



Resuscitation of eight-year VBNC *Providencia stuartii*

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Abstract

Providencia stuartii was stressed by incubation in soil microcosms for eight years. After five years of starvation, no culturable strain was observed. Incubation of samples in nutrient-rich broth medium not supplemented with growth factors, however, allowed resuscitation of VBNC cells so that subsequent plating yielded observable colonies for significantly extended periods of time. Recovery of VBNC *Providencia stuartii* was obtained by incubation in nutrient broth even after eight years of starvation. To see whether the samples contained the same strain of *Providencia stuartii* inoculated eight years ago, the complete 16S rRNA gene was PCR amplified and sequenced from initial, stressed and revived strains of *Providencia stuartii*. The 16S rRNA gene sequences from eight-year stressed strains were homologous with a high degree of similarity to the GenBank reference strain and were identical to each other.

Key words: *Providencia stuartii*, soil, stress, resuscitation.

Introduction

Enterobacteria are important food-borne pathogens that represent an increasingly significant public health issue in industrialized countries. The problem, at least in part, is that these organisms can persist for long periods in the environment in a heavily stressed state known variously, and often contentiously, as viable but non-culturable (VNC) ⁹. These heavily stressed microorganisms show only very weak metabolic activity, often at the very limits of detection, and they lose the ability to form colonies on non-selective plating media or to grow in non-selective broth media. Nevertheless, in the case of non-culturable populations of pathogenic bacteria in the environment (soil or water, etc.) or associated with bacteriological spoilage of human foods and animal feeds, they may still be capable of causing disease if ingested by a susceptible animal host ¹¹. We previously demonstrated that VBNC *Salmonella* persisted for many years in seawater microcosms; recovery was obtained in nutrient broth⁵ and in embryonated chicken eggs ². We demonstrated also that VBNC *Vibrio cholerae* resisted in seawater for many years and resuscitation was obtained by long-term incubation in nutrient broth ⁴. We observed also that *Bacillus* spores persisted for many years in crystal salt; recovery was obtained by incubation in nutrient broth ³. The aim of this study was to see if *Providencia stuartii* could persist as other bacteria studied for many years in environment microcosms.

Material and Methods

Strain and inoculum preparation: *Providencia stuartii* was kindly provided by Professor Amina Bakhrouf, Pharmacy University of Monastir. Before experiments, agar plates (1% tryptone, 1% sodium chloride, 1.5% agar) were inoculated with *Providencia stuartii* incubated for 18 hours at 37°C. A loopful of

the resulting growth was resuspended in 150 ml of autoclaved physiologic water amended with 0.1% (wt/vol) tryptone and incubated at room temperature (23±1°C) for 18 hours. Cells were then harvested by centrifugation (3,100 x g, 10 min, room temperature), the supernatant was aspirated, and the remaining pellet was resuspended in autoclaved physiologic water (pH 7.4). This washing process was repeated five times in order to prevent the carryover of medium nutrients. Finally, the pellet was resuspended in sterilized physiologic water for subsequent inoculation of microcosms.

Experimental design: Microcosm experiments were performed by using the soil collected in Ksour Essef, Tunisia. Soil microcosms were made by inoculating 500 g batches of heat-sterilized (2 h at 134°C) soil. The bacterial cells were added to sterilized soil until a density of 10⁻⁸ bacteria g⁻¹. We used 30 microcosms containing infected soil and 30 microcosms containing only sterilized soil used as control. Each experiment in this work was duplicated. Microcosms were stored at 20±2°C. At intervals, 25-g samples of soil were suspended in 250 ml of 0.85% (wt/vol) NaCl (saline), shaken thoroughly for 30 min at room temperature and serially diluted in saline. Duplicate 0.1-ml aliquots were plated for culturable counts on nutrient agar.

In vitro resuscitation of VBNC state: When colonies were no longer observed by plate counting, 10-g samples of soil were suspended in 100 ml of nutrient broth and incubated with shaking at 37°C for 24 h before plating on selective and non-selective agar. Incubation was done at 37°C with shaking for three months. Every 48 hours 0.1 ml of incubated solution was inoculated into 90 ml of nutrient broth. Control microcosms were followed as

infective microcosms and no *Providencia stuartii* germ was found even after three months of reviviscence in nutrient broth which confirmed the absence of contamination.

