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Resuscitation of Seventeen-year Stressed *Salmonella typhimurium*

Dhiaf Amel[1], Bakhrouf Amina[2]

[1] Cabinet Dr Allaya, Ouled Salah 5116, Ksour Essef, Tunisia
[2] Laboratoire de Recherche en Microbiologie
Santé Hôpital de circonscription de Ksour Essef, 5180 Tunisia

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Abstract

Salmonella enterica serovar *typhimurium* was stressed by incubation in seawater microcosms for seventeen years. The microcosms were prepared in such a way as to allow progressive evaporation of the water. Despite being introduced into the sterile seawater at very high concentrations, the *Salmonella* rapidly declined to levels undetectable by plate counts on nutrient agar. After two years of starvation, about half of the seawater volume had evaporated from each microcosm, and salt crystals appeared. Inoculation of the salty suspension did not result in any culturable strains in selective and non-selective media. However, incubation of samples in nutrient-rich broth, without supplemental growth factors, allowed resuscitation of stressed cells, yielding colonies that remained viable for extended periods of time. After three years the total volume of water had evaporated from each microcosm, and only salt crystals remained. These microcosms were then incubated at room temperature for seventeen years. Resuscitation of VBNC *Salmonella* from the salt crystals was conducted in vitro and in vivo; recovery occurring after incubation in both nutrient broth and in mice. Recovery occurred in the *Salmonella*

[1] Corresponding author e-mail: *Hamadi.allaya@topnet.tn*

administered orally into the mice, but not in those administered by intra-peritoneal injection. The identity of initial, stressed and revived strains was confirmed by DGGE analysis and sequencing of 16S RNA gene fragments.

INTRODUCTION

Salmonella species are important food-borne pathogens that represent increasingly significant public health issues in industrialized countries. The problem, at least in part, is that this organism can persist for long periods in the environment in a heavily stressed state known variously, and often contentiously, as viable but nonculturable (VBNC) (Roszak & Colwell 1987, Rahman et al. 1996, Oliver 1995, Sylvester et al. 2001) or not immediately culturable (Kell et al. 1998). This phenomenon has been demonstrated for many bacterial species, including significant human pathogens such as *Vibrio cholerae, Shigella dysenteriae, Escherichia coli* O157:H7, and *Campylobacter jejuni* (Oliver 2000). Recovery of culturable cells from a population of nonculturable cells would provide convincing support for the VBNC hypothesis. The appearance of large numbers of culturable cells after the addition of nutrients to populations of nonculturable cells has been reported to occur via a process termed resuscitation (Kaprelyants et al. 1993, Oliver et al. 1993). The purpose of this study was to investigate the maintenance of viability of *Salmonella* Typhimurium in salt crystals for many years. The strain was experimentally stressed by exposure in seawater microcosms. Microcosms were prepared in a way that permitted a progressive evaporation of water, leading the appearance of salt crystals. The resuscitation process was conducted after long-term incubation in nutrient broth, oral administration to mice, and by intraperitonial injection into mice. Colony morphologies and the biochemical activities of stressed and recovered cells were subsequently documented.

MATERIALS AND METHODS

Strain and inoculum preparation

S. typhimurium LT4 was kindly provided by A. Bakhrouf, Pharmacy University of Monastir. Initially agar plates (1% tryptone, 1% sodium chloride, 1.5% agar) were inoculated with *S. typhimurium* LT4 and incubated for 18 hours at 37°C. A loopful of the resulting growth was resuspended in 150 ml of autoclaved 0.2 µm filtered seawater 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 × *g*, 10 min, room temperature), the supernatant aspirated, and the remaining pellet resuspended in 0.2-µm-filter-sterilized, autoclaved phosphate-buffered saline (pH 7.4). This washing process was repeated five times in order to prevent carry over of medium nutrients. Finally, the pellet was resuspended in phosphate-buffered saline for subsequent inoculation of microcosms. Seawater was filtered through a 0.6-µm-pore-size polycarbonate filter (Osmonics), and the filtrate used as an inoculum. Epifluorescence microscopy of DAPI (4',6'-diamidino-2-phenylindole)-stained inoculum showed no detectable protists or algae. Most viruses present in the seawater sample presumably passed into the inoculum, but we did not attempt to detect them. The DAPI method does not stain viruses intensely enough for detection.

