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TOWARDS THE BIOTECHNOLOGICAL EXPLOITATION OF MARINE MICROORGANISMS: INVESTIGATION ON CULTIVATION, PRESERVATION AND GENETIC MANIPULATION

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Abstract

Microorganisms are the most diverse and abundant living organisms on Earth, inhabiting different and heterogeneous terrestrial and aquatic habitats and establishing interactions with plant, animal and human hosts. Particularly, marine microorganisms represent an intriguing reservoir of genetic and functional diversity, the products of which could be exploited in many different industrial sectors; for instance, they can produce bioactive natural compounds, pharmaceutical agents or enzymes useful in industrial applications (Dionisi et al., 2012; Ferrer et al, 2005, Bhatnagar et al., 2010). However, marine microorganisms are still largely uncultured, and thus unexploited, due to the lack of current efficient isolation and/or cultivation methods (Joint et al., 2010). Recently, the awareness that marine microorganisms represent a fascinating source of bioactive molecules is encouraging the interest from the scientific community, as also highlighted by last calls in the EU programs and related funded projects. In this scenario, understanding the genetic manipulation systems to be applied to marine microorganisms represents an important issue too. Genetic manipulation systems are generally aimed to obtain new improved strains expressing desired genetic determinants or to insert genetic variants encoding for specific phenotypic changes. Particularly, one of the most interesting applications in the field of environmental microbiology is to exploit genetic manipulation methodologies to explore the enormous undiscovered source of genetic information associated to the environmental samples (e.g. by the construction of metagenomics libraries in functional metagenomics) and to study the biotechnological potential of cultured microorganisms (e.g. to investigate gene functions) (Lam et al., 2015). Since the increasing interest on this topic, a part of this PhD thesis has been devoted to a thorough analysis of the literature in order to present in a review the genetic manipulation methodologies hitherto applied on marine strains. In particular, the attention has been directed to natural and artificial transformation (especially electroporation) and conjugation, since they have been successfully applied to marine strains.

The aim of my PhD project was to investigate those aspects that could affect the biotechnological exploitation of marine bacteria, i.e. cultivation of novel strains, cryopreservation of isolated ones and their genetic manipulation. First, I evaluated the ability of several marine bacteria to enter and exit from the state defined as “viable but non culturable” (VBNC), since this state has been proposed as one of the putative reasons for the uncultivability of environmental microorganisms. A second part of the work has been dedicated to the comparison of the protection property of several compounds, conventional or not, used as bacterial cryo-protectants, in long-term conservation experiments and testing different marine strains. Then, I assessed the capability of selected strains with biotechnological interest to be genetically manipulated. Finally, last part of the work has been devoted to the genome sequencing of a marine bacterium isolated from the interface of the deep hypersaline anoxic basin Discovery, located in the Mediterranean Sea.

Viable but non culturable (VBNC) state induction and resuscitation of marine bacteria

VBNC state is a physiological condition in which bacterial cells are alive but no longer able to grow on laboratory media (Oliver, 2005). These cells retain the capability to restart growth upon "resuscitation" under particular conditions and with favorable environmental parameters. Various stress factors, such as starvation and incubation at non-optimal temperatures, can induce VBNC state in a wide range of bacteria, including human pathogens. It has been proved that VBNC cells can resist to multiple treatments (Nowakowska and Oliver, 2013): since they could easily bypass microbiological safety controls due to their lack of cultivability, they represent a potential threat for human health. On the other side, only a few number of environmental bacteria, have been characterized in relation to VBNC state. For these reasons, deepening the knowledge on the mechanisms of entrance and resuscitation from VBNC state is of great interest, especially in the light of the need to bring into culture new microorganisms.

Aim of this work was to evaluate the ability of selected marine isolates to enter into VBNC state and to exit from it, *i.e.* to "resuscitate". A sub-collection of bacterial strains isolated from symbiotic assemblages associated to mangrove crabs has been used, including a reference strain (*Vibrio harveyi* BAA-1117^{TM}). The loss of cultivability of four strains belonging to different *Vibrio* species (i.e. *V. harveyi*, *V. fortis*, *V. hepatarius* and *V. nereis*) has been followed during starvation in seawater at 4°C for 54 days. By means of flow cytometry, cell morphological changes have been described: two of them, *V. harveyi* and *V. fortis,* have been studied until the cells entered in VBNC. In both species, during entrance in VBNC state, cell dwarfing, decrease in DNA quantity and a peculiar green fluorescence (when the cells have been stained with propidium iodide) have been observed. All these features have been partially or totally reverted when cells resuscitated after incubation at 30°C.