PCR amplification of the 16S rDNA: Almost the complete 16S rDNA gene was amplified initially using eubacterial primers Eub 9_27 and Eub 1542. A second nested PCR was then realized using the PCR products as template with GC-clamped P3 primer and P2 primer for subsequent DGGE analysis according to Muyzer *et al.*⁸. PCR was made in a total volume of 50 µl containing 5 pmol of each primer, 1.5 mM MgCl₂, PCR buffer 1X (10 mM Tris-HCl; 50 mM KCl, pH 8.3), dNTP 200 µM each one and 1 U of Taq polymerase (Roche). In the first amplification the PCR program was composed by an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 40°C for 45 s and extension at 72°C for 90 s. In the nested PCR with the GC-clamped primer a touchdown program was used. Initially the DNA was denatured at 94°C for 5 min; the next 20 cycles were composed by denaturation at 94°C for 30 s, annealing at 65°C for 45 s and extension at 72°C for 90 s, decreasing the annealing temperature in 0.5°C in each cycle; ten additional cycles were then realized with the same times but with a constant annealing temperature of 55°C, ending with a final extension at 72°C for 5 min. Nucleotide sequences of primers used in this study are listed in Table 1.

Table 1. Primers used.

5'-GAG TTT GAT CCT GGC TCA G-3'
EUB9-27f 5'-AGA AAG GAG GTG ATC CAG CC-3'
EUB1542r 5'-ATT ACC GCG GCT GCT GG-3'
P2 (534r) 5'-C GCC CGC CGC GCG CGG CGG GCG GGG
CGG GGG CAC GGG GGG -3' + 5'-CCT ACG GGA GGC AGC AG-3'
GC-clamp + 341f = P3

Denaturing gradient gel electrophoresis (DGGE): DGGE was performed with the D-Gene System (BioRad) in polyacrylamide gels (8% of 37:1 acrylamide-bisacrylamide mixture in 0.5X TAE buffer, 0.75 mm thick, 16 cm x 10 cm) with a gradient of 30 to 60%, according to the manufacturer's guidelines. Gels were run in 0.5X TAE buffer at 200 V and constant temperature of 60°C for 5 h. The DGGE gels were stained by silver staining and scanned using a HP Scanjet 5470c.

Cloning and sequencing: PCR products to be cloned were amplified with *Pfu* DNA polymerase (Promega) in a total reaction volume of 50 µl containing 25 pmol of each primer, 1.5 mM MgCl₂, PCR buffer 1X (20 mM Tris-HCl; 10 mM KCl; 10 mM (NH₄)₂SO₄; 2 mM MgSO₄; 0.1% Triton X-100; 1 mg/ml nuclease-free BSA) and 250 µM of each dNTP. Thermal cycling was carried out by an initial denaturation at 94°C for 2 min, hot start at 80°C, followed by 25 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 73°C for 3 min, with a final extension at 73°C for 5 min. PCR reactions from 3 replicates were combined, concentrated and purified in a Multiscreen plate (Millipore Inc.). For cloning the Zero Blunt PCR cloning kit (Invitrogen) was used according to the manufacturer's instructions. From each strain 5 clones were selected, and the insert size in the plasmid was checked by PCR with primers flanking the cloning side of the vector and agarose electrophoresis. Clones carrying the right size insert were one-

shot sequenced using the BigDye terminator v2.1 cycle sequencing kit with primer 341f (Lane 1991) in an ABI 377 genetic analyzer (Applied Biosystems). The sequences of about 300 to 500 bp in length were used for an initial identification of the strains using the match program BLAST¹³ on the NCBI's homepage (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

Results

Decline of *Providencia stuartii* level in sterile soil: Averages of logarithmic results found for the thirty microcosms were calculated every month (Fig. 1). Fig. 1 shows the responses of *Providencia stuartii* to incubation in soil. Strains of *Providencia stuartii* inoculated into sterile soil at 20°C and monitored for nearly one year and four months, displayed declining plate counts and accumulation of large numbers of non-culturable cells. The results demonstrated that *Providencia stuartii* cells could enter the VBNC stage, while no colony growth was observed. The numbers of viable cells of *Providencia stuartii* strain decreased slightly over time (Fig. 1).

In vitro reviviscence of VBNC *Providencia stuartii*: After five years of starvation, no culturable cells were observed in selective and non-selective media. After 48 hours of incubation, turbidity was observed in nutrient broth. From this medium, normal colonies with regular periphery appeared on non-selective media. After two months of reviviscence in nutrient broth, stressed cells acquired the ability of growing in selective medium. Colonies morphology and biochemical activities were also acquired.

After eight years of starvation, reviviscence assay gave the same results described above. Control microcosms were followed as infective microcosms and no *Providencia stuartii* germ was found even after three months of reviviscence in nutrient broth which confirmed the absence of contamination.

