

Resuscitation of eleven-year VBNC *Citrobacter*

Amel Dhiaf, Amina Bakhrouf and Karl-Paul Witzel

ABSTRACT

Citrobacter freundii strain WA1 was stressed by incubation in seawater microcosms for eleven years. After two 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 *Citrobacter freundii* was obtained by incubation in nutrient broth even after eleven years of starvation. To see whether the samples contained the same strain of *Citrobacter freundii* inoculated 11 years ago. The complete 16S rRNA gene was PCR amplified and sequenced from initial, stressed and revived strains of *Citrobacter freundii* strain WA1. The 16S rRNA gene sequences from eleven-year stressed strains were homologous with a high degree of similarity to the GenBank reference strain and were identical to each other.

Key words | *Citrobacter freundii* strain WA1, resuscitation, seawater, stress

Amel Dhiaf (corresponding author)
Cabinet Dr Allaya, Ouled Salah 5116, Ksour, Essef,
Tunisia
Tel.: 00216098560041
Fax: 00216073461000
E-mail: dhiafamel@yahoo.fr

Amina Bakhrouf
Cabinet Dr Allaya, Ouled Salah 5116, Ksour,
Essef,
Tunisia

Karl-Paul Witzel
Department of Evolutionary Genetics,
Max-Planck Institute for Evolutionary Biology,
Ploen
Germany

INTRODUCTION

Enterobacteria are important food-borne pathogens which 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 nonculturable (VNC) (Oliver 1995; Rahman *et al.* 1996). 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 nonselective plating media or to grow in nonselective broth media. Nevertheless, in some instances these organisms may cause disease (Sylvester *et al.* 2001). We previously demonstrated that VBNC *Salmonella* persisted for many years in seawater microcosms, recovery was obtained in nutrient broth (Dhiaf *et al.* 2001) and in embryonated chicken eggs (Dhiaf & Bakhrouf 2004). We demonstrated also that VBNC *Vibrio cholerae* persisted in seawater for many years and resuscitation was obtained by long-term incubation in nutrient broth (Dhiaf & Bakhrouf 2005a,b). We observed also that *Bacillus* spores persisted

for many years in crystal salt, recovery was obtained by incubation in nutrient broth (Dhiaf & Bakhrouf 2005a,b). The aim of this study is to see if *Citrobacter* could persist as other bacteria studied for many years in environmental microcosms.

METHODS

Citrobacter freundii strain WA1 was kindly provided by P^r A. Bakhrouf, Pharmacy University of Monastir. The strain was grown in Trypticase soy broth (bioMérieux) at 37°C for 18 h, centrifuged (4,000 × g for 10 min), washed in NaCl solution (salinity, 9‰) two times, and resuspended in NaCl solution (salinity, 9‰).

Microcosm experiments were performed by using the seawater collected: (Monastir beach, Tunisia); Pyrex beakers (1 litre) acted as microcosms. Each contained 300 ml of autoclaved seawater. The bacterial cells were suspended in the autoclaved seawater until a density of 10⁹ bact/ml.

doi: 10.2166/wh.2008.131

Pyrex beakers were covered with sterilized cotton protected with sterilized cellulose paper. We used 30 microcosms containing bacterial suspension and 30 microcosms containing only sterilized seawater, used as control. The different microcosms were set up as replications and each and all of them were used for plate counting, total and viable cell counts. The different microcosms were stored in a protected area at the laboratory of microbiology, Pharmacy University of Monastir. Storage was done in the dark without shaking. Temperature varied from 20°C to 30°C. The total cell number was determined by direct counting after 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, Saint Quentin Fallavier, France) staining (Porter & Feig 1980). Briefly, 2% formalin-fixed samples (1 ml) were filtered using 0.2- μ m-pore-size polycarbonate black filters (Dominique Dutscher S.A., Brumath, France) and stained for 20 min with a 2.5- μ g/ml DAPI solution. The filters were rinsed with sterile ultrapure water, mounted on a glass slide, and viewed using low-fluorescence immersion oil under an Olympus epifluorescence microscope with UV excitation.

After two years of starvation colonies were no longer observed 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-fold-concentrated nutrient broth. Incubation was done at 37°C with shaking for seven months. Every 48 hours 60 ml of incubated solution was inoculated into 90 ml of 1.67-fold-concentrated nutrient broth. After two years of starvation, the inoculation of suspension from each microcosm did not give any culturable strain. After two months of resuscitation in nutrient broth, stressed cells determined the cultivability, colonies morphology and biochemical activities were also determined.

After two and eleven years of starvation, resuscitation assay gave the same results described above. Control microcosms were followed as infective microcosms and no *Citrobacter freundii* strain WA1 strain was found even after seven months of resuscitation in nutrient broth which confirmed the absence of contamination. As demonstrated by the results presented in this study, the resuscitation was succeeded by incubation in nutrient broth. The techniques which have been reported to resuscitate nonculturable cells are nutrient addition, temperature shifts, and nutrient

addition in the presence of culturable cells (Sylvester *et al.* 2001). The present study adds to literature that VBNC forms of *Citrobacter freundii* strain WA1 could persist for many years (eleven years) in seawater microcosms and could be resuscitated by incubation in nutrient broth.

To see whether the samples contained strains of *Citrobacter freundii* strain WA1 inoculated eleven years ago, we used PCR amplification of the 16S rDNA followed by Denaturing gradient gel electrophoresis (DGGE) then Cloning and sequencing. The Genomic DNA extraction was realized as follows; colonies of at least 1 mm diameter from each isolate were individually picked from the culture plates. Bacterial cells were transferred into microfuge tubes containing 100 μ l suspension of 5% Chelex-100 sodium form (100–200 mesh) in sterile TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0] buffer. Samples were vortex-mixed, boiled for 15 min, and then centrifuged for 5 min at 12,000 g. The supernatant was stored at 4°C for further analysis. One microlitre was added to the PCR assay (Walsh *et al.* 1991).

PCR amplification of the 16S rDNA was realized as follows. Almost the complete 16S rDNA gene was amplified initially using eubacterial primers Eub 9_27 5'-GAG TTT GAT CCT GGC TCA G-3' and Eub 1542 5'-AGA AAG GAG GTG ATC CAG CC-3'. PCR was made in a total volume of 50 μ l containing 5 pmol of each primer, MgCl₂ 1.5 mM, PCR buffer 1X (10 mM Tris-HCl; 50 mM KCl, pH 8.3), dNTP 200 μ M 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.

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 \times 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 and 5 h. The DGGE gels were stained by silver staining and scanned using an HP scanjet 5470c.

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, MgCl₂ 1.5 mM, 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 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 BLASTN on the NCBI's homepage (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

RESULTS AND CONCLUSIONS

The results mentioned (Figure 1) confirmed the identity of all strains tested. The patterns were reproducible and

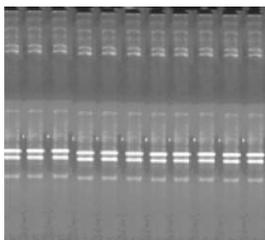


Figure 1 | DGGE of 16S RNA gene fragments.

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 identity tested produced similar band patterns indicating that they may have similar copies of 16S rDNA.

The sequences for isolated cell clones were deposited in the EMBL sequence database. The results confirmed that the cells studied related to *Citrobacter freundii* strain WA1.

The results obtained in this study demonstrated that VBNC *Citrobacter freundii* strain WA1 persisted for many years in seawater microcosms. Recovery was obtained after incubation in nutrient broth.

Lane 1: *Citrobacter freundii* strain WA1 initial strain; **Lane 2:** *C. freundii* starved for two years in seawater microcosms and resuscitated for 48 hours in NB; **Lane 3:** *C. freundii* starved for two years in seawater microcosms and resuscitated for ten days in NB; **Lane 4:** *C. freundii* starved for two years in seawater microcosms and resuscitated for one month in NB; **Lane 5:** *C. freundii* starved for two years in seawater microcosms and resuscitated for two months in NB; **Lane 6:** *C. freundii* starved for eleven years in seawater microcosms and resuscitated for 48 hours in NB; **Lane 7:** *C. freundii* starved for eleven years in seawater microcosms and resuscitated for ten days in NB; **Lane 8:** *C. freundii* starved for eleven years in seawater microcosms and resuscitated twenty days in NB; **Lane 9:** *C. freundii* starved for eleven years in seawater microcosms and resuscitated for one month in NB; **Lane 10:** *C. freundii* starved for eleven years in seawater microcosms and resuscitated for two months in NB; **Lane 11:** *C. freundii* starved for eleven years in seawater microcosms and resuscitated for two months in NB.

REFERENCES

- Dhiaf, A. & Bakhrouf, A. 2004 Recovery in embryonated chicken eggs of viable but non-culturable *Salmonella*. *Food Agr. Environ. J.* 2(2), 104–107.
- Dhiaf, A. & Bakhrouf, A. 2005a Resistance of *Bacillus* spores in salt crystals. In *Proceedings of the Fifth International Conference on the Mediterranean Coastal Environment*, (ed. E. Ozhan), Vol 1. pp. 301–303. MEDCOST 1, Turkey.

- Dhiaf, A. & Bakhrouf, A. 2005b Adaptation of *Vibrio Cholerae* to extreme seawater stress. In *Proceeding of the Fifth International Conference on the Mediterranean Coastal Environment*, (ed. E. Ozhan), Vol 1. pp. 304–306. MEDCOST 1, Turkey.
- Dhiaf, A., Chnity, N. & Bakhrouf, A. 2001 Adaptation of *Salmonella* to extreme seawater stress. In *Proceeding of the Fourth International Conference on the Mediterranean Coastal Environment* (ed. E. Ozhan). MEDCOST 1, pp. 760–766.
- Oliver, J. D. 1995 **The viable but non-culturable state in the human pathogen *Vibrio vulnificus***. *FEMS Microbiol. Lett.* **133**, 203–208.
- Porter, K. G. & Feig, Y. S. 1980 The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.* **25**, 943–948.
- Rahman, I., Shahamat, M., Chowdhury, M. A. R. & Colwell, R. R. 1996 Potential virulence of viable but nonculturable *Shigella dysenteriae* type 1. *Appl. Environ. Microbiol.* **62**, 115–120.
- Sylvester, D. M., Taylor, R. & LaHann, T. R. 2001 Viable but nonculturable bacteria: a public health threat? *Infect. Dis. Rev.* **3**, 70–82.
- Walsh, P. S., Metzger, D. A. & Higuchi, R. 1991 Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTechniques* **10**, 506–513.

First received 16 October 2007; accepted in revised form 28 January 2008. Available online March 2008