Since oxidative stress has been demonstrated to be related to VBNC, the response of VBNC cells in presence of H_2O_2 has been evaluated. VBNC cells demonstrated to be very sensitive to oxidative stress and hydrogen peroxide exposition as low as 0.007 mM resulted in a permanent damage of the cells. Resuscitation from the VBNC state resulted in a gradual increase of strain resistance to H_2O_2, up to 0.2 mM.

In conclusion, VBNC and related phenomenon may be not easily attributable to a unique pathway even in the context of a single genus. Moreover, this study provides hints on one putative reason for the uncultivability of marine microorganisms, indicating starvation, temperature and oxidative stress as relevant environmental parameters, which can have a role in inducing or preventing VBNC phenomenon.

Cryopreservation of marine bacteria

Long term preservation of microorganisms is a mandatory issue for microbiological research and it can be accomplished by different methods, such as cryo-preservation, lyophilization and liquid-nitrogen cryo-preservation, all dealing with the application of ultra-low temperatures to the microbial cells. In this work I focused the attention of cryo-

preservation method with the aim to compare the protection property of several compounds, conventionally or not used as bacterial cryo-protectants, in long-term conservation experiments. Several strains encompassing different phylogenetic taxa have been incubated at -80°C in 96-wells plates with cryo-protectant molecules i.e. glycerol (20 % v/v), skimmed milk (10% v/v), ethylene glycol (10% v/v), betaine (5% v/v) and urea/glucose (100 mmol l^{-1}). After 6, 12, 18 and 24 months aliquots have been thawed and vital cells counted by plating. PERMANOVA analysis has been applied to statistically evaluate the decrease in vitality with cryo-preservation time.

The results demonstrated that all the applied molecules could preserve the vitality of the strains at some extent until 24 months of storage at -80°C. However, in most of the cases, the vitality has been reduced with time, indicating that incubation periods of several years could lead to the complete loss of the strain. Any of the cryo-preservation agents demonstrated higher cryo-protection efficiency on all the tested strains, indicating that the best preservation efficiency is dependent upon the specific combination of strain/molecule applied. If long-term preservation efficiency is desired, our results indicate that several agents have to be tested, in order to evaluate the best agent for each specific strain.

Genetic manipulation of bioactive marine and crab-associated bioactive strains

Bacterial genetic engineering aims to introduce DNA into bacterial cells in order to express new traits or to characterize genes of interest in a biotechnological perspective or for a basic study (Keasling, 2012). Genetic manipulation procedures foresee the introduction of genes into a host cell, after being inserted in cloning vectors - plasmids are the mostly used ones - through different transfer systems. Vectors containing optical markers are useful to easily verify the success of the genetic manipulation: The marker commonly used in microbial genetic engineering to monitor gene expression or to localize particular cells or proteins are fluorescent proteins (e.g. green fluorescent protein, GFP) (Southward & Surette., 2002). One of the most applied artificial methods developed to insert foreign DNA into bacterial cells is transformation, which includes chemotransformation and electroporation. Although both methods have been demonstrated efficient to manipulate a large number of bacteria, they are nevertheless not successful with a range of marine strains (Piekarski et al., 2009, Hamashima et al., 1990), mostly hampered by the presence of NaCl in the bacterial growth medium(Wang et al., 2015).

Aim of this work was to assess the capability of selected strains with biotechnological interest to be genetically manipulated. In particular, artificial transformation by electroporation and conjugation have been employed. Specifically, we focused our attention on the following strains:

i) *Halomonas aquamarina* 9B, isolated from the seawater-brine interface of the deep hypersaline anoxic basin Bannock (De Vitis et al., 2015); ii) *Halomonas boliviensis* M2, isolated from mangrove crabs; iii) *Pseudomonas plecoglossicida* PG21, isolated from

mangrove crabs; and iv) *Halomonas axialensis* M10, isolated from mangrove crabs. The first strain has been selected since its enantioselective esterase activity on a key intermediate for prostaglandin synthesis, respectively (De Vitis et al., 2015). M2, PG21 and M10 have been selected for their ω-transaminase (ω-TA) activity.

