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Survival of *Vibrio fluvialis* in seawater under starvation conditions

Ben Kahla-Nakbi Amel*, Besbes Amine, Bakhrouf Amina

Laboratoire d'Analyse et de Contrôle des Polluants Chimiques et Microbiologiques de l'Environnement, Faculté de Pharmacie de Monastir- Rue Avicenne, 5000 Monastir, Tunisia

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Summary

The viability of *Vibrio fluvialis* in seawater microcosms, with and without sediment was investigated. The strain survived as culturable bacteria for at least 1 year and the expression of its virulence factors was maintained. In microcosms containing sediment *Vibrio fluvialis* was more stable. Viable but nonculturable (VBNC) cells of *Vibrio fluvialis* were able to resuscitate to the culturable state up to 6 years of incubation in marine sediment. These cells recuperate their initial biochemical characteristics after 3 months of incubation in marine broth. Amplified 16S ribosomal DNA (rDNA) restriction analysis (ARDRA) was used to confirm that it is the same strain of *Vibrio fluvialis* which resists in all microcosms during a long period of time.

Introduction

Vibrio fluvialis is a halophilic pathogen frequently found in marine environments or marine products. Currently, *V. fluvialis* is known to cause serious infections because its clinical symptoms of gastroenteritis are very similar to those caused by *V. cholerae*. This became even more serious after the recent characterisation of an enterotoxigenic El Tor-like haemolysin in *V. fluvialis*, which represents one of the virulence factors of *V. cholerae* (Kothary et al., 2003).

Usually, lack of nutrient is the most common environmental stress which microorganisms routinely encounter in natural ecosystems. However, it was found that *Vibrio* spp. can survive for a long time during starvation by sequential changes in cell physiology and gradual changes in morphology (Morita, 1993; Östling et al., 1993; Albertson et al., 1990). Moreover, it was reported that some species develop the so-called viable but

Abbreviations: i, strain before incubation in seawater microcosms; M, month; P, period of incubation of sediment in marine broth; R, resistant; S, sensible; ms, strain incubated in marine sediment (after 8 months of incubation in microcosms); sw, strain incubated in seawater (after 8 months of incubation in microcosms); VBNC, viable but nonculturable; W, week

*Corresponding author. Tel.: +216 21108431; fax: +216 461 830.
E-mail address: Nakbi_amel@yahoo.fr (B.K.-N. Amel).

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nonculturable (VBNC) state in response to certain stress conditions (Biosca et al., 1996; Jiang and Chai, 1996; Oliver and Wanucha, 1989). It has been proposed that the VBNC state is an adaptative strategy of microorganisms against stress from which cells may be able to recover once optimal conditions are restored (Huq and Colwell, 1995; Nybroe, 1995; Oliver, 1995). VBNC state was described for many *Vibrio* species [*V. anguillarum*, *V. campbellii*, *V. cholerae*, *V. fischeri*, *V. harveyi*, *V. mimicus*, *V. natriegens*, *V. parahaemolyticus*, *V. proteolyticus*, *V. vulnificus* (McDougald et al., 1998)].

To our knowledge, none of the reported studies in the literature has addressed the *V. fluvialis* VBNC state in seawater during a long period of time. The aim of the present work was to investigate the effect of marine stress on the behaviour of *V. fluvialis* incubated in seawater microcosms. Resuscitation experiments were conducted to establish the resistant profile of this species.

Material and methods

Bacterial strains and growth conditions

A strain of *V. fluvialis* was isolated during an epizootic event in the Tunisian aquaculture centre from internal organs of diseased sea bass (*Dicentrarchus labrax*), reared in tanks with marine water. The strain was first identified by API 20E (Bio-Mérieux) strips and further characterised by using morphological, physiological, and biochemical tests (Holt et al., 1994). Stock cultures were frozen at −80 °C with 20% (vol/vol) glycerol, and the strain was routinely cultured on tryptic soy agar or in tryptic soy broth supplemented with 1% NaCl (TSA-1 and TSB-1, respectively) (Difco) at 22 °C.

Survival assay

Two experimental microcosms were constructed: one containing only seawater and the other containing seawater supplemented with sediment (1:4, V/V). Water and sediment were taken from Tunisian coast, Monastir (salinity 4%, pH 8), transported in cold storage containers and immediately sterilised (Magariños et al., 1994). For starvation experiments, a 24 h culture in marine broth was centrifuged at 12000*g*, 4 °C, 15 min, and the pellet was washed three times with sterile seawater. Then the cells were inoculated into Erlenmeyer flasks containing 500 ml of sterile seawater or seawater plus sediment to a concentration of approximately 10^7–10^8 colony forming units (CFU) per ml. All microcosms were incubated in a static state at ambient temperature.

Enumeration techniques

Microcosms were sampled daily during the first week, weekly during the first 3 months, and then once a month. Plate counts of culturable cells of all samples were determined by the drop plate method (Hoben and Somasegaran, 1982), using marine agar (MA). The plates were incubated at room temperature for 24–48 h.

Biochemical activities and exoenzymes expression

Biochemical activities of the initial cells and the cells incubated in seawater and marine sediment were studied with API 20E (Bio-Mérieux), and exoenzymes were tested with the API Zym System (Bio-Mérieux) containing 19 substrates. The activities of other enzymes were determined following inoculation of cultures onto TSA-1 to which the following substrates had been added: 0.2% starch for amylase, 1% skim milk for caseinase activity, 1% gelatine for gelatinase, 1% Tween 80 for lipase, 5% egg yolk for phospholipase (lecithinase) and 5% human blood cells for haemolysin.

Antimicrobial susceptibility tests

Sensitivity to antimicrobials was performed by disk diffusion test according to the method recommended by the National Committee for Clinical Laboratory Standards. The following drugs were used: oxytetracycline (30 μg), flumequine (30 μg), chloramphenicol (30 μg), oxolinic acid (2 μg), trimethoprim-sulphametoxazole (25 μg), nitrofurantoin (30 μg), erythromycin (15 μg), furazolidone (50 μg), kanamycin (30 μg), cefotaxime (30 μg), vancomycin (30 μg), clindamicyn (2 μg) and ampicillin/sulbactam (20 μg). Values in brackets indicate the charge per disk.

Resuscitation of VBNC cells

Starved cells of *V. fluvialis* incubated in marine sediment microcosms at a static state at room temperature were resuscitated after 6 years by addition of nutrient broth and culturability in marine agar and biochemical activities using the API 20E galleries (Bio-Mérieux) tested during 3 months.