institute of Bio-Energy Research

Ankur Agrawal
DIBER: DRDO Defence Institute of Bio-Energy Research

Veena Pande
Kumaun University

Madhu Bala
DIBER: DRDO Defence Institute of Bio-Energy Research

Research Article

Keywords: VBNC, Ralstonia pseudosolanacearum, Phytopathogen, Isopropanol, UV-C stress, Psychrophilic Stress

DOI: https://doi.org/10.21203/rs.3.rs-312710/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

*Ralstonia pseudosolanacearum* and other members of *Ralstonia solanacearum* species complex (RSSC) causes the disease bacterial wilt in many crops of economic importance. The organism is known to form Viable But Non Culturable cells (VBNC). VBNCs resuscitate invitro during the “resuscitation window” period and are infectious Previous studies have identified nonresuscitatable VBNCs in various bacterial genus including RSSC, however their infectivity was not elucidated and described. In this work, VBNCs of two *Ralstonia pseudosolanacearum* strains were generated by exposing the microcosms to psychrophilic stress, UV-C radiation and 70% isopropanol. Both resuscitatable and nonresuscitatable VBNCs were observed in psychrophilic and UV-C stressed microcosms. The nonresuscitatable VBNCs generated at psychrophilic temperature were found infective. Based on resuscitation properties, nonresuscitatable VBNCs can be considered as a different VBNC type from resuscitatable VBNCs.

Introduction

*Ralstonia solanacearum* species complex (RSSC) is a soil, seed and water borne bacterial phytopathogen which causes the disease bacterial wilt across different botanical families (Hayward 1994). Based on genomic differences, RSSC has been reclassified into three different species: *Ralstonia solanacearum*, *Ralstonia pseudosolanacearum* and *Ralstonia syzygii* (Safni et al. 2014). *Ralstonia pseudosolanacearum* (*R. pseudosolanace arum*) infects the host through root epidermis and colonizes xylem, resulting in wilting and subsequent plant death. In the environment, the bacterium survives in reservoir plants, soil and water as metabolically dormant Persister cells and Viable But Non Culturable cells (VBNC) (Alvarez et al. 2010). Persisters and VBNCs share molecular characteristics, regulated by different molecular switches (Bamford et al. 2017). VBNCs require special agents like catalase for invitro resuscitation, while persisters can be resuscitated simply by reversing the induced stress; hence VBNCs are considered to be in deeper state of dormancy than persisters (Ducret et al. 2014; Ayrapetyan et al. 2015; Ayrapetyan et al. 2018).

Many bacterial species are known to enter VBNC state. Unlike their culturable counterparts, VBNCs possess enhanced tolerance towards various physico-chemical stresses (Weichart and Kjelleberg 1996; Rowe et al. 1998; Wong and Wang 2004; Lleo et al. 2007; Oliver 2010). *R. pseudosolanacearum* and other members of RSSC enter VBNC state under nutrient starvation, psychrophilic stress and heavy metal exposure (van Elsas et al. 2000; van Elsas et al. 2001; van Overbeek et al. 2004; Alvarez et al. 2008; Kong et al. 2014; Stevens et al. 2018). The important characters of RSSC VBNCs are cell dwarning, reduction in nutrient transport, high or low ATP levels, reduced respiration rate and reduced macromolecular synthesis. Genes which are essential for the organism’s survival are only expressed during the VBNC state. The important transcribed genes are *mobA, rfbE, stx1, Omp, Dps* and 16S rDNA. Among them, the transcriptome of 16S rDNA (16S rRNA) is used as a marker for cell viability (Lleo et al. 2000; Lleo et al. 2001; Yaron and Matthews 2002; Kong et al. 2004).
Resuscitation or reverse culturability is the most important phenotypic character used to differentiate culturable cells from VBNCs. Resuscitation happens during the “resuscitation window” period under the influence of external resuscitating agent like catalase. Beyond this period, VBNCs may remain viable, but are considered nonresuscitatable, noninfectious and nonpathogenic (Pinto et al. 2011; Pinto et al. 2015). VBNCs nonresuscitatable with catalase has been observed in members of RSSC incubated in sterile artificial soil (Kong et al. 2014). However, their infectivity is unknown. The existence of such infectious VBNCs will have profound implications on understanding the pathogen's life cycle and its detection. In this work, the formation of stress induced nonresuscitatable VBNCs in *R. Pseudosolanacearum* and their infectivity has been evaluated.

**Materials And Methods**

**Bacterial strains and growth conditions**

Two *R. Pseudosolanacearum* isolates DIBER115 (NCBI Accession number MG266193) and DIBER118 (NCBI Accession number MG266203) isolated from tomato plants were used in this study. They belong to Phylotype-I of RSSC which are taxonomically reclassified as *R. pseudosolanacearum* (Prior and Fegan 2005; Safni et al. 2014). Both the strains were grown at 28°C in casamino acid peptone glucose (CPG) broth for 48 hours and counted in CPG agar (Kelman A. 1954).

**Induction of VBNC state at psychrophilic temperature**

To induce VBNC state, CPG grown microcosms were pelleted, water washed and resuspended in sterile distilled water and aliquoted into batches of 10 ml to prevent contamination during periodical investigations. The microcosms were given psychrophilic stress at 4°C for 480 days. The transformation was monitored periodically by direct viable plate count (DVC) and resuscitation assays. Whereas, the viability status and infectivity were monitored by quantifying the 16S rRNA transcriptomes and inoculation assays respectively (van Overbeek et al. 2004; Lahtinen et al. 2008).