Three of the four abovementioned strains have been successfully manipulated by conjugation or electroporation. A GFP protein coding gene has been chromosomally inserted by conjugation into the chromosome of *H. axialensis* M10 and *H. aquamarina* 9B, taking advantage of a mini Tn7 transposition system. Negative results have been reported for *H. boliviensis* M2. *P. plecoglossicida* PG21 using conjugation. On the contrary, the strains have been successfully transformed by electroporation, by inserting the plasmid pHM2-GFP. To our knowledge, this is the first time that a crab-associated *Pseudomonas* species has been transformed through electroporation. In conclusion, these systems could be used in further studies that foresee the genetic manipulation of the strains

Sequence genomes of marine bacteria

Sequencing the entire microbial genome is important for i) generating accurate reference genomes, ii) microbial identification, iii) comparative genomic studies and iv) retrieving novel functional genes of biotechnological interest**.**

In this work the genome sequence of the marine bacterium *Virgibacillus* sp. Strain 21D, isolated from the seawater-brine interface of the deep hypersaline anoxic basin Discovery in the Mediterranean Sea, has been performed. This strain grows in presence of NaCl and $MgCl_2$ concentrations up to 9% and 9.52%, respectively, with optimum growth at 6-9% NaCl and 4.76% $MgCl_2$; pH optimum (8-9) and temperature optimum (30-37°C) for growth have been also determined. The genome was sequenced by the use of PacBio platform, and a total number of 117,330 reads has been retrieved with a mean subread length of 8,461 bp and N50 of 11,849 bp. One circular contig has been obtained, constituted by 4,263,520 bp. The genome showed a GC% of 36.6, with 3,915 CDS, 63 tRNA and 18 rRNA. Automatic annotation of the genome showed that it contains genes coding for proteins related to the production and secretion of compatible solutes, molecules which could help the bacterium to thrive osmotic stresses typically of extreme environments. Genes coding for osmotically activated L-carnitine/choline ABC transporters, glycine betaine transporters, choline uptake and an ectoine synthase were identified in *Virgibacillus* sp. 21D genome. Genes involved in iron acquisition and metabolism have been, moreover, retrieved: the presence of these genes in 21D suggested its strain adaptation to marine conditions characterized by iron depletion

Conclusions

Microorganisms are the most diverse life form present of Earth (Solden et al., 2016). Particularly, marine bacteria provide a reservoir of bioactive secondary metabolites that are ultimately useful for humans. Unraveling methods for their cultivation and preservation in laboratory, along with the establishment of efficient genetic manipulation systems can have important repercussions on their biotechnological exploitation.

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Chapter I

Introduction

Microorganisms are the most diverse and abundant living form on Earth. They inhabit different and heterogeneous habitats, including environments indicated as "extreme" ones, characterized by severe and inhospitable -at least for the vast majority of organisms- physico-chemical conditions, e.g. polar sea, cold and hot deserts and hydrothermal vents (Dalmaso et al., 2015). Deep-sea hypersaline anoxic basins, or DHABs, are an example of extreme environments: they are highly saline lakes on the seafloor, without oxygen and with high hydrostatic pressure (Pachiadaki et al., 2014). Several DHABs have been found in the Mediterranean Sea, located at a depth of more than 3000 m below sea level. Due to the high density of the brines, mixing of these water masses with the overlying deep-sea water (average density: 1.03 kg m^{-3}) is restricted. High salt concentrations up to saturation, high pressure exceeding 300 atm, and a lack of oxygen make these basins ones of the harshest environments on ourplanet, defining new parameters for the limits of life (Pachiadaki et al., 2014).

Microorganisms inhabiting extreme ecosystems, namely "extremophiles", can overcome harsh conditions, since their peculiar metabolism and physiology, which make them also an interesting source of biotechnological potential (Morozkina et al., 2010). Many extremophiles (or molecules derived from them) have been investigated in order to be exploited in industrial applications. As instance, one of the most a known and useful product in molecular biology is DNA polymerase (namely Taq polymerase): derived from the extremophile *Thermus aquaticus*, it is universally used in the polymerase chain reaction (Gelfand et al., 1989). However, a limited knowledge of methods to cultivate extremophiles in laboratory hampers largely their exploitation, thus influencing the possible discovery of novel compounds with invaluable pharmaceutical, industrial and ecological potentials.