PCR-DGGE analysis: The results mentioned (Figs 1 and 2) confirmed the identity of all strains tested. The patterns were reproducible and characteristic for each strain tested, indicating that there was interstrain sequence divergence. This observation indicates that there were DNA molecules with slightly different melting behaviors, possibly caused by incomplete extension of

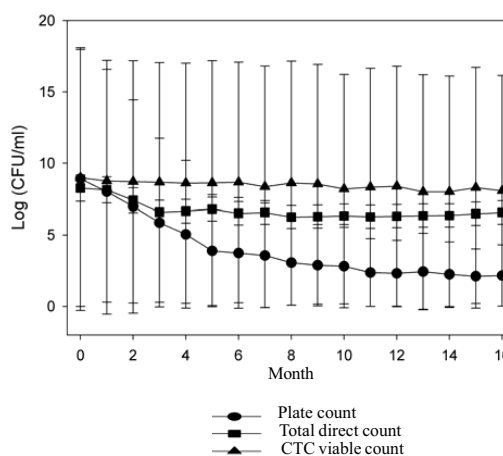


Figure 1. Entrance of logarithmic-phase cells of *Providencia stuartii* into the VBNC state incubated in soil: the average and standard deviation of total direct count, CTC viable count and plate count.

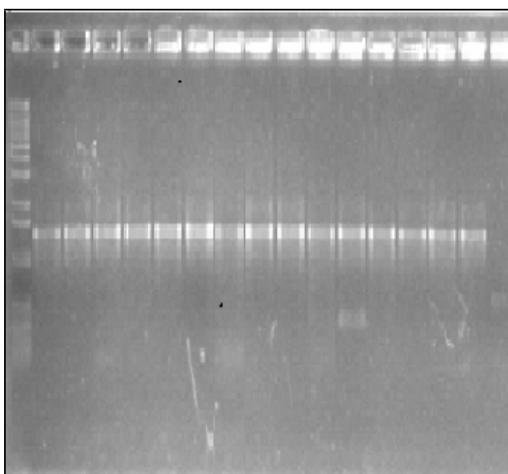


Figure 2. PCR of 16S RNA gene fragments of *Providencia stuartii* strain.

the same template due to the GC clamp. The strains having the same identity tested produced similar band patterns indicating that they may have similar copies of 16S rDNA (Fig. 3).

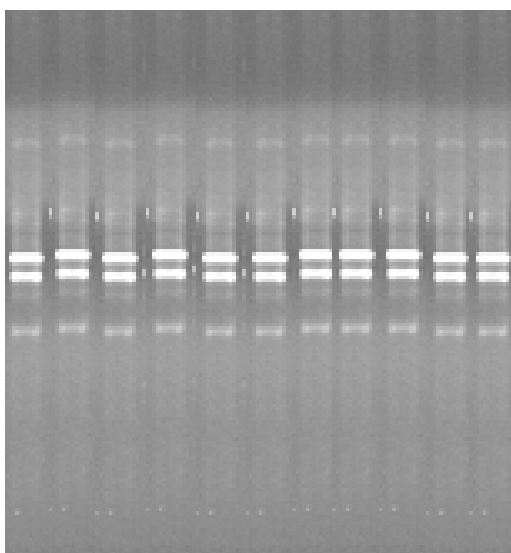


Figure 3. DGGE of 16S RNA gene fragments of stressed and revived *Providencia stuartii* strain. Lane 1: *P. stuartii* initial strain; Lane 2: *P. stuartii* starved for 5 years in soil microcosms and resuscitated for 48 hours in NB; Lane 3: *P. stuartii* starved for 5 years in soil microcosms and resuscitated for 10 days in NB; Lane 4: *P. stuartii* starved for 5 years in soil microcosms and resuscitated for one month in NB; Lane 5: *P. stuartii* starved for 5 years in soil microcosms and resuscitated for 2 months in NB; Lane 6: *P. stuartii* starved for 8 years in soil microcosms and resuscitated for 48 hours in NB; Lane 7: *P. stuartii* starved for 8 years in soil microcosms and resuscitated for 10 days in NB; Lane 8: *P. stuartii* starved for 8 years in soil microcosms and resuscitated 20 days in NB; Lane 9: *P. stuartii* starved for 8 years in soil microcosms and resuscitated for one month in NB; Lanes 10 and 11: *P. stuartii* starved for 8 years in soil microcosms and resuscitated for 2 months in NB.

Nucleotide sequence accession numbers: The sequences for isolated cell clones were deposited in the EMBL sequence database. The sequences for isolated cell clones were deposited in the EMBL sequence database. The results confirmed that the cells studied related to *Providencia stuartii*.