**UV-C radiation and isopropanol treatment**

To induce VBNCs by UV-C and isopropanol, water washed CPG grown cells were resuspended in 10 ml of sterile distilled water or 70% isopropanol. For UV-C treatment, the microcosms were irradiated with different UV-C fluencies using UV strata linker 2400 in “energy” mode (Strata gene, USA). The instrument emits radiation at a wavelength of 254 nm with a total power output of 75 watts. Briefly, 10 ml of microcosms in open petriplates were exposed to 200 mJ/cm², 1000 mJ/cm² and 2500 mJ/cm² of UV-C fluencies. During the exposure, the suspension was stirred slowly using magnetic stirrer to facilitate uniform radiation exposure.

In another set of experiment, alcohol stress was provided using 70% isopropanol for 5 and 10 minutes using standard microbiological methods. After exposing the microcosms to different stresses, the
microcosms were washed once and resuspended in equal amount of sterile distilled water and processed for different examinations.

**Confirmation of VBNC state**

VBNC cells are distinguished from culturable cells based on two key characteristics: nonculturability, resuscitatability and viability. In this work, nonculturability was confirmed by the absence of visible colonies in direct plate counting (DVC) technique. The resuscitatability and cell viability was determined by resuscitation assay using catalase and by quantifying 16S rRNA using Reverse-transcription quantitative polymerase chain reaction. 16S rRNA is a potential viability marker for psychrophilic stressed and UV-C exposed microcosms (Lahtinen et al. 2008; Lothigius et al. 2010; Kong et al. 2014; Li et al. 2017; Yang et al. 2019). But the method gives overestimated viable counts with isopropanol exposed samples (Sheridan et al. 1998; Ju et al. 2016; Santander et al. 2019). Therefore, an alternate method, BacLight™ bacterium viability kit which measures the cell membrane integrity was chosen for isopropanol stressed microcosms (Grey and Steck, 2001; Um et al. 2013).

Briefly, 1 ml of the sample aliquots was pelleted at 5000g for 10 minutes and processed separately for DVC, resuscitation assay, inoculation assay and cell viability assays. Direct viable count was performed as follows. The cell pellet was resuspended in 1ml of sterile distilled water and serially diluted in sterile water. 100µl of the dilutions were spread plated on CPG agar plates and the resultant colonies were counted and expressed in logarithmic numbers. Untreated control cells were also pelleted and processed by the same method as test microcosms.

**Resuscitation assay**

Resuscitation or reverse culturability is considered as a fundamental property of pathogenic VBNCs. All pathogens in VBNC state have to resuscitate exvivo for host invasion and establishing disease (Du et al. 2007; Ramamurthy et al. 2014; Imamura et al. 2015; Zhao et al. 2017). However, for achieving invitro resuscitation in microbial media, various environmental and chemical stimuli are required (Pinto et al. 2011). For RSSC, catalase is found to be superior than root exudates and other resuscitating agents, hence selected for this work (van Overbeek et al. 2004; Kong et al. 2014). For resuscitation, the pelleted cells from 1 ml samples were resuspended in 1 ml of sterile distilled water supplemented with 1000u/ml and 5000 u/ml of catalase (HiMedia Labs, Mumbai) and incubated at 28°C for five days and 21 days respectively (van Overbeek et al. 2004; Kong et al. 2004). After incubation, serial dilutions were performed and 100µl of the microcosms were plated onto CPG plates and monitored for colony formation. The resultant colonies were counted and expressed in logarithmic numbers.

**RT-qPCR**

As nonresuscitatable VBNCs cannot be grown and quantified in microbial culture media, reverse-transcription quantitative polymerase chain reaction (RT-qPCR) was performed to determine the viability status of microcosms and quantify them. Briefly, pellet from the 1ml samples was resuspended in 1ml of
TRIZOL reagent (Sigma Chemicals) and RNA extraction was performed as per manufacturer’s instructions. The qPCR involving BRYT Green® dye chemistry was performed using 1-step RT-qPCR reagents (Promega Corporation, USA) after removing residual DNA using DNase. The primer pairs Rp16F (5’-CCTGGCTCAGATTGAACGCT-3’) and Rp16R (3’-CTCCTATAGCATGAGGCCTT-5’) designed against the conserved sequences of 16S rDNA gene of RSSC was used for gene amplification. The resulting 210bp PCR product of the above primers were ligated in plasmid pTZ57 R/T (Thermo Fisher) and transformed into E. coli M15 cells as per the manufacturer’s instructions. After extraction and subsequent quantification, 10-fold serial dilutions of the cloned plasmid were made and used as standard for quantifying 16S rRNA (Jain et al. 2012). The RT-qPCR reaction was performed and the results were analysed in CFX96 Real-Time System and CFX manager respectively (Bio-Rad, Hercules, CA, USA). Dead cells prepared by exposing live cells to 2500 mJ/cm² of UV-C radiation were used as negative control.

Inoculation assay

Inoculation assay was performed before catalase treatment on bacterial wilt susceptible tomato seeds ICAR H-86 as per the procedure developed by Singh and his colleagues (Singh et al. 2018). The assay is economic in terms of space, labour, time, cost, required bacterial inoculum and is at par with soil drench assay (Wu et al. 2019; Gracia et al. 2019). Briefly, 1 ml of stress exposed microcosms and the controls were pelleted and resuspended in 1 ml of sterile distilled water to prepare a cell suspension. Thereafter, the roots of five tomato seedlings in two leaf stage were dipped in the cell suspension for 10 minutes. After air drying for 5 minutes, the roots of the seedlings were immersed in 1.5 ml of sterile distilled water for incubation. The setup was maintained in a controlled environment with 65-70% humidity and temperature of 30°C for 7 days. The disease severity was determined on 7th day according to the following key: 0 = No symptoms; 1= One leaf wilted; 2 = Two leaves wilted; 3= Plant dead. The Disease index (DI) was calculated by the formula DI=∑RT/4N, where: R = Disease severity scale; T = Number of wilted plants in each category and N = Total number of tested plants (Mishra et al. 2019). To double verify infection and bacterial colonization, the causative agent was reisolated from seedlings showing disease symptoms and confirmed by PCR (Prior and Fegan 2005).

