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Resuscitation of ten-year VBNC *Klebsiella oxytoca*

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Abstract: *Klebsiella oxytoca* was stressed by incubation in seawater microcosms for ten years. Despite being introduced into sterile seawater, the added *Klebsiella oxytoca* declined to levels undetectable by plate counts on nutrient agar. 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 viable but non culturable (VBNC) cells so that subsequent plating yielded observable colonies for significantly extended periods of time. Recovery of VBNC *Klebsiella oxytoca* was obtained by incubation in nutrient broth even after ten years of starvation. To see whether the samples contain the same strain of *Klebsiella oxytoca* inoculated 10 years ago, the complete 16S rDNA gene was PCR amplified and sequenced from initial, stressed and revived strains of *Klebsiella oxytoca*. The 16S rDNA gene sequences from ten-year stressed strains were homologous in a high degree of similarity to the GenBank reference strain and were identical to each other.

Résumé : *Reviviscence de cellules viables mais non cultivables de* Klebsiella oxytoca*. Klebsiella oxytoca* a été stressée pendant dix ans dans des microcosmes d'eau de mer. Nous avons observé que le stress marin induit une évolution vers l'état viable non cultivable (VNC). Après deux ans d'incubation, aucune cellule cultivable n'a été observée. Le processus de reviviscence des formes VNC de *Klebsiella oxytoca,* stressées pendant dix ans, a été obtenu suite à une incubation plus ou moins prolongée en bouillon nutritif. Pour identifier les différentes souches de *Klebsiella oxytoca*, le gène ADNr 16S a été amplifié par PCR et séquencé. En se référant aux données de GenBank, les souches initiales ainsi que celles stressées pendant dix ans sont homologues et présentent un haut degré de similarité.

Keywords: *Klebsiella oxytoca* ● Seawater ● Stress ● Resuscitation ● Nosocomial infections

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Introduction

Bacteria of the genus *Klebsiella* are a frequent cause of nosocomial infections (Horan et al., 1988). Their non-clinical habitats encompass the gastrointestinal tract of mammals as well as environmental sources such as soil, surface waters and plants (Bagley, 1985). Environmental isolates have been described as being indistinguishable from human clinical isolates with respect to their biochemical reactions and virulence (Matsen et al., 1974). While the medical significance of *Klebsiella* obtained in the natural environment is far from clear, such habitats are thought to be potential reservoirs for the growth and spread of these bacteria, which may colonize animals and humans (Knittel et al., 1977). 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) (Bogosian et al., 1998). Recovery of culturable cells from a population of nonculturable cells would provide convincing support for the VBNC hypothesis (Kaprelyants et al., 1993). The purpose of this study was to investigate the maintenance of viability of *Klebsiella oxytoca* in seawater for extended period of time. The strain was experimentally stressed by exposure in seawater microcosms for ten years from 8th January 1994 to 10th January 2004. Resuscitation process was realized after incubation in nutrient broth.

Material and Methods

Strain and inoculum preparation

Klebsiella oxytoca was kindly provided by Professor Amina Bakhrouf, Pharmacy University of Monastir. The strain was grown in Trypticase soy broth (bioMérieux, France) at 37°C for 18 h, centrifuged (4,000 *g* for 10 min), washed three times, and resuspended in NaCl solution (salinity = 9).

Experimental design

Seawater was collected in Pyrex beakers (1 litre) from the surface of Monastir beach, Tunisia. Pyrex beakers acted as microcosms, each contained 300 ml of autoclaved seawater. The bacterial cells were suspended in the autoclaved seawater until a density of 10^9 bact.ml^{-1}. We used 30 microcosms containing bacterial suspension and 30 microcosms containing only sterilized seawater as control. The different microcosms were set up as replications and all of them were used for plate counting, total and viable cell counts, and *in vitro* and *in vivo* analysis. The different microcosms were stored in protected area at laboratory of microbiology, Pharmacy University of Monastir. Storage was done in the dark without shaking. Temperature varied from 20°C to 30°C.

Colony enumeration

Cell culturability was determined using nutrient agar plates (Difco Laboratories). Colonies identity was confirmed by Api 20 E (bioMérieux, France). CFU.ml^{-1} 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 nutrient agar plates. Colonies were counted after incubation for 48 h at 37°C. 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 increase or change the number of colonies. These tested were duplicated and performed during the first period of starvation which consisted in seventeen months.

Total cell count

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 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 ultrapure 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); briefly, 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. A minimum of 20 microscope fields were counted.

Monitoring entry to the VBNC state

Entry to the VBNC state was monitored using CFU.ml^{-1} enumeration and total and viable counts, as described above. Mean and standard deviation of the thirty microcosms were calculated for each sampling time (17

months). A 1-way ANOVA test was calculated for each counting method over time. Statistics were carried out using the statistical package included in Excel and the statistical software MacAnova.

Molecular analysis

To see whether the samples contain strains of *Klebsiella oxytoca* inoculated 10 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 following: 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 µl 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 following. 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') (Stackebrandt & Liesack, 1993). PCR was made in a total volume of 50 µl containing 5 pmol of each primer, $MgCl_2$ 1.5 mM, 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 for DGGE 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 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 and 5 h. The DGGE gels were stained by silver staining and scanned using a HP scanjet 5470c. For DGGE, products of about 200 bp were generated with the primers P2/P3 according to Muyzer et al. (1993).

PCR products to be cloned were amplified with *Pfu* DNA polymerase (Promega) using the oligonucleotide primers Eub9-27F and Eub1542R (Stackebrandt & Liesack, 1993) in a total reaction volume of 50 µl containing 25 pmol of each primer, $MgCl_2$ 1.5 mM, PCR buffer 1x (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. 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

Decline of Klebsiella oxytoca *level in sterile seawater*

Mean values of the colony enumeration, total-cell counting, and viable-cell counting were calculated every month (Fig.1). Figure 1 shows the responses of *Klebsiella oxytoca* to incubation in seawater. Strains of *Klebsiella oxytoca* inoculated into sterile seawater at 20°C and monitored for nearly one year and four months, displayed declining plate counts (colony enumeration) and accumulation of large numbers of nonculturable cells. The results demonstrated that *Klebsiella oxytoca* cells could enter the VBNC stage, while no colony growth was observed. The numbers of viable cells *Klebsiella oxytoca* strain decreased slightly over time (Fig. 1).

The decline in number of colonies according to the colony enumeration counting method was statistically significant ($F = 342$, $p < 0.001$). The decline according to the viable-cells counting method was also statistically significant ($F = 342$, $p < 0.001$), whereas values obtained for the total-cell counting method did not change significantly over time ($F = 1.540$, $p > 0.05$).

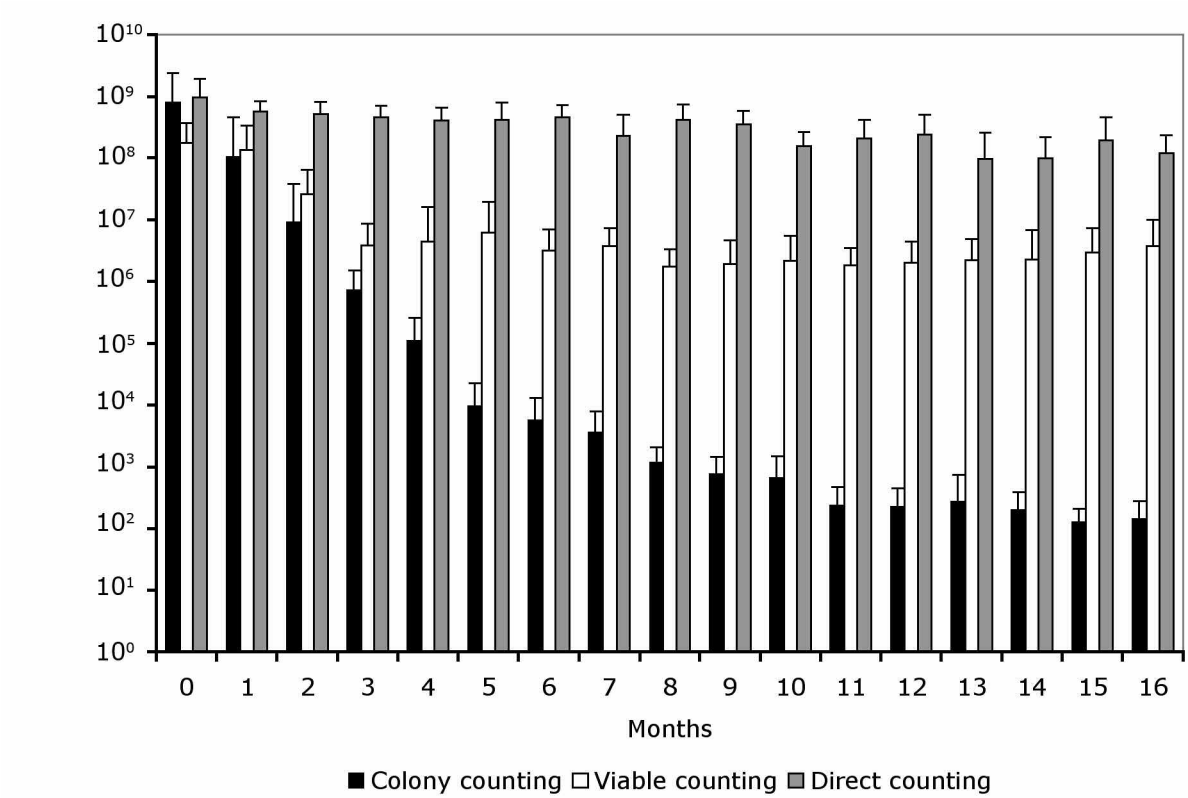

Figure 1. *Klebsiella oxytoca.* Entrance of logarithmic-phase cells into the VBNC state incubated in seawater. The standard deviation was calculated with the 30 microcosms prepared for each counting method.

Figure 1. *Klebsiella oxytoca.* Entrée dans l'état viable non cultivable des cellules en phase logarithmique incubées dans l'eau de mer. L'écart-type est calculé pour chaque méthode de comptage à partir des 30 microcosmes.

In vitro *reviviscence of VBNC* Klebsiella oxytoca

When 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 hour, 60-ml of incubated solution was inoculated into 90 ml of 1.67-fold-concentrated nutrient broth. After two years of starvation, we attempted to study whether starved *Klebtiella* remained cultivable and if resuscitation could be obtained. The inoculation of suspension from each microcosm did not give any cultivable strain. After two months of resuscitation of VBNC *Klebsiella oxytoca* in nutrient broth, stressed cells determined the cultivability, colonies morphology and biochemical activities. After three and ten years of starvation, resuscitation essay gave the same results described above. Control microcosms were followed as infective microcosms and no *Klebsiella oxytoca* germ was found even after seven months of resuscitation in nutrient broth which confirmed the absence of contamination.

PCR-DGGE analysis

Results (Fig. 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 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.

Sequence analyses

The sequences for isolated cell clones were analyzed by BLAST. The results confirmed that the cells were related to *Klebsiella oxytoca*. Sequences were 100% identical to each other, and have as best match in the BLAST results the sequence of *Klebsiella oxytoca* Accession Number AF319525.3.

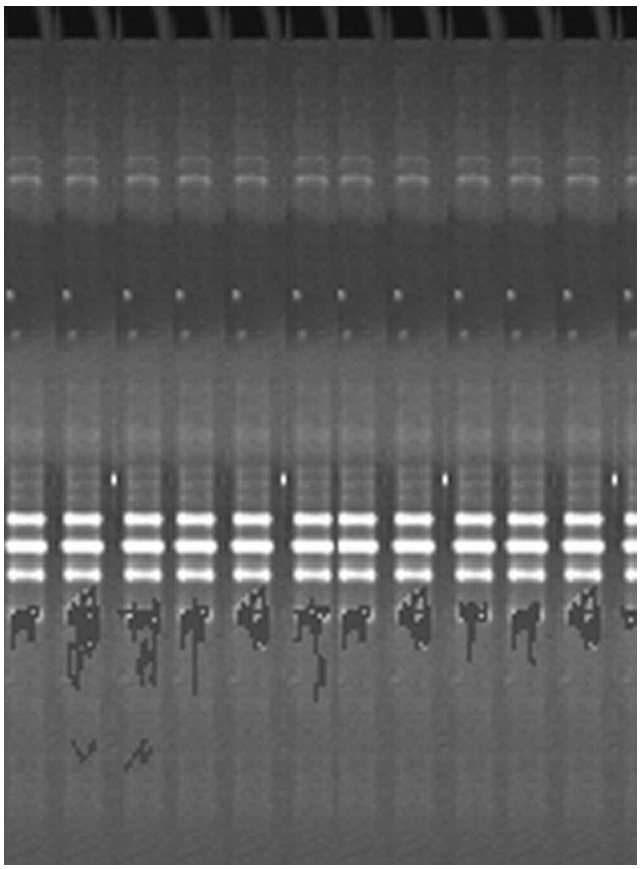

Figure 2. *Klebsiella oxytoca.* Denaturing gradient gel electrophoresis of 16S RNA (rDNA?) gene fragments. **Lane 1**: initial strain. **Lane 2**: strain starved for 2 years in seawater microcosms and resuscitated for 48 hours in NB. **Lane 3**: strain starved for 2 years and resuscitated for 2 months in NB. **Lane 4**: strain starved for 3 years and resuscitated for 48 hours in NB. **Lane 5**: strain starved for 3 years and resuscitated for 3 months in NB. **Lane 6**: strain starved for 10 years and resuscitated for 48 hours in NB. **Lane 7**: strain starved for 10 years and resuscitated for 72 hours in NB. **Lane 8**: strain starved for 10 years and resuscitated for one week in NB. **Lane 9**: strain starved for 10 years and resuscitated for one month in NB. **Lane 10**: strain starved for 10 years and resuscitated for 2 months in NB. **Lane 11**: strain starved for 10 years and resuscitated for 3 months in NB.

Figure 2. *Klebsiella oxytoca.* Electrophorèse sur gradient de gel denaturant de fragments de gène 16S ADNr. **Piste 1**: souche initiale. **Piste 2**: souche stressée pendant 2 ans dans des microcosmes d'eau de mer et revivifiés pendant 48 h dans le milieu nutritif (NB). **Piste 3**: souche stressée pendant 2 ans et revivifiée pendant 2 mois dans NB. **Piste 4**: souche stressée pendant 3 ans et revivifiée pendant 48 h dans NB. **Piste 5**: souche stressée pendant 3 ans et revivifiée pendant 3 mois dans NB. **Piste 6**: souche stressée pendant 10 ans et revivifiée pendant 48 h dans NB. **Piste 7**: souche stressée pendant 10 ans et revivifiée pendant 72 h dans NB. **Piste 8**: souche stressée pendant 10 ans et revivifiée pendant une semaine dans NB. **Piste 9**: souche stressée pendant 10 ans et revivifiée pendant un mois dans NB. **Piste 10**: souche stressée pendant 10 ans et revivifiée pendant 2 mois dans NB. **Piste 11**: souche stressée pendant 10 ans et revivifiée pendant 3 mois dans NB.

Discussion

Three different methods were used to monitor the cells of *Klebsiella oxytoca* stressed in seawater: plate count, DAPI staining, and CTC viable count (Cappelier et al., 1997). Strains of *Klebsiella oxytoca* inoculated into sterile seawater at 20°C and monitored for nearly one year and four months, displayed declining plate counts and accumulation of large numbers of nonculturable cells. This result has been interpreted in other studies as an indication that nonculturable cells were still viable and thus in the VBNC state (Barer et al., 1993). The results demonstrated that *Klebsiella oxytoca* cells could enter the VBNC stage, while no colony growth was observed. After two years of starvation, we attempted to study whether starved *Klebtiella* remained culturable and if resuscitation could be obtained. Resuscitation of VBNC *Klebsiella oxytoca* was obtained in nutrient broth, stressed cells determined the cultivability, colonies morphology and biochemical activities.

After three and ten years of starvation, resuscitation essay gave the same results described above. As demonstrated by the results presented in this study, the resuscitation was succeeded by incubation in nutrient broth. 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, (Sylvester et al., 2001). The present study adds to literature that VBNC forms of *Klebsiella oxytoca* could persist for ten years in seawater microcosms and could be resuscitated by incubation in nutrient broth.

We confirmed in this study the identity of stressed and revived strains by DGGE and the sequencing of 16S rDNA 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 following by sequencing of 16S rDNA were optimized for the identification of stressed and revived *Salmonella.*

The results obtained in this study demonstrated that VBNC *Klebsiella oxytoca* resisted for ten years in seawater microcosms. Recovery was obtained after incubation in nutrient broth.

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