Discussion

The results obtained in this investigation have been interpreted as an indication that non-culturable cells were still viable and thus in the VBNC state^{1,10}. The VBNC state is a particular condition that bacteria may undergo when environmental conditions are not suitable for normal cell growth and division. Under these conditions, bacteria are unable to form colonies in normal growth media but are still viable and endowed with metabolic activity. The present study adds to literature that VBNC forms of *Providencia stuartii* could persist for many years (eight years) in agricultural soil and could be resuscitated by incubation in nutrient broth. As demonstrated in this study, the resuscitation was succeeded by long-term incubation in nutrient broth.

Workers in the VBNC field have addressed experiments with various resuscitation techniques in an attempt to return non-culturable cells to a state in which they do exhibit discernible population increases. The techniques that have been reported to resuscitate non-culturable cells are nutrient addition, temperature shifts and nutrient addition in the presence of culturable cells^{6,7,12}. According to nutrient broth incubation, stressed cells acquired progressively the initial colonies morphology and biochemical activities. After two months of incubation revived cells acquired the capacity of growing in selective medium. In fact, nutrient addition and temperature shift are reportedly effective methods for rescuing bacterial populations from the VBNC state⁶.

We confirmed in this study the identity of stressed and revived strains by DGGE and the sequencing of 16S RNA gene fragments. Fingerprinting techniques such as DGGE allow reproducible comparisons of DNA profiles obtained from microbial communities to be made⁸. As such, an additional advantage of DGGE is that selected bands can be sequenced and the presence of a particular bacterium can be monitored. In the present study, DGGE following by sequencing of 16S rDNA was optimized for the identification of stressed and revived *Providencia stuartii*.

DGGE, sequencing of 16S RNA gene fragments and multiplex PCR confirmed that VBNC *Providencia stuartii* resisted in soil microcosms for eight years and could be resuscitated *in vitro* system. The present study adds to literature that VBNC forms of *Providencia stuartii* could persist for long time in natural environment and could be resuscitated *in vivo* and *in vitro*.

References

- Barer, M.R., Gribbon, L.T., Harwood, C.R. and Nwoguh, C.E. 1993. The viable but nonculturable hypothesis and medical bacteriology. *Rev. Med. Microbiol.* **4**:183–191.
- Dhiaf, A. and Bakhrouf, A. 2004. Recovery in embryonated chicken eggs of viable but non-culturable *Salmonella*. *J. Food, Agriculture and Environment* **2**(2):104-107.
- Dhiaf, A. and Bakhrouf, A. 2005. Resistance of *Bacillus* spores in salt crystals. In Ozhan, E. (ed.). Proceedings of the Fifth International Conference on the Mediterranean Coastal Environment, MEDCOST 1, Vol 1.

- ⁴Dhiaf, A. and Bakhrouf, A. 2005. Adaptation of *Vibrio cholerae* to extreme seawater stress. In Ozhan, E. (ed.). Proceedings of the Fifth International Conference on the Mediterranean Coastal Environment, MEDCOST 1, Vol 1.
- ⁵Dhiaf, A., Chnity, N. and Bakhrouf, A. 2001. Adaptation of *Salmonella* to extreme seawater stress. In Ozhan, E. (ed.). Proceedings of the Fifth International Conference on the Mediterranean Coastal Environment, MEDCOST 1, 23-27 October, 2001, pp. 760-766.
- ⁶Kaprelyants, A.S., Gottschal, J.C. and Kell, D.B. 1993. Dormancy in non-sporulating bacteria. FEMS Microbiol. Rev. **104**:271–286.
- ⁷Kaprelyants, A.S., Mukamolova, G.V. and Kell, D.B. 1994. Estimation of dormant *Micrococcus luteus* cells by penicillin lysis and by resuscitation in cell-free spent culture medium at high dilution. FEMS Microbiol. Lett. **115**:347–352.
- ⁸Muyzer, G., De Waal, E.C. and Uitterlinden, A.G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59**:695-700.
- ⁹Oliver, J. D. 1995. The viable but non-culturable state in the human pathogen *Vibrio vulnificus*. FEMS Microbiol. Lett. **133**:203-208.
- ¹⁰Oliver, J.D. and Kjelleberg, S. 1993. Formation of Viable but Nonculturable Cells Starvation in Bacteria. Plenum Press, New York, N.Y., pp. 239–272.
- ¹¹Sylvester, D. M., Taylor, R. and LaHann, T. R. 2001. Viable but nonculturable bacteria: A public health threat? Infect. Dis. Rev. **3**:70-82.
- ¹²Votyakova, T.V., Kaprelyants, A.S. and Kell, D.B. 1995. Influence of viable cells on the resuscitation of dormant cells in *Micrococcus luteus* cultures held in an extended stationary phase: The population effect. Appl. Environ. Microbiol. **60**:3284–3291.
- ¹³Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucl. Acids Res. **25**:389-3402 (<http://www.ncbi.nlm.nih.gov/BLAST/>).