Epifluorescence microscopy

Epifluorescence microscopy was performed to determine the membrane integrity of isopropanol treated microcosms using Live/Dead BacLight bacterium viability kit containing the dyes SYTO9 and Propidium iodide (PI). The staining was performed as per the manufacturer’s instructions. The stained cells were viewed in Olympus BX63 microscope using image analysis software Olympus Cellsens Dimension (Version 1.16). The SYTO9 and PI channels were super imposed using the same software to view membrane integrity.

Data analysis

The data presented in this study are the means of two experiments. The difference between sample duplicates were analysed by two-tailed Student’s t test. The percentage of nonresuscitatable VBNCs were
calculated by comparing the 16S rRNA copy number against the control by the formula: \[ \frac{(RNA \ copies \ of \ N^{th} \ day/sample \ treatments \times 100)}{RNA \ copies \ of \ N^{th} \ day/control} \].

## Results

### Confirmation of VBNCs after psychrophilic stress

The formation of VBNC is genus, strain and stress dependent. Microcosms of RSSC incubated at psychrophilic temperatures lose their culturability within 150 days (van Overbeek et al. 2004; Stevens et al. 2018). In this investigation, the total loss of culturability was noticed on 180\(^{th}\) day in both the strains. The average DVC of \textit{R. pseudosolanacearum} strains DIBER115 and DIBER118 dropped respectively from log 8.01 (p=0.08) and log 8.33 (P=0.2) CFU/ml on day 0 to below detection in 180 days. However, log 7.25 (p=0.47) and log 8.28 (p=0.5) cfu/ml of microcosms were resuscitated using catalase, confirming the transformation of culturable microcosms into resuscitatable VBNCs within 180 days (Fig. 1). During the period, the disease index dropped from 100% to 50% and 63.3% respectively for the strains. On prolonged incubation, the resuscitatability gradually declined and was totally lost on 360\(^{th}\) day. However, some cells were found expressing 16S rRNA, confirming the cells viability. These nonresuscitating cells are termed as nonresuscitatable VBNCs. To calculate its percentage, the 16S rRNA copy numbers on N\(^{th}\) day were compared with 0\(^{th}\) day. On 420\(^{th}\) day, the nonresuscitatable VBNC population was 0.252% and 7.8% for DIBER115 and DIBER118 respectively. Hereafter, nonresuscitatable cells were observed throughout the study period. On 480\(^{th}\) day it was 0.15% and 3.43% for DIBER115 and DIBER118 respectively (Fig. 1). These results confirm the existence of infectious nonresuscitatable VBNCs and their survival over a period of time.

To the best of our knowledge, the infectivity of nonresuscitatable VBNCs was not reported previously in any bacterial genera. In this work, varying degrees of wilting symptoms were observed on tomato seedlings inoculated with nonresuscitatable VBNCs. On 360\(^{th}\) day, the disease index was 65.3% and 33.3%, and on 480\(^{th}\) day it was 33.3% and 40% respectively for the strains DIBER115 and DIBER118 (Fig. 1). The infection was double confirmed by reisolating the pathogen from all wilted seedlings. These results confirm that nonresuscitatable VBNCs can also remain infectious over a period of time.

### Confirmation of VBNCs after UV-C treatment

To investigate, whether the formation of nonresuscitatable VBNCs is restricted to psychrophilic stress or in generally associated with other stresses, we choose two stress inducers, UV-C and isopropanol.

Most of the studies on UV-C were performed below the fluence of 200 mJ/cm\(^2\) for water disinfection studies (Hinjen et al. 2006). At fluence of 200 mJ/cm\(^2\), no colonies were observed in DVC (Table 1). However, log 6.389 (P=0.1) and log 6.34 (P=0.4) cfu/ml of cells resuscitated, confirming the transformation of culturable microcosms into resuscitatable VBNCs. These resuscitatable VBNCs were found infectious, which was confirmed by reisolating the pathogen from all wilted seedlings (Disease...
Index; DIBER115 - 40%, DIBER118 - 33%). These results confirm that UV-C induced resuscitatable VBNCs remain infectious. At fluence of 1000 mJ/cm², both the strains failed to resuscitate and infect the seedlings. To explore the possible formation of nonresuscitatable VBNCS at this fluence, the live/dead status of the microcosms were investigated by RT-qPCR. Presence of modest number of viable cells (0.37 % in DIBER115 and 8.5 % in DIBER118) was noticed, confirming the existence nonresuscitatable VBNCS state. At higher fluence of 2500mJ/cm², all microcosms were eliminated as no transcriptomes were detected (Table 1).

**Confirmation of VBNCS state after isopropanol treatment**

Alcohols are used at a concentration of 60 - 90% (v/v) to eliminate spores, bacteria and viruses (McDonnell and Russell 1999). In this work, isopropanol, the common laboratory disinfectant was tested at a concentration of 70% (v/v). After 5 minutes of exposure, the average plate count of *R. pseudosolanacearum* strains DIBER115 and DIBER118 respectively dropped from log 6.39 (P=0.51) and log 6.28 (P=0.10) CFU/ml to below detection. However, when resuscitated with catalase, log 4.16 (P=0.07) and log 4.21 (P=0.33) cfu/ml of cells formed colonies, confirming the transformation of culturable microcosms into resuscitatable VBNCS. However, these VBNCS failed to infect the seedlings, concluding the loss of pathogenicity after isopropanol exposure (Table 1). In epifluorescence microscopy, most of the 5 min exposed cells were found double stained (>95%), where SYTO9 and PI simultaneously stain individual cells confirming damage to membrane integrity (Berney et al. 2007) (Fig. 2). The loss of infectivity observed was due to this membrane damage. After 10 minutes of exposure, both the strains failed to resuscitate and extensive loss of membrane integrity confirming cell death.