Experimental design

Microcosms were conducted in Pyrex beakers (1 dcm^3) using seawater collected from Monastir beach, Tunisia. Each beaker contained 300 cm^3 of sterilized seawater, into which the bacterial cells were suspended**.** 30 experimental microcosms were inoculated with bacterial suspension, and 30 microcosms containing only sterilized seawater were used as controls. Each experiment in this work was duplicated.

Colony enumeration

Cell culturability was determined using marine agar (MA) plates (Difco Laboratories). CFU (Colony Forming Units) were enumerated either by filtering duplicate 1- or 10-ml assay aliquots through 0.22-µm-pore-size filters (Millipore Corp.) and placing each filter on MA plates. Colonies were counted after incubation for 48 h at 37°C. The detection limit of the plate counts was 0.05 CFU ml^{-1}. Incubation of plates for an additional 5 days at 37°C or for 3 days at three different incubation temperatures (20, 30, or 37°C) did not change the number of colonies.

Total cell count

Total cell numbers were determined by direct counting after DAPI (Sigma-Aldrich, Saint Quentin Fallavier, France)-staining (Porter and Feig 1980). Briefly, 2% formalin-fixed samples were filtered using 0.2-µm-pore-size polycarbonate black filters (Dominique Dutscher S.A., Brumath, France) and stained for 20 min with a 2.5-µg ml^{-1} DAPI solution. The filters were rinsed with sterile ultra pure water, mounted on a glass slide, and viewed using low-fluorescence immersion oil under an Olympus epifluorescence microscope with UV excitation.

Viable-cell count

Viable-cell counts were determined using the 5-cyano-2,3-ditolyl tetrazolium chloride (CTC; Polysciences Europe, Eppelheim, Germany) method (Rodriguez et al. 1992). Bacteria with a functioning electron transport chain reduce the CTC in CTC-formazan, forming a red fluorescent precipitate in the cell membrane. Samples were incubated with 3.0 mM CTC, in the presence of 0.025% yeast extract, at room temperature and then fixed with formalin (2% final concentration). Samples were stained with DAPI, as described above. Cells exhibiting red precipitate under green excitation were counted.

Monitoring entry to the VBNC state

Entry into the VBNC state was monitored using CFU enumeration and total and viable counts, as described above.

In vitro resuscitation of VBNC state

When colonies were no longer observable by direct viable counting of 0.1- and 0.5-ml samples, 60-ml samples of water microcosms were inoculated into 90 ml of 1.67 × concentrated nutrient broth (NB), which were incubated at 37°C, with shaking, for seven months. Every 48 hours 0.1 ml of incubated solution was inoculated into 90 ml of NB. Control microcosms were followed in the same manner as infected microcosms and no *Salmonella* were found even after seven months of incubation in NB, confirming the absence of contamination.

In vivo resuscitation essay

In vivo recovery of cells was undertaken using mice as model animals. 30 groups of six six-week-old, male, BALB/c were obtained from the University of Pharmacy of Monastir and maintained in animal facilities for the duration of the experiment. 10 groups of mice were administered an oral dose of 0.2 ml of seawater suspension using a stainless steel gavage needle. 10 other groups were infected by intraperitoneal injection with approximately 10^3 total bacterial cells in 100 µl. The last group was taken as a control. The spleens and livers of dead mice were removed for detection of culturable *Salmonella*. Faeces were collected daily from all animals, weighed, initially diluted (1:10) in phosphate-buffered saline (PBS), and serially diluted for plating.

DNA extraction

Cells were harvested by centrifugation (6,000× *g* for 60 min at 4°C) and resuspended in 400 µl of double-distilled water. The DNA was extracted with Chelex 100 (Walsh et al. 1991).

PCR amplification of the 16S rDNA

Almost the complete 16S rDNA gene was amplified using eubacterial primers Eub 9_27 and Eub 1542. A nested PCR was then carried out, using the fist round PCR products as templates, with P2 and GC-clamped P3 primers for subsequent DGGE analysis, as described in Muyzer et al. (1993). The 50 µl PCR reactions contained 5 pmol of each primer, 1.5 mM $MgCl_2$, 1× PCR buffer (10 mM Tris-HCl; 50 mM KCl, pH 8.3), 200 µM each dNTP and 1 U of Taq polymerase (Roche). The program of the first PCR had an initial denaturation at 94^{o}C for 5 min and then 35 cycles of: denaturation at 94°C for 30s, annealing at 40°C for 45s, extension at 72°C for 90s. A touchdown program was used for the nested PCR. The DNA was denatured at 94°C for 5 min followed by 20 cycles of denaturation at 94°C for 30s, annealing starting at 65°C, then decreasing by 0.5°C in each cycle, for 45s, and extension at 72°C for 90s. This was followed by ten cycles with the same times but a constant annealing temperature of 55°C, and 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 using the D-Gene System (BioRad) in polyacrylamide gels (8% of 37:1 acrylamide:bisacrylamide mixture in 0.5× TAE buffer, 0.75 mm thick, 16 × 10 cm) with a gradient of 30 to 60%

denaturant, according to the manufacturer's guidelines. Gels were run in 0.5× TAE buffer at 200 V and a constant temperature of 60°C for 5 h. DGGE gels were stained using silver stain and scanned using an 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 $MgCl_2$, 1× PCR buffer (20 mM Tris-HCl; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 2 mM $MgSO_4$; 0.1% Triton X-100; 1 mg ml^{-1} nuclease-free BSA) and 250 µM of each dNTP. The thermal cycle had an initial denaturation at 94°C for 2 min, a hot start at 80°C, then 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 checked by PCR using primers flanking the cloning site of the vector. 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). Sequences of about 300 to 500 bp in length were used for an initial identification of the strains using the match program BLAST (Altschul et al. 1997) on the NCBI's homepage (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi).

RESULTS

Decline of Salmonella levels in sterile seawater

Strains of *Salmonella typhimurium* LT2 gradually became nonculturable during prolonged incubation in sterile seawater at 20°C. Fig. 1 shows changes in the viability of the strain exposed to sterile seawater. Despite being introduced into the sterile seawater at very high levels, the *Salmonella* rapidly declined to levels undetectable by plate counts on nutrient agar.

In vitro revival of VBNC Salmonella typhimurium

After two years of starvation, about half of the seawater volume had evaporated from each microcosm and salt crystals had appeared. The inoculation of salty suspension from each microcosm did not result in any culturable strains on a non-selective medium (nutrient agar plates). However, after incubation in NB for 48-hours a biofilm was clearly visible on culture tube

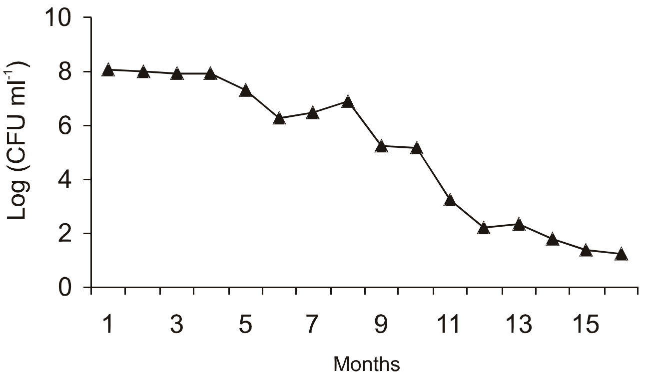

Fig. 1. Decline in number of *Salmonella typhimurium* colony forming units (CFU) in sterile seawater at 20°C.

walls, and the medium started to become turbid. When this medium was plated onto nutrient agar mucous colonies with irregular peripheries appeared. In Api 20 E no biochemical activity was observed. After one month of revival in NB smooth colonies with regular peripheries were observed in association with the mucous colonies. With time in the NB incubation the mucous phenotype decreased and the smooth phenotype increased (Fig. 2).

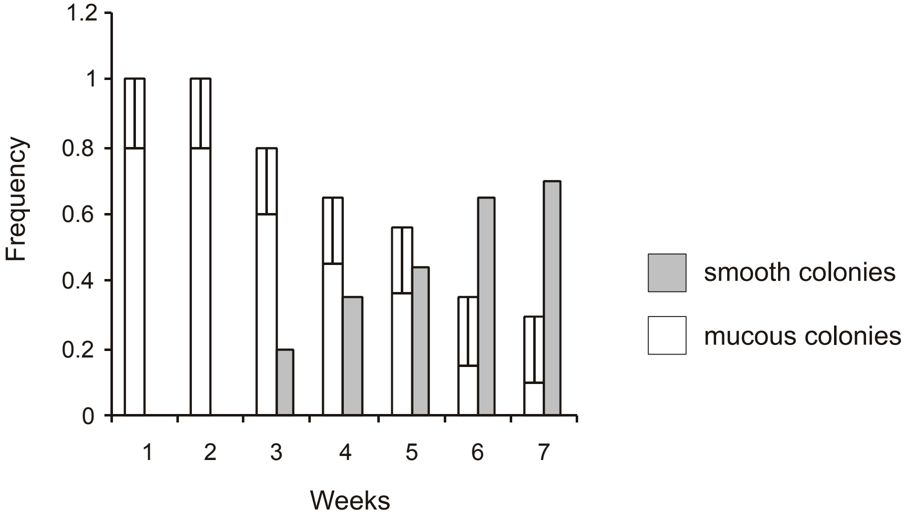

Fig. 2. Evolution of stressed *Salmonella* phenotype when revived in nutrient broth.

After two months of revival in NB, stressed cells acquired the ability to grow in selective media, colony morphology and biochemical activities were acquired.

After three years no water remained in the microcosms, and only salt crystals were visible. Incubation of 1 g of salt in 100 cm^3 of NB led to the formation of biofilms on the upper surfaces of culture tube walls after 48 hours. The mucous phenotype was also observed, but subsequently disappeared during the two and a half month incubation. Colony morphology and biochemical profile of Api 20 E were acquired by three months after resuscitation. After seventeen years of starvation, a revival essay gave the same results as described above. Control microcosms were treated the same as infected microcosms, and no *Salmonella* was observed after seven months of incubation in NB, confirming the absence of contamination.

In vivo revival of VNC Salmonella typhimurium

Mice faeces were observed to be free of *Salmonella* for a week prior to the beginning of the experiment. One day after mice were administered an oral dose of the starved cells *Salmonella* was observed in mice faeces and organs (stomach, large and small intestines, liver and lungs). In SS medium the percentage of *Salmonella* increased, reaching 100% after four days (Fig. 3). *Salmonella* was not observed in the faeces or organs of control mice. Recovery of VBNC *Salmonella* was not observed following intra-peritoneal injection into mice.

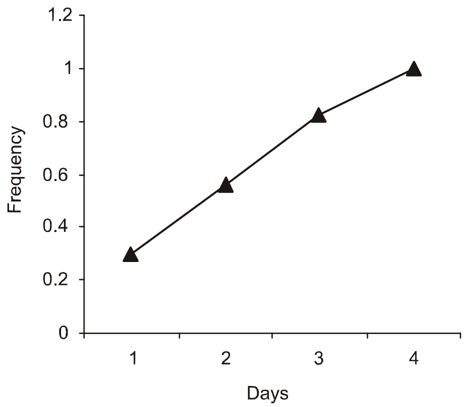

Fig. 3. Evolution of revived *Salmonella* in mice faeces.

PCR-DGGE analysis

The results of the PCR and DGGE confirmed the identities of all strains tested (Fig. 4, 5 a and b). The DGGE 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 the same template due to the GC clamp. The strains having the same sequence identity produced similar banding patterns, indicating that they may have similar copies of 16S rDNA.

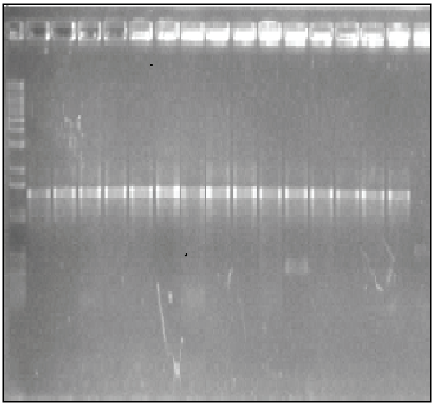

Fig. 4. PCR of 16S RNA gene fragments of *Salmonella* strains.

a b

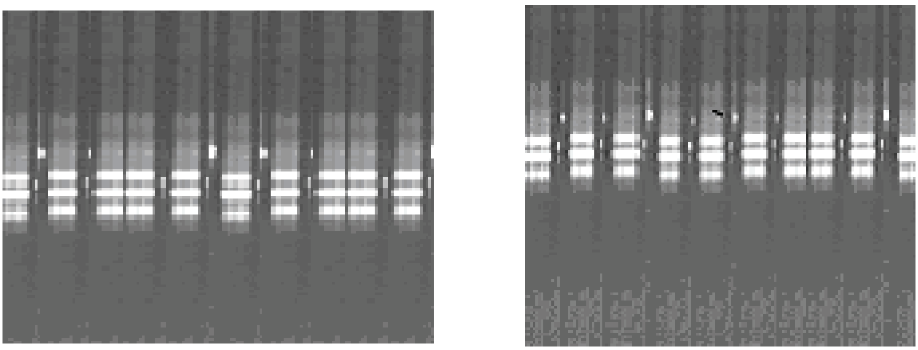

Fig. 5. DGGE of 16S RNA gene fragments of stressed (a) and revived (b) *Salmonella* strains.

Lane 1: *Salmonella typhimurum* initial strain; **Lane 2**: *S. typhimurium* starved for two years in seawater microcosms and resuscitated for 48 hours in NB; **Lane 3**: stressed *Salmonella* revived for ten days in NB (giving mucous colonies); **Lane 4**: stressed *Salmonella* revived for one month in NB (giving smooth colonies); **Lane 5**: stressed *Salmonella* revived for one month in NB (giving mucous colonies); **Lane 6:** stressed *Salmonella* revived for two months in NB (growing in selective medium SS); **Lane 7**: stressed *Salmonella* revived for 24 hours in digestive tract of mice; **Lane 8:** stressed *Salmonella* revived for 48 hours in digestive tract of mice; **Lane 9**: stressed *Salmonella* isolated from mice liver; **Lane 10**: stressed *Salmonella* isolated from mice lungs.

Nucleotide sequence accession numbers

Sequence data of clones generated from these experiments were deposited in the EMBL sequence database, accession number AE008857.1. The results confirmed that the cells studied were closely related to *Salmonella typhimurium* LT4.

DISCUSSION

Strains of *Salmonella* inoculated at high levels into sterile seawater at 20°C and monitored for well over a year displayed declining plate counts and accumulation of large numbers of nonculturable cells. Total cell counts remained constant at the initial inoculation level, while plate counts indicated that the number of viable and culturable cells dropped to less than 1 CFU per ml in about one year and four months (Fig. 1). Similar results from other studies have been interpreted as indications that nonculturable cells were still viable and thus in the VBNC state (Barer et al. 1993, Oliver 1993). 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. This study demonstrates successful resuscitation of such cells by long-term incubation in nutrient broth. Other experiments in this field have varied resuscitation techniques in attempts to return nonculturable cells to states in which they exhibit population increases. The techniques that have been reported to resuscitate nonculturable cells are nutrient addition, temperature shifts, and nutrient addition in the presence of culturable cells (Kaprelyants et al. 1993, Kaprelyants et al. 1994, Votyakova et al. 1995). The present study adds to literature that VBNC forms of *Salmonella* can persist for many (seventeen) years in salt crystals and subsequently be resuscitated by incubation in NB. Incubation in NB resulted in the appearance of a biofilm on the upper surface of cultures after 48 hours. The biofilm concept has drawn attention to the ecological and biotechnological importance of bacteria (Costerton et al. 1987, Costerton et al. 1985). Biofilms are highly organized communities of cells (Davies et al. 1998). Like the cells of tissues that communicate via autocrine and paracrine stimulation, cells of microbial biofilms release chemical compounds that act in concert, reaching threshold densities that signal the initiation of coordinated cellular differentiation events (Miller and Bassler 2001, Parsek and Greenberg 1994, Singh et al. 2000). Mucous colonies were obtained from incubated nutrient broth. In Api 20 E no biochemical activity was observed.

There is unequivocal evidence that bacteria respond to changes in their environment by profound phenotypic variations in enzymatic activity, cell wall composition (Shand et al. 1985), and surface structure (Anwar et al. 1985). Rice et al. (1992) discovered that *V. cholerae* O1 from the Peru epidemic was able to shift to a phenotype having wrinkled or rugose colony morphology. Morris et al. (1996) also reported that *V. cholerae* can shift to a rugose colony morphology associated with the expression of an amorphous exopolysaccharide (EPS) that promotes cell aggregation, and they also confirmed that rugose strains displayed resistance to killing by chlorine and complement-mediated serum bactericidal activity. They also indicated that these rugose strains cause human disease. In fact, compared with the smooth variant, rugose cells displayed increased resistance to chlorine, salt, and oxidative stress (Wai et al. 1998) and had the ability to form biofilms (Mizunoe et al. 1999).

Following nutrient broth incubation, stressed cells progressively acquired initial colony morphologies and biochemical activities. After two months of incubation revived cells acquired the capacity of growing in selective medium. Nutrient addition and temperature shifts have been reported to be effective methods for rescuing bacterial populations from the VBNC state (Kaprelyants et al. 1993).

Resuscitation of cells tested by intra-peritoneal injection into mice was not observed in this study, but resuscitation of ingested cells was clearly observed in the digestive tract, occurring more rapidly than in the in vitro system. Nonculturable *Vibrio cholerae* and *V. vulnificus* have been reported recovering in rabbit and mouse intestines, respectively (Porter and Feig 1980, Rodriguez et al. 1992). However, a recent report indicated that NaCl-stressed *E. coli* O157:H7 did not recover in the mouse intestine. Simultaneous losses of culturability and pathogenicity have been reported in *Campylobacter jejuni*, despite a high level of viability in the cellular population tested (Medema et al. 1992). However, certain studies have demonstrated that VBNC cells retain some pathogenic effects (Pommepuy et al. 1996, Rahman et al. 1994, Rahman et al. 1996) and it is suspected that the cells reverse into a culturable state (Colwell et al. 1996, Steinert et al. 1997). In this study we confirmed the identity of stressed and revived strains by DGGE and sequencing of 16S RNA gene fragments. Fingerprinting techniques such as DGGE allow reproducible comparisons of DNA profiles obtained from microbial communities to be made (Muyzer et al. 1998). 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 followed by sequencing of 16S rDNA were optimized for the identification of stressed and revived *Salmonella.*

CONCLUSION

The results obtained in this study demonstrated that VBNC *S. typhimurium* can persist for many years in salt crystals. Recovery was obtained after long-term incubation in nutrient broth and also in mice when administered orally, but not by intra-peritoneal injection.

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