Natural resources, such terrestrial herbs and marine macroalgae, have been used in traditional medicine to treat many diseases because of their accessibility and availability. However, it is in the middle part of XX century that scientists initiated to investigate oceans looking for new molecules and, at present, the research is still in progress (Romano et al., 2016). Today, the discovery of bacterial bioactive compounds to satisfy human needs as pharmaceutical and therapeutic agents is a cutting-edge and compelling research topic: the development of molecular biology tools, automated high throughput screening methodologies, extract-based screening methods, and bioinformatics provide more opportunities to unveil the compounds associated to marine environment (Reen et al., 2015).

Recent advances in metagenomics and bioinformatics provide estimates about the hidden compounds associated to bacteria to be exploited (Reen et al., 2015).

By metagenomics, which enables the direct access to total DNA from a given environment (eDNA) without prior culturing, genomes of uncultured bacteria became accessible. In particular, metagenomic studies revealed that marine microorganisms originated from extreme environments are an interesting source of novel genes (Dalmaso et al., 2015). However, learning function of new genes and pathways from pure sequence data is challenging and cultivation of bacteria in laboratory remains an essential part to understand the bacterial physiology and ecological role (Prakash et al., 2013).

Hitherto, more than 28,175 marine natural products have been discovered from marine organisms (crawford et al. 2016), ranging from derivatives of amino acids and nucleosides, to macrolides, porphyrins, terpenoides and aliphatic cyclic peroxides, to sterols (Thakur et al. 2003). Particularly, sponges are considered a golden mine for the discovery of therapeutic molecules, including anticancer compounds, e.g. "Cytarabine" used to treat acute myelocytic leukemia (Sagar et al., 2010), and "Eribulin" developed from the metabolite halichondrin B produced by the sponge *Halichondria okadai* (Hirata et al., 1986). Interestingly, new researches have demonstrated that many intriguing bioactive metabolites extracted from sponges are instead produced by microbial symbionts (Noyer et al., 2011). For example, in a taxonomic survey of the bacteria associated to the marine sponge *Arenosclera brasiliensis*, different genera have been classified among which *Bacillus, Shewanella, Pseudovibrio* (Rua et al., 2014); antimicrobial assays on the isolates revealed that one third of them produced antibiotics against *Bacillus subtilis*, suggesting a protective role toward the sponge (Rua et al., 2014). Moreover, another interesting application of marine bacteria could be found in the bioremediation of heavy metal and hydrocarbon-contaminated marine environments. For example, *Marinobacter* and *Alcanivorax*, indigenous microbes in oil-contaminated environments, have been identified as main players of alkane degradation in sediments and thereby they could play an important role in the treatment of the polluted zone following a bioremediation strategy (Barbato et al., 2016).

As mentioned before, although the recent advance in genomics and bioinformatics and their huge impact on the research orientation in microbial and biotechnological fields, cultivation of bacteria remains of high importance (Prakash et al., 2013). Marine bacteria are highly diverse and almost 99% of them have been considered uncultivated, hampering the application of traditional screening methods to discover bioactive metabolites. Nutrient rich agar media are not the optimal way to cultivate bacteria in laboratory: the majority of marine bacteria fail to be isolated in such common media, demanding specific growth media including small signalling molecules or the presence of other bacteria, due to some positive interactions that they can establish affecting positively their growth (Prakash et al., 2013).

Different cultivation methods have been employed to attempt the cultivation of unculturable bacteria. To this regard, one of the strategies foresees to encounter the growth requirements of not-yet cultured bacteria by mimicking the bacterial natural environment. As instance, Kaeberlein and colleagues (2012) created a diffusion chamber placed in the environment: the system encloses the bacteria within a semipermeable chamber, in a way that cells are unable to pass through the membrane barrier, whilst growth factors can diffuse from outside. The diffusion-chamber-based approach led to the isolation of species from rarely cultivated groups, such as *Deltaproteobacteria, Verrucomicrobia, Spirochaetes*, and *Acidobacteria* (Bollmann et al., 2007). The challenge in such systems is to separate the isolates of interest from the growing bacterial population. Another method for culturing recalcitrant bacteria has been performed with the SAR11 clade of *Alphaproteobacteria,* ubiquitous bacteria in marine water. Giovannoni and his group separate SAR11 from other isolates in the environmental sample based on the fact that SAR11 was one of the most abundant organism in their samples. They used fresh Oregon coast seawater as a growth medium,and they diluted their samples several dilutions till 'extinction',therefore they were able to separate the bacteria of this clade into the wells of microtiter plates and grow them in pure culture(Rappé et al., 2002).