**Discussion**

Members of RSSC incubated at 24°C are known to survive and remain infectious up to four years (Alverez et al. 2008). However, cold stressed microcosms in experimental settings were found simultaneously losing culturability and infectivity within 150 days. Hence VBNCS induced at psychrophilic temperature and the threat posed by them were considered ephemeral (van Overbeek et al. 2004; Alverez et al. 2010). Contrary to this, studies in natural water bodies indicated the presence of infective nonresuscitating RSSC VBNCS populations surviving winter months (Caruso et al. 2005). This scenario can only be explained by the existence of infectious VBNCS surviving the psychrophilic temperature. In this work, a substantial population remained infectious and resuscitatable during the “resuscitation window”. Later, approximately ≤3% of initial microcosms survived as infectious nonresuscitatable VBNCS (Figure 1). These results suggest that both resuscitatable and nonresuscitatable VBNCS surviving psychrophilic stress can remain infectious. These nonresuscitatable VBNCS populations may have a role in pathogens survival mechanism in the environment. This hypothesis is further strengthened by previous observation of nonresuscitatable VBNCS in artificial soil (Kong et al. 2014).
Similar to our results with UV-C, resuscitatable VBNCs has been previously observed at fluences 172-200 mJ/cm² in *E. coli*, *Pseudomonas sp*, *Aeromonas sp* and *S. aureus* (Zhang et al. 2015; Zhang et al. 2018; Guo et al. 2019). However, the infectivity of these strains was not elucidated. The crucial observation in our study is the infectivity exhibited by UV-C (200 mJ/cm²) exposed microcosms (Table 1). Although >99% of culturable microcosms transformed into VBNCs, only few cells were in a capacity to induce infection as evidenced by the low disease index observed (≤40%). UV-C damages nucleic acids and cellular membranes, which impairs the pathogens ability to infect their host (Nebe von Caron et al. 2000; Nocker et al. 2007; Pawlowski et al. 2011; Santos et al. 2013; Kim et al. 2017). At lower fluencies below 330 mJ/cm², majority of cells resist UV-C induced membrane and DNA damages (Schenk et al. 2011; Zhang et al. 2015). This explains the successful infection observed in tomato seedlings after irradiation with 200 mJ/cm² fluence (Table 1). Similarly, a subpopulation of cells is known to survive higher fluencies of UV-C (800 mJ/cm²) irradiation (Guo et al. 2019). In that capacity, it can be hypothesized that some cells surviving 800 mJ/cm² can also survive 1000 mJ/cm² fluence due to tailing effect (Schenk et al. 2011). Accordingly, in our experiments ≤0.3% of cells survived as nonresuscitatable VBNCs (Table 1). At higher fluences of 1000 mJ/cm², the acquired DNA and membrane damage may have crossed a critical threshold which is beyond the competence of cellular repair mechanisms (Rastogi et al. 2010). This accounts for the loss of infectivity observed at higher doses observed.

Our results raise a crucial question; why 16S rRNA transcriptomes were detected from cells having heavily damaged nucleic acids (1000 mJ/cm²)? First; in RSSC, the number of 16S rRNA encoding operons varies between 3 to 9 (Salanoubat et al. 2002; Cao et al. 2013; Remenant et al. 2010). Though the exact number of rRNA operons in the strains DIBER 115 and DIBER 118 is unknown, it can be speculated that, minimum of 3 copies of them may exist. In such a scenario the probability of 16S rDNA and its transcriptomes surviving a lethal UV-C is more than a gene having a single copy. Secondly, a preferential DNA damage prevention system equivalent to AidB protein (protects and repair housekeeping genes from alkalyting agents) can also be proposed (Rippa et al. 2011). To our knowledge, such selective mechanism for high energy radiation has not been elucidated. In the light of the above, we assume that the phenomenon encountered is more likely due to the presence of multiple copies of 16S rDNA genes.

Isopropanol is wildly used as sanitizer and disinfectant in laboratory, domestic and hospital settings. Immersing scalpels, scissors and other tools used for nursery operations in aqueous solution containing 70% isopropanol (v/v) for 10 minutes is an established phytosanitising procedure (Gebel et al. 2019; Wilkinson et al. 2014). Several disinfectants containing surfactants and salts were known to induce VBNC formation in many bacterial genera (Robben et al. 2018). However, the status of isopropanol is unknown. In our investigations, we observed 0.6% of culturable cells survived isopropanol by transforming into resuscitatable VBNCs after 5 minutes of exposure. However, these resuscitatable VBNCs failed to initiate any infection in tomato seedlings. This is due to the membrane damage caused by isopropanol, as evidenced by epifluorescence microscopy (Fig.2) (Ingram 1986; Mc Donnell and Russell 1999; Maillard 2002; Russell 2002; Ou et al. 2017). Hence, it can be hypothesised that isopropanol induces both membrane damage and VBNC formation. This result supports the efficacy of isopropanol
as an effective disinfectant (Table 1). Meanwhile, cells exposed to low temperature retain membrane integrity and other molecular mechanisms involved in pathogenesis (Pawlowski et al. 2011). Consequently, the VBNCs formed at psychrophilic temperature were successful in initiating infection and wilting (Table 1).

Three key conclusions can be drawn based on our results. First; apart from psychrophilic stress, isopropanol and UV-C also induces VBNC formation in R. pseudosolanacearum. Second; nonresuscitatable VBNCs are formed in response to psychrophilic stress and high fluences of UV-C. Third; damaged membranes restrict VBNCs ability to infect their host. According to microbial dormancy continuum hypothesis, the free toxin (Reactive Oxygen Species-ROS) to antitoxin ratio (T:A) determines the state of viability and dormancy in bacteria. VBNC cells tend to have higher T:A ratio compared to persisters and culturable cells, consequently require an external antitoxin (catalase) in-proportionate to ROS for promoting resuscitation (Ayrapetyan et al. 2015; Rotem et al. 2010). Accordingly, for nonresuscitatable VBNC, the requirement for antitoxin has to be in-proportionate to toxins accumulated over time. To validate the hypothesis, microcosms were exposed to 5000U/ml of catalase for 21 days (results not shown), as observed in 1000U/ml treatments, only nonresuscitatable VBNCs survived. These ‘nonresuscitatable’ forms are considered as “short intermediate transiting phase” between VBNCs and dead cells. We hypothesize that this phase is not at all a “short intermediate transiting phase”; rather it is a “different type” due to the cells prolong survival and infectivity noticed at psychrophilic temperature. Therefore, to differentiate it from the classical resuscitating VBNCs belonging to “resuscitation window” type, these VBNCs can be considered as “nonresuscitatable VBNCs”. Briefly the terms “resuscitatable VBNCs” and “nonresuscitatable VBNCs” denotes two different phenotypic variations revealed during catalase treatment. For practical purposes, they can be rechristened as Type-1 VBNC, which are resuscitatable and Type-2, which are nonresuscitatable. In such a scenario, a crucial question remains unanswered; whether Type-2 VBNCs are continuum of Type-1 VBNCs or stochastically formed within growing cultures? This can only be answered after unlocking the rudimentary molecular mechanisms associated with these phenotypic variations.

The formation of nonresuscitatable VBNCs (Type-2) in response to various stresses and their ability to infect and initiate infections emphasise the importance of these cells in the life cycle and survival of R. pseudosolanacearum. Previous studies and our limited experiments emphasise the possibility of infective nonresuscitatable VBNCs existing in the environment (Caruso et al. 2005). This also incites the necessity to develop alternative VBNC detection strategies for environmental samples as classical resuscitation assays and RT-PCR targeting 16s RNA are indeed nonspecific procedures when dealt with environmental samples having heterogenous microbial populations. In such scenario it is essential to identify and validate specific R. pseudosolanacearum transcriptomes for identifying VBNCs from the environment.

Declarations

Acknowledgements
The authors wish to express their sincere thanks to Director DIBER, DRDO Haldwani and Co-ordinator, Department of Biotechnology, Bhimtal. Kumaun University, Nainital, India for providing the facilities, support and encouragement. Help provided by Mr. Dinesh Pathak during isolation of \textit{R. pseudosolanacearum} strains is duly acknowledged. Research fellowship by DRDO and DST to Neha Faridi and Shalini Bhatt is also acknowledged.

**Funding**

Funding for this work was provided under Defence Research and Development Organisation (Project Number: DIB76).

**Author information**

**Affiliations**

Defence Institute of Bio-Energy Research (DIBER), DRDO, P.O. Arjunpur, Haldwani, Uttarakhand, Pin-263139, India.

Neha Faridi, Samuel Merwyn, Shalini Bhatt, Ankur Agarwal, Madhu Bala

Department of Biotechnology, Kumaun University, Bhimtal, Nainital, Uttarakhand, Pin-263001, India.

Neha Faridi, Veena Pande

**Contributions**

Conceptualization and designing of experiments: SM, NF, AA, VP and MB. Experiments and wet lab work: NF, SM and SB. Data analysis: SM, NF; Contribution of reagents/materials/analysis tools: NF, SM, SB, AA and MB. Manuscript preparation: NF, SM and AA. The final version of the manuscript was reviewed and agreed by all the authors.

**Corresponding author**

Correspondence to Samuel Merwyn

**Ethics declarations**

**Conflict of interest**

The authors declare no conflict of interest.

**Ethical statement**

This study does not describe any experimental work related to human.
Consent to participate
Not applicable.

Consent for publication
Not applicable.

References

1. Alvarez B, Biosca EG, Lopez MM (2010) On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. 267–279. In Mendez-Vilas (ed), *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, vol 1. Formatex Research center, Badajoz, Spain.

2. Alvarez B, Lopez MM, Biosca EG (2008) Survival strategies and pathogenicity of *Ralstonia solanacearum* Phylotype II subjected to prolonged starvation in environmental water microcosms. *Microbiology* 154 (11): 3590-3598. https://doi:10.1099/mic.0.2008/019448-0.

3. Ayrapetyan M, Williams T, Oliver JD (2018) Relationship between the viable but non-culturable state and antibiotic persister cells. *J Bact* 200 (20):e00249-18. https://doi.org/10.1128/JB.00249-18.

4. Ayrapetyan M, Williams TC, Oliver JD (2015) Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends Microbiol* 23(1):7–13.

5. Bamford RA, Smith A, Metz J, Glover G, Titball R, Pagliara S (2017) Investigating the physiology of viable but non-culturable bacteria by microfluidics and time-lapse microscopy. *BMC Biol* 15: 1-12. https://doi.org/10.1186/s12915-017-0465-4.

6. Berney M, Hammes F, Bosshard F, Weilenmann HU and Egli T (2007) Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight Kit in combination with flow cytometry. *Appl Environ Microbiol* 73:3283-3290. https://doi.org/10.1128/AEM.02750-06.

7. Cao Y, Tian B, Liu Y, Cai L, Wang H, Lu N, Wang M, Shang S, Luo Z, Shi J (2013) Genome sequencing of *Ralstonia solanacearum* FQY_4, isolated from a bacterial wilt nursery used for breeding crop resistance. *Gen announ*1. https://doi.org/10.1128/genomeA.00125-13.

8. Caruso P, Palomo J, Bertolini J, Alvarez B, Lopez MM, Biosca EG (2005) Seasonal variation of *Ralstonia solanacearum* biovar 2 populations in a Spanish river: Recovery of stressed cells at low temperatures. *Appl Environ Microbiol* 71(1):140-148. https://doi.org/10.1128/AEM.71.1.140-148.

9. Du M, Chen J, Zhang X, Li A, Li Y, Wang Y, (2007) Retention of virulence in a viable but nonculturable *Edwardsiella tarda* isolate. *Appl Environ Microbiol* 73(4): 1349–1354.

10. Ducret A, Chabalier M, Dukan S (2014) Characterization and resuscitation of ‘non-culturable’ cells of *Legionella pneumophila*. *BMC Microbiol* 14(1): 31-10.

11. García RO, Kerns JP, Thiessen, L. (2019). *Ralstonia solanacearum* species complex: A quick diagnostic guide. *Pla Heal Prog* 20(1), 7-13.
12. Gebel J, Gemein S, Kampf G, Pidot SJ, Buetti N, Exner, M. (2019) Isopropanol at 60% and at 70% are effective against ‘isopropanol-tolerant’ Enterococcus faecium. J Hosp Infect 103: e88-e91.

13. Grey BE, Steck TR (2001) The viable but nonculturable state of Ralstonia solanacearum may be involved in long-term survival and plant infection. Appl Environ Microbiol 67(9): 3866-3872.

14. Guo L, Ye C, Cui L, Wan K, Chen S, Zhang S, Yu X (2019) Population and single cell metabolic activity of UV-induced VBNC bacteria determined by CTC-FCM and D2O-labeled Raman spectroscopy. Environ Int 130: 104883.

15. Hayward CA (1994) Systematics and phylogeny of Pseudomonas solanacearum and related bacteria, 123-135. In A.C Hayward and G.L Hartman (ed.), Bacterial wilt: the disease and its causative agent, Pseudomonas solanacearum. CAB International.

16. Hijnen WA, Beerendonk EF, Medema GJ (2006) Inactivation credit of UV radiation for viruses, bacteria and protozoan (oo) cysts in water: A review. Water Res 40:3–22.

17. Imamura D, Mizuno T, Miyoshi SI, Shinoda S (2015) Stepwise changes in viable but nonculturable Vibrio cholerae cells. Microbiol Immunol 59(5):305–310.

18. Ingram LO (1986) Microbial tolerance to alcohols: Role of the cell membrane. Trends Biotechnol 4(2): 40-44.

19. Jain N, Merwyn S, Rai GP, Agarwal GS (2012) Real-time polymerase chain reaction assay for rapid and sensitive detection of anthrax spores in spiked soil and talcum powder. Folia Microbiol 57(3): 237-242.

20. Ju W, Moyne. A-L, Marco ML (2016) RNA-based detection does not accurately enumerate living Escherichia coli O157:H7 cells on plants. Front Microbiol 7:223.

21. Kelman A, (1954) The relationship of pathogenicity of Pseudomonas solanacearum to colony appearance in a tetrazolium medium. Phytopathol 44 (12).

22. Kong GH, Bae YJ, Lee JH, Joo JH, Jung JE, Chung E, Lee WS (2014) Induction of the viable but non-culturable state of Ralstonia solanacearum by low temperature in the soil microcosm and its resuscitation by catalase. Plos one. 9: e109792. https://doi.org/10.1371/journal.pone.0109792.

23. Kong SI, Bates TC, Hulsmann A, Hassan H, Smith BE, Oliver JD (2004) Role of catalase and oxyR in the viable but non-culturable state of Vibrio vulnificus. FEMS Microbiol Ecol. 50(3): 133–142.

24. Lahtinen SJ, Ahokoski H, Reinikainen JP, Gueimonde M, Nurmi J, Ouwehand AC, Salminen SJ (2008) Degradation of 16S rRNA and attributes of viability of viable but non-culturable probiotic bacteria. Lett Appl Microbiol 46(6):693–8.

25. Li R, Tun HM, Jahan M, Zhang Z, Kumar A, WGD, Farenhorst A, Khafipour E (2017) Comparison of DNA-, PMA-, and RNA-based 16S rRNA illumina sequencing for detection of live bacteria in water. Sci Rep 7, 5752 (2017). https://doi.org/10.1038/s41598-017-02516-3.

26. Lleo MM, Benedetti D, Tafi MC, Signoretto C, Canepari P (2007) Inhibition of the resuscitation from the viable but non-culturable state in Enterococcus faecalis. Environ Microbiol 9 (9): 2313–2320.
27. Lleo MM, Bonato B, Tafi MC, Signoretto C, Boaretti M, Canepari P (2001) Resuscitation rate in different enterococcal species in the viable but non-culturable state. *J Appl Microbiol* 91(6): 1095-1102.

28. Lleo MM, Pierobon S, Tafi MC, Signoreto C, Canepari P (2000) mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. *Appl Environ Microbiol* 66(10): 4564-4567.

29. Lothigius Å, Sjöling Å, Svennerholm AM, Bölín I (2010) Survival and gene expression of enterotoxigenic *Escherichia coli* during long-term incubation in sea water and freshwater. *J Appl Microbiol* 108(4):1441-1449.

30. Maillard JY (2002) Bacterial target sites for biocide action. *J Appl Microbiol* 92:16 S-27S.

31. Mc Donnell, G., Russell, A.D. (1999) Antiseptics and disinfectants: activity, action, and resistance. *Clin Microbiol Rev* 12(1): 147–179.

32. Nebe von Caron G, Stephens PJ, Hewitt CJ, Powell JR, Badley RA (2000) Analysis of bacterial function by multi-colour fluorescence flow cytometry and single cell sorting. *J Microbiol Meth* 42(1):97-114.

33. Nocker A, Sossa KE, Camper AK. (2007) Molecular monitoring of disinfection efficacy using propidium monoazide in combination with quantitative PCR. *J Microbiol Methods* 34(4): 415–425.

34. Oliver JD (2010) Recent findings on the viable but non-culturable state in pathogenic bacteria. *FEMS Microbiol Rev* 34(4): 415–425.

35. Ou F, McGoverin C, Swift S, Vanholsbeeck F (2017) Absolute bacterial cell enumeration using flow cytometry. *J appl Microbiol* 123(2):464-477.

36. Pawlowski DR, Metzger DJ, Raslawsky A, Howlett A, Siebert G, Karalus RJ, Whitehouse, CA (2011) Entry of *Yersinia pestis* into the viable but nonculturable state in a low-temperature tap water microcosm. *Plos One* 6(3): e17585. https://doi.org/10. 1371/journal.pone.0017585.

37. Pinto D, Almeida V, Almeida SM, Chambel L (2011) Resuscitation of *Escherichia coli* VBNC cells depends on a variety of environmental or chemical stimuli. *J Appl Microbiol* 110(6): 1601–1611.

38. Pinto D, Santos MA, Chambel L (2015) Thirty years of viable but non-culturable state research: Unsolved molecular mechanisms. *Crit Rev Microbiol* 41(1): 61-76.

39. Prior P, Fegan M (2005) How complex is the *Ralstonia solanacearum* species complex. “*Ralstonia solanacearum* species complex?” 449-461. In C. Allen, P. Prior, and A.C. Hayward (ed.), Bacterial wilt disease and the *Ralstonia solanacearum* species complex. APS Press, St. Paul, MN.

40. Ramamurthy T, Ghosh A, Pazhani GP, Shinoda S (2014) Current perspectives on viable but non-culturable (VBN C) pathogenic bacteria. *Front Publ Healt* 2:103. doi: 10.3389/fpubh.2014.00103.

41. Rastogi, R.P., Kumar, A., Tyagi, M.B., Sinha, R.P. (2010) Molecular mechanisms of ultraviolet radiation-induced DNA damage and repair. *J Nucleic Acids* 2010. https://doi.org/10.4061/2010/592980.
42. Remenant B, Coupat-Goutaland B, Guidot A, Cellier G, Wicker E, Allen C, Fegan, M, Pruvost O, Elbaz M, Calteau A, Salvignol G (2010) Genomes of three tomato pathogens within the *Ralstonia solanacearum* species complex reveal significant evolutionary divergence. *BMC genomics* 11(1): 1-16.

43. Rippa V, Duilio A, di Pasquale P, Amoresano A, Landini P, Volkert MR. (2011) Preferential DNA damage prevention by the *E. Coli AidB* gene: A new mechanism for the protection of specific genes. *DNA repair* 10(9): 934-941.

44. Robben C, Fister S, Witte AK, Schoder D, Rossmanith P, Mester P (2018) Induction of the viable but non-culturable state in bacterial pathogens by household cleaners and inorganic salts. *Sci Rep* 8(1): 1-9.

45. Rotem, E, Loinger A, Ronin I, Levin-Reisman I, Gabay C, Shores N, Balaban NQ (2010) Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. *PNAS USA* 107(28): 12541-12546.

46. Rowe TM, Dunstall G, Kirk R, Loughney FC, Cooke LJ, Brown RS (1998) Development of an image system for the study of viable but non-culturable forms of *Campylobacter jejuni* and its use to determine their resistance to disinfectants. *Food Microbiol* 15(5): 491–498.

47. Russell AD (2002) Mechanisms of antimicrobial action of antiseptics and disinfectants: an increasingly important area of investigation. *J Antimicrob Chemother* 49(4): 597–599.

48. Safni I, Cleenwerck I, De Vos P, Fegan M, Sly L, Kappler U (2014) Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: Proposal to amend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygyii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. Solanacearum* phytype IV strains as *Ralstonia syzygii* subsp. *Indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R.solanacearum* phytype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. *Int J Syst Evol Micr* 64(9): 3087–3103.

49. Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M, Billault A, Brottier P, Camus JC, Cattolico L, Chandler M (2002) Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* 415(6871): 497-502.

50. Santander RD, Meredith CL, Acimovic SG (2019) Development of a viability digital PCR protocol for the selective detection and quantification of live *Erwinia amylovora* cells in cankers. *Sci Rep* 9(1): 1-17.

51. Santos AL, Oliveira V, Baptista I, Henriques I, Gomes NC, Almeida A, Correia A, Cunha Â (2013) Wavelength dependence of biological damage induced by UV radiation on bacteria. *Arch Microbiol* 195(1): 63-74.

52. Schenk M, Raffellini S, Guerrero S, Blanco GA, Alzamora SM (2011) Inactivation of *Escherichia coli, Listeria innocua* and *Saccharomyces cerevisiae* by UV-C light: study of cell injury by flow cytometry. *LWT-Food Sci Tech* 44(1):191-198.
53. Sheridan GEC, Masters CI, Shallcross JA and Mackey BM (1998) Detection of mRNA by Reverse Transcription-PCR as an Indicator of Viability in *Escherichia coli* cells. *Appl Environ Microbiol*. 64(4): 1313–1318.

54. Singh N, Phukan T, Sharma PL, Kabyashree K, Barman A, Kumar R, Sonti RV, Genin S, Ray, SK (2018) An innovative root inoculation method to study *Ralstonia solanacearum* pathogenicity in tomato seedlings. *Phytopathology* 108(4): 436-442.

55. Stevens L, Vander Zouwen P, Van Tongeren C, Kastelein P, Vander Wolf J (2018) Survival of *Ralstonia solanacearum* and *Ralstonia pseudosolanacearum* in drain water. *Bull OEPP* 48(1): 97-104.

56. Um HY, Kong HG, Lee HJ, Choi HK, Park EJ, Kim ST, Murugiyavan S, Chung E, Kang KY and Lee SW (2013) Altered gene expression and intracellular changes of the viable but nonculturable state in *Ralstonia solanacearum* by copper treatment. *Plant Path J* 29:374-385.

57. van Elsas JD, Kastelein P, van Bekkum P, van der Wolf JM, de Vries PM, van Overbeek, LS (2000) Survival of *Ralstonia solanacearum* biovar 2, the causative agent of potato brown rot, in field and microcosm soils in temperate climates. *Phytopathology* 90(12):1358-1366.

58. van Elsas JD, Kastelein P, De Vries PM, van Overbeek LS (2001) Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* biovar.2 in irrigation water. *Can J Microbiol* 47(9): 842-854.

59. van Overbeek LS, Bergervoet JHW, Jacobs FHH, van Elsas JD (2004) The low-temperature-induced viable but non-culturable state affects the virulence of *Ralstonia solanacearum* biovar 2. *Phytopathology* 94(5): 463-469.

60. Weichart D, Kjelleberg S (1996) Stress resistance and recovery potential of culturable and viable but non-culturable cells of *Vibrio vulnificus*. *Microbiology* 142(4), 845–853.

61. Wilkinson KM, Landis TD, Haase DL, Daley BF Dumroese RK (2014) Tropical nursery manual: a guide to starting and operating a nursery for native and traditional plants. *Agriculture Handbook 732. Washington, DC. US Department of Agriculture, Forest Service* 376: 138.

62. Wong CH, Wang P (2004) Induction of viable but non-culturable state in *Vibrio parahaemolyticus* and its susceptibility to environmental stresses. *J Appl Microbiol* 96(2): 359–366.

63. Yang C, Sun W, Ao, X (2019) Using mRNA to investigate the effect of low-pressure ultraviolet disinfection on the viability of *E. coli*. *Front Environ Sci Eng* 13(2): 1-8.

64. Yaron S, Matthews K (2002) A reverse transcriptase-polymerase chain reaction assay for detection of viable *Escherichia coli* O157: H7: investigation of specific target genes. *J Appl Microbiol*. 92(4): 633–640.

65. Zhang S, Guo L, Yang K, Zhang Y, Ye C, Chen S, Yu X, Huang W, Cui L (2018) Induction of *Escherichia coli* into a VBNC state by continuous-flow UVC and subsequent changes in metabolic activity at the single-cell level. *Front Microbiol* 9:1–11.

66. Zhang, S, Ye C, Lin, H, Lv. L, Yu X (2015). UV disinfection induces a VBNC state in *Escherichia coli* and *Pseudomonas aeruginosa*. *Environ Sci Technol* 49(3):1721-1728.
Tables

Table 1: Effect of UV-C and Isopropanol on microcosms

| Strains   | Assays | Control | UV-C Fluence | Isopropanol (70% v/v) |
|-----------|--------|---------|--------------|------------------------|
|           |        |         | 200 mJ/Cm²   | 1000 mJ/Cm²            | 2500 mJ/Cm² | 5 min     | 10 min    |
|           |        |         | 200 mJ/Cm²   | 1000 mJ/Cm²            | 2500 mJ/Cm² | 5 min     | 10 min    |
| DIBER115  | DVC    | 6.39±0.012 | 0 | 0 | 0 | 0 | 0 |
|           | RC     | 6.44±0.043 | 6.38±0.006 (87) | 0 | 0 | 4.16±0.038 (0.5) | 0 |
|           | 16S    | 6.63±0.033 | NP | 4.20±0.029 (0.3) | 0 | NP* | NP* |
|           | DI (%) | 100 | 40 | 0 | 0 | 0 | 0 |
| DIBER118  | DVC    | 6.28±0.35 | 0 | 0 | 0 | 0 | 0 |
|           | RC     | 6.34±0.034 | 6.38±0.067 (100) | 0 | 0 | 4.21±0.039 (0.6) | 0 |
|           | 16S    | 5.65±0.04 | NP | 4.57±0.09 (8.3) | 0 | NP* | NP* |
|           | DI (%) | 100 | 33.3 | 0 | 0 | 0 | 0 |

DVC: Direct Viable Plate Count; RC: Cell count after Resuscitation; 16S: 16S rRNA copy number; DI: Disease Index; 0: No microbial count detected; NP: Not Performed; All values are expressed in Log except pathogenicity Index; * 16S transcriptome detection is not recommended for isopropanol treated bacterial cells; Values in brackets denote the percentage of cfu counts/copy numbers to the control.

Figures
Figure 1

Survival of *R. pseudosolanacearum* strains in psychrophilic temperature

Figure 2

2a

2b
Epifluorescence microscope observations of SYTO9 and PI stained DIBER 118 strain. 2a: DIBER118 Control cells: Cells with intact membrane appear green due to SYTO9 staining and cells with damaged membrane appears red due to PI staining. 2b: DIBER118 exposed to 70% isopropanol for 5 minutes: Arrow indicates the double stained cells where both SYTO9 and PI fluoresce in equal intensity.