MaCuMBA

Marine Microorganisms: Cultivation Methods for Improving their Biotechnological Applications

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Deliverable D3.7

Culturing protocols minimizing VBNC state in DHABs isolates

Organisation name of lead contractor: UMIL

Due date of deliverable: M30.

Actual submission date: M36. We obtained an extension to M36.

Revision: V.1

Project co-funded by the European Commission within the Seventh Framework Programme (2007-2013)	
Dissemination Level	
PU Public	**X**
PP Restricted to other programme participants (including the Commission Services)	
RE Restricted to a group specified by the consortium (including the Commission Services)	
CO Confidential, only for members of the consortium (including the Commission Services)	

List of reviewers

Issue	Date	Implemented by
v.1	16.07.2015	UMIL
v.2	24.07.2015	G Le Blay
v.3	27.07.2015	UMIL

Indicate any document related to this deliverable (report, website, ppt etc) and give file name

** Please attach deliverable documents and any additional material if needed.*

Summary

Objective:

The aim of this deliverable was to verify if MaCuMBA isolates were able to enter into a state known as "viable but nonculturable" (VBNC). We studied in particular a subcollection of bacterial strains isolated from symbiotic assemblages associated to mangrove crabs. VBNC state describes the condition for which the normal bacterial growth on routine bacteriological media is limited (Oliver, 2010). In this condition, bacteria lose their growth capability on suitable media, maintaining however their viability and metabolic activity. The information obtained with the completion of this deliverable could be used to improve cultivation approach experiments, in order to avoid bacteria to enter into a dormancy state, resulting in a lack of cultivation, or as a base to develop novel strategies that allow the bacteria to exit from the VBNC state (resuscitation).

Rationale and experiment description:

In the literature, many bacteria have been described as able to enter into VBNC state. One of the most known bacterial genus for its capacity to enter VBNC state is *Vibrio*, with *Vibrio vulnificus* and *V. cholerae* as the most investigated species. In our collection, several isolates belonging to the species *V. natriegens*, *V. fortis* and *V. hepatarius* have been selected for the experimentations. Besides these isolates, reference strains belonging to *V. harveyi* BAA-1117^{TM}, *V. natriegens* ATCC14048 and *V. anguillarum* 90-11-287 species have been also included. The strains have been then used for the preparation of microcosms in artificial seawater. Briefly, after an overnight growth at 30°C in rich TSB medium (tryptic soy broth) supplemented with 2.5% NaCl, a fixed number of cells for each strain has been inoculated in sterile artificial seawater to mimic starvation. Cultures were then moved to 4°C. At time 0 (beginning of the experiment) and after fixed experimental times, microcosms have been sampled to measure live, dead and cultivable cells. Measurements of viable and dead cells have been done with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes®, Life Technologies, Italy). VBNC fraction has been calculated by subtracting cultivable cells (obtained by plate counts) to viable ones.

Once the strains' ability to enter into the VBNC state was verified, the next step was to find which stimuli could "resuscitate" them from their dormancy state. Resuscitation from the VBNC state occurs when cells return culturable on routine media. To prove that true resuscitation happens is challenging: a simple growth of a small fraction of undetected culturable cells among the VBNC state-induced population, could indeed be confused as resuscitated cells (Oliver, 2010). To gain information on this topic, we decided to apply some of the most common and studied factors promoting resuscitation, i.e. the reversal of the stress (incubation of cultures at room temperature; Oliver, 2010) or the addition of bacterial supernatants (Ayrapetyan et al., 2014; Mukamolova et al., 1998). VBNC induced cells have been exposed to room temperature with or without the addition of bacterial supernatants of selected bacteria (S4: *Pseudoalteromonas* sp. UU89, S2: *V. harveyi* BAA-1117, S8: *Vibrio fortis* UU24). Aliquots taken from stored microcosms at 4°C have been incubated at room temperature for 2 or 24 hours. After these periods, they have been plated on MA (marine agar) or TSA (tryptic soy agar) and colonies have been counted. Aliquots have been also exposed to different treatments, e.g. addition of TSB or addition of supernatants of selected bacteria.

Results:

VBNC state induction. The microcosm containing the bacterium *V. harveyi* BAA-1117 showed the most interesting results. Indeed, a reduction of cultivation, both on MA (marine agar) and TSA (tryptic soy agar) media was obtained, with a strong reduction of cultivation on TSA. Interestingly, the colony growth rate was also affected (Fig. 1A).

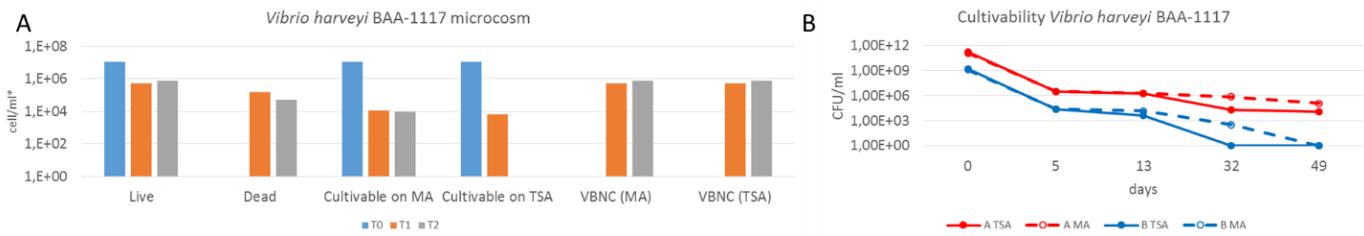

Fig. 1. A) Measurements of live, dead, cultivable (both on MA and TSA) and VBNC-state induced (both on MA and TSA) cells. Vertical axis*: for cytofluorimetric measurements related to live or dead counts we refer to cells/ml; for cultivable measurements we refer to CFU/ml; the estimation of VBNC cells has been obtained by subtracting the number of CFU/ml to the number of live cells/ml measured by cytofluorimetry. T1: 87 days from T0; T2: 122 days from T0. B) Cultivability of *V. harveyi* BAA-1117 according to the initial concentration of cells (A: 1.7×10^{11} cells/ml; B: 1.7×10^{9} cells/ml) along the time course of the experiment on different media (TSA and MA). "A TSA" and "A MA" refer to cultivability measurements on TSA and MA, respectively, starting from inoculum A; "B TSA" and "B MA" refer to cultivability measurements on TSA and MA, respectively, starting from inoculum B.

As reported in Figure 1, after 122 days (T2), no colonies were retrieved on TSA plates. In particular, Figure 1B highlights the decrease of cultivability along the time on the different media (TSA and MA) in a second microcosm inoculated with *V. harveyi* BAA-1117.

Resuscitation. The main result regards the resuscitation of *V. harveyi* BAA-1117 -which entered in the VBNC state after 122 days at 4°C, as shown in Figure1- by exposing the cells to room temperature for 2 hours. After this incubation, the cells were plated on MA and TSA media. While on MA no difference in colony number was found between aliquots plated before and after the incubation at room temperature, plating on TSA resulted in a significant increase of colony numbers after the thermal stimulus, inexplicable as the simple growth of an undetectable fraction of cultivable cells. Apparently, the addition of bacterial supernatants did not influence or ameliorate the cultivation rates. Exposure to room temperature for 24 hours resulted in an increase of cultivable cells, probably due to the growth of cultivable cells, not to a true resuscitation (Figure 2).

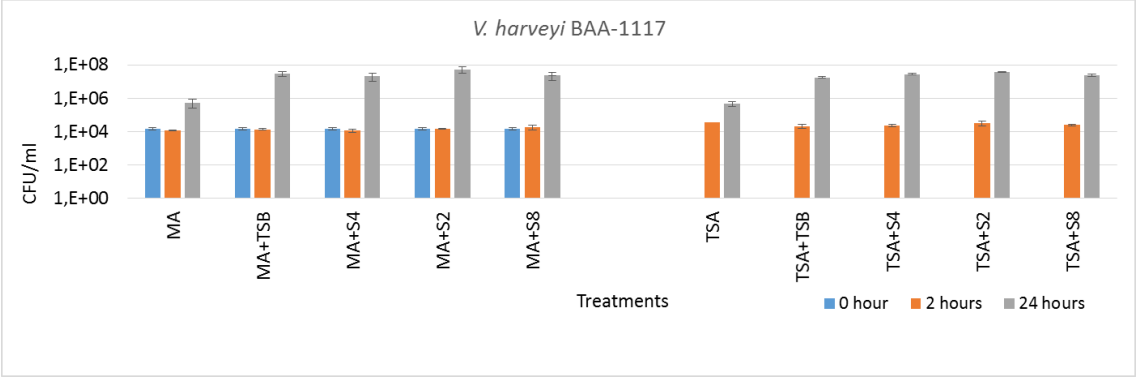

Fig.2 Count measurements of *V. harveyi* BAA-1117 (in CFU/ml) after exposition of aliquots to room temperature for 0, 2 and 24 hours applying different treatments. Aliquots have been plated on MA (left portion) or TSA (right portion). Treatments were: no addition of supernatant, addition of TSB, addition of supernatants of selected bacteria (S4, S2 and S8).

Conclusions

It has been demonstrated that the phenomenon of entrance in the VBNC state could occur in marine bacterial isolates. In particular, cell incubation at 4°C for 122 days was able to induce a significant decrease in the cultivability of selected strains. This is a useful information if related to sampling operations. In fact, the downshift of the temperature (at 4°C) typically used in sample storage might induce cells to enter in the VBNC state, in turn negatively affecting the subsequent isolation efficiency. On the other hand, it has been found that a subsequent exposition of VBNC state-induced cells to room temperature for a minimum of 2 hours might induce cell resuscitation and increase cell cultivability.

References:

Oliver J.D. 2010 Recent findings on the viable but nonculturable state in pathogenic bacteria. FEMS Microbiol Rev 34: 415–425.

Ayrapetyan M., Williams T.C., Oliver J.D. 2014. Interspecific Quorum Sensing Mediates the Resuscitation of Viable but Nonculturable *Vibrios*. Appl. Environ. Microbiol. 80:2478.

Mukamolova G.V., Yanopolskaya N.D., Kell D.B., Kaprelyants A.S. 1998. On resuscitation from the dormant state of *Micrococcus luteus*. Antonie Van Leeuwenhoek 73(3):237-43.

This deliverable has been initially scheduled for month 30, but an extension of the deadline to month 36 has been agreed with the project Coordinator. This extension did not affect any other deliverable or related deliverable.

Partner(s) involved in Deliverable production: UMIL (partner 15) has worked on this Deliverable.