Other approach to growing uncultured bacteria uses the bacteria themselves to determine the particular aspect of the environment that is important to their growth rather than adding the entire environment to the medium. Graf and coworkers used high-throughput sequencing of RNA transcripts (RNA-seq) to determine that an uncultured *Rikenella*-like bacterium in the leech gut was utilizing mucin as a carbon and energy source (Bomar et al., 2011). Moreover, the bacterial growth can be stimulated by the presence of other bacteria. As instance, it has been identified that the co-culture of an uncultivated bacterium with another bacterium, called “helper”, induces the growth of the former since the latter compensates the auxotrophy of the former, i.e. a missing metabolite that cannot be synthetized by itself. For example, the growth of *Maribacter polysiphoniae* KLE1104 is dependent from the presence of a strain of *Escherichia coli*, since the latter secretes an enterbactin siderophore, a class of small molecules that are able to solubilize oxidized iron (Fe^{3+}) and thereby make this essential nutrient available to KLE1104 cells (D’Onofrio et al., 2010). Furthermore, it has been identified that adding a low concentration of the 5-amino-acid peptide LQPEV to a standard medium makes cultivable an uncultivable *Psychrobacter* sp. strain MSC33 (Nichols et al., 2010). Other methods of cultivation in enclosed fermenters and bioreactors with controllable growth conditions, such as temperature, aeration, medium composition, pH, and other parameters have been also employed (Dalmaso et al., 2015)

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Aim of the work

Marine microorganisms represent a reservoir of genetic diversity and bioactive secondary metabolites that can be exploited in many different industrial applications. The main bottleneck in exploiting the biotechnological potential associated to marine microorganisms relies on their uncultivability or poor growth on standard nutrient media, due to several intrinsic and extrinsic reasons, including the lack of information about their growth requirements and conditions. The vast majority of marine microorganisms are still largely uncultured and thus unexploited. In addition, several aquatic microorganisms have been described as able to enter in the "viable but non culturable" (VBNC) state, which may further hamper their isolation and cultivation. Unraveling the methods for an efficient cultivation of marine microorganisms in laboratory, along with the establishment of efficient genetic manipulation systems and long term storage procedures, will have an important impact on their exploitation in a biotechnological perspective.

The aim of my PhD project was to give a contribute to the study of the biotechnological potential associated to marine microorganisms. The thesis intended to investigate some of the aspects that could affect the biotechnological exploitation of marine bacteria, i.e. cultivation, cryopreservation and genetic manipulation. Specifically, these topics are here delineated in succession in chapters II to V and are followed by a short final chapter summarizing the general conclusions of the work developed during the three years of the PhD.

Chapter I presents a general introduction which states the general topics of the thesis.

Chapter II focuses on the ability of different strains belonging to *Vibrio* genus to enter in the VBNC state, since this state could have a role in the uncultivability of marine microorganisms. In addition, the ability to exit from this state is evaluated in two of the strains.

Chapter III is devoted to the study of the cryopreservation property of several compounds in long-term conservation experiments, considering marine bacterial strains that belong to different taxa. Preserving marine strains in an efficient way preventing the loss of their characteristics and genetic information is a crucial step of ordinary laboratory practices.

Chapter IV is composed of two parts. Part I describes the genetic manipulation methods hitherto used in marine bacteria. Genetic manipulation of marine strains is a keystone to unravel their biotechnological potentials. The methods that have been employed so far, including natural and artificial transformation and conjugation, are reviewed here, highlighting the limiting factors of the different techniques with the aim to increase genetic manipulation efficiency; Part II focuses on the genetic manipulation of different selected marine strains. In particular, strains able to synthetize esterase and transaminase enzymes have been considered. Their capability to be genetically manipulated has been assayed by tagging their genomes with a gene cassette encoding for a green fluorescent protein (GFP) or by introducing plasmids encoding for the GFP.

Chapter V depicts the genome sequence of a halophile marine bacterium, isolated from Discovery, a deep hypersaline anoxic basin in the Mediterranean Sea, in order to exploit the genetic determinants with biotechnological potentials and to unveil genes involved in the bacterial adaptation to osmotic stress conditions typical of extreme environments.

Chapter VI outlines the conclusions of this work and the perspectives for further researches.

Chapter II

The Viable but Non Culturable State follows different pathways in two Vibrio species

Abstract

Vibrios are Gram-negative, marine bacteria. Many of them are pathogens transmitted by raw or undercooked seafood, or by exposure to contaminated marine water. This genus is one of the most studied concerning the Viable But Not Cultivable (VBNC) state. In this particular physiological state, which can be induced by starvation in seawater at 4°C, *Vibrio* cells are no longer cultivable in standard media agar plates, but they can "resuscitate" if incubated at 30°C prior to plating.

In this work, we characterized by means of flow cytometry the morphological changes in four *Vibrio* strains (belonging to the species *V. harveyi, V. fortis, V. hepatarius and V. nereis*) during the incubation in cold seawater microcosms (4°C) for 50 days. *V. harveyi* and *V. fortis* microcosms have been further studied until the cells entered in VBNC state from which their resuscitation has been also characterized through time. Finally, the relationship between resuscitation and oxidative stress has been investigated. In all the strains, although with different modes, VBNC-induction has been characterized by cell dwarfing, decrease in DNA quantity and the appearance of a peculiar green fluorescence when the cells have been stained with propidium iodide. These features have been partially or totally reverted when cells resuscitated. Moreover, cultivability of VBNC-induced cells can be rescued, at least in part, by plating the VBNC cells in presence of catalase. Indeed, hydrogen peroxide at concentrations as low as 0.007 mM prevents resuscitation and a prolonged exposure to H_2O_2 concentrations, far under the concentration inhibiting normal growth, permanently damaged VBNC cells, which were not able to resuscitate anymore. Finally, we assessed that during the process of resuscitation these cells gradually increase their resistance to H_2O_2. The time and mode of the processes differed in the two tested strains, proving that "VBNC" state may include many phenomena in different bacteria and not easily attributable to a unique pathway also in the context of a single genus.

Keywords: VBNC, *Vibrio*, Flow Cytometry, Hydrogen Peroxide, Catalase

Introduction

The Viable But Not Cultivable (VBNC) state is a physiological condition in which bacterial cells are alive but no longer able to grow on laboratory media. These cells retain the capability to restart growth upon "resuscitation" under particular conditions and if the environmental parameters are favorable (Oliver, 2005). Various stress factors, such as starvation and incubation at non-optimal temperatures, can induce the VBNC state in a wide range of bacteria, many of which are human pathogens (Oliver, 2010). It has been proved

that VBNC cells can resist to multiple treatments and easily bypass microbiological safety controls due to their lack of cultivability, representing thus a potential threat for human health (Nowakowska and Oliver, 2013; Li et al., 2014). On the other side, a number of environmental bacteria, with a perspective high biotechnological potential, are yet poorly characterized due to cultivation issues that could be caused by VBNC state. For these reasons, deepening the knowledge on the mechanisms of entrance into VBNC and resuscitation is of great interest both from a medical and from a biotechnological point of view.

VBNC state has been extensively studied in the genus *Vibrio*, a group of Gram-negative, marine bacteria (Oliver, 2005). Many of them are pathogenic (e.g. *V. vulnificus*, *V. cholerae*, *V. haemolyticus*) and they are transmitted by eating raw or undercooked seafood, or by exposure to contaminated marine water. Besides in infected humans, they are typically found in estuarine environments, associated to shellfish and marine arthropods (O'Neil et al., 1992; Lutz et al., 2013). As instance, the appearance of a VBNC form within *Vibrio* genus has been mainly detected in *V. vulnificus*, shortly after incubation in cold seawater and the resuscitation of these cells has been described upon exposure to higher temperatures prior to plating (Whitesides and Oliver, 1997). More recent studies in *V. vulnificus* explained, at least partially, these phenomena. On one hand, the loss of cultivability is connected with a significant decrease in the periplasmic catalase KatG expression, and consequently in catalase activity, induced by starvation and low temperature (Kong et al., 2004). Therefore, the growth in rich media that contain a certain amount of hydrogen peroxide, e.g. Brain Heart Infusion, is hampered (Kong et al., 2004). On the other hand, the quorum-sensing molecule AI-2, as well as cell-free supernatants form *V. vulnificus* itself, *V. paraheamolyticus* or *Escherichia coli* can trigger the resuscitation process in both environmental seawater samples and experimental VBNC microcosms (Ayrapetyan et al., 2014). However, studies in other *Vibrio* species suggest that the mechanisms behind the loss of cultivability could be far more complicated. In *V. cholerae*, following the induction of VBNC with the same protocol above mentioned, the resuscitation is not always possible with the same stimuli, but depending on the age of the VBNC microcosms it is necessary to add catalase or human colon cells extract, or co-cultivate the bacteria with colon cells to obtain colonies again (Imamura et al., 2015). Thus, the authors conclude that, rather than a single property, the VBNC state in *V. cholerae* is a condition that continuously changes over time (Imamura et al., 2015). This view is supported by recent articles in which the transcriptional profile of *V. harveyi* at different stages after incubation in seawater without nutrients is evaluated. Transcriptional changes are detected early within the first 12 hours of incubation (Montánchez et al., 2014), and, with time, cells (which are still cultivable) become smaller and adapt their gene expression again (Kaberdin et al., 2015). The reduction in size, together with the decrease in DNA quantity, has been extensively described also in VBNC-induced *V. parahaemolyticus* (Falcioni et al., 2008).

As regards to the relationship between entrance to VBNC and resistance to hydrogen peroxide, the literature so far did not come to an exhaustive explanation. On one hand, VBNC-induced cells of *V. vulnificus* are reported to be highly sensitive to hydrogen

peroxide, which is suspected to be the main reason for their inability to grow on BHI (Kong et al., 2004). On the other hand, VBNC-induced cells of the same species, at least for one of the two strains tested by Nowakowska and Oliver (2013), appear more resistant to this stress than a log-phase culture, as they can resuscitate after a 60 min of challenge with H_2O_2. During the initial stages of starvation, *V. harveyi* appears to up-regulate the genes for resistance to oxidative stress (Montánchez et al., 2014). This seems to be necessary for the detoxification of the hydrogen peroxide endogenously produced as a side product of the lipid turnover, which in this phase is enhanced to allow the modification of the cell envelope and shape.

Although several studies have been performed on vibrios with regard to the VBNC transition, to our knowledge no direct comparison has been tempted between species. Therefore, it is not clear if the results obtained with particular strains can be generalized and if the mechanisms and causative agents of this transition are always the same. Moreover, the interesting results obtained on the role of catalase and hydrogen peroxide by Kong and coworkers (2004) need to be validated in other strains to assess if the ones described are a general VBNC entrance mechanism or rather a specific pathway of *V. vulnificus.* As for the entrance in VBNC, the process for resuscitation has not been described yet with the aim to compare different species (Kong et al., 2004).

In this study, we characterized the morphological changes in four *Vibrio* species (*V. harveyi*, *V. nereis*, *V. fortis* and *V. hepatarius*) during the incubation in cold seawater microcosms for 54 days. *V. harveyi* and *V. fortis* microcosms have been further studied until they entered in VBNC and their resuscitation has been induced by incubation at 30°C for different times before plating. Finally, the relationship between resuscitation and the oxidative stress generated by hydrogen peroxide has been investigated.

Materials and Methods

***Vibrio* strains**

For this study we used one reference strain and three environmental isolates. The reference strain was *Vibrio harveyi* BAA-1117^{TM} (luxN::tn5Kan), mutated in the LuxN receptor, which is involved in the quorum sensing mediated by homoserin-lactones. *Vibrio hepatarius* UU21, *Vibrio fortis* UU24 and *Vibrio nereis* M5 have been isolated from the gills of mangrove crabs of the species *Uca urvillei*. Briefly, gills have been dissected in sterility from crabs and smashed in sterile physiological solution (0.9% NaCl). Serial dilutions have been then plated on Marine Agar (Difco) plates added with 100 ppm cycloheximide to avoid eukaryotic growth. Random colonies have been selected and purified. Their identity has been established by amplification of a fragment of the 16S rRNA gene using primers 27F (5′-TCG ACA TCG TTT ACG GCG TG-3′) and 1495R (5′-CTA CGG CTA CCT TGT TAC GA-3′‘) (Mapelli et al., 2013). 16S rRNA gene fragments have been then sequenced (Macrogen, Korea) and obtained sequences have been aligned against the public databases at the National Centre for Biotechnology Information (NCBI) using BLASTn (Altshul et al., 1990).

Vibrio microcosms' preparation

The strains have been grown overnight in Triptic Soy Broth with added 2.5% sodium chloride (w/vol) (TSB-NaCl). This culture has been used to inoculate fresh TSB-NaCl in a 1:100 ratio. As soon as the culture reached the exponential phase (O.D. 0.3-0.5), the cells have been washed twice in sterile Artificial Sea Water (ASW) prepared dissolving 40 g of sea salts (Sigma-Aldrich) in 1L of deionized water. Cells have been counted in a Thoma chamber and diluted in ASW to reach the concentration of approximately 10^7 cells/ml in glass vials. The microcosms have been stored at 4°C in the dark.

CFU counting

An aliquot from each microcosm has been serially diluted up to 1:10,000. Four drops containing 10 µl of each dilution were plated on TSB-NaCl. Colonies in each drop were counted after overnight incubation at 30°C.

Resuscitation

Resuscitation of the VBNC bacteria has been achieved by incubating the cells at 30°C for different time spans prior to plating.

Assessment of VBNC state

In order to verify that the observed increase in cultivable cells was due to resuscitation and not to the growth of an originally undetectable non-VBNC population, we applied the following procedure. An aliquot of the microcosm has been serially diluted and all the dilutions, as well as the undiluted sample, have been plated on TSB-NaCl agar plates to verify the absence of growth. Then, we incubated the dilutions at 30°C to induce resuscitation. The following day, we plated each dilution on TSB-NaCl and counted the colonies (after a further overnight incubation). Assuming that, if the increase in CFU count was due to growth rather than to resuscitation, at least one non-VBNC cell was initially present in each dilution from which we cultivated cells, we calculated the theoretical initial concentration of these non-VBNC cells in the undiluted sample, and we verified that this value was higher than our detection limit.

Viability staining

Two aliquots of 100 µl have been withdrawn from each microcosm and stained separately with Syto9 or propidium iodide from the LIVE-DEAD BacLight kit (Invitrogen). The staining solutions have been prepared by adding 3 µl of each dye to 1 ml milliQ water; then, 100 µl of the staining solutions have been mixed with each microcosm aliquot. Samples have been analyzed after 45 min incubation at 30°C in the dark.

Flow cytometry

A BD Accuri-C6 device has been used. For each sample, 30,000 events have been acquired, and an unmarked sample from each microcosm has been always included in the analysis. The excitation laser was set at 488 nm. Green fluorescence signal of Syto9 has been detected with a 530/30 nm filter, while red fluorescence from propidium iodide has been detected with a 585/40 nm filter

Growth in the presence of hydrogen peroxide

Growth curves of *V. harveyi* and *V. fortis* have been obtained using a Tecan microplate reader. Ten µl of the cell suspension have been inoculated in 90 µl TSB-NaCl medium in each well of a 384-well microplate using an Ep-Motion liquid handler (Eppendorf). Different concentrations of H_2O_2 have been achieved by adding to each well 2 µl of hydrogen peroxide solution at the appropriate concentrations prepared diluting 30% hydrogen peroxide (Sigma-Aldrich, Milan, Italy) in deionized water. The plates have been incubated at 30°C for a minimum of 24 hours and the optical density has been measured every 15 minutes. We calculated the growth curves by averaging the O.D. measurements of 4 to 8 identical wells and subtracting the average O.D. value of negative controls (non –inoculated wells). Since the differences between experimental conditions have been best visualized as the number of grown wells rather than in the shape of the growth curves, we also performed an end-point analysis counting the number of grown wells at the end of the incubation time.

This experimental setup has been applied to study the sensitivity to hydrogen peroxide of the microcosms at day three (inoculum: 10 µl of the microcosm for each well, H_2O_2 concentrations from 0 to 0.8 mM, scale intervals: 0.1), of the microcosms at different resuscitation times (inoculum: 10 µl microcosm, H_2O_2 concentrations from 0 to 0.25 mM, intervals: 0.05) and of the growing cells of *V. harveyi* and *V. fortis* (inoculum: 10 µl of overnight culture diluted to 10^7 cell/ ml, H_2O_2 concentrations from 0 to 0.6, intervals: 0.025).

Plates treated with catalase

Catalase from bovine liver has been purchased from Sigma Aldrich, dissolved in phosphate buffer pH 7 at the concentration of 1 mg/ml and filter-sterilized. In order to obtain the same catalase activity per plate as described by Kong et al. (2004), 1 ml of catalase solution has been poured on each TSB-NaCl plate. The plates have been dried in a biosafety cabinet and immediately inoculated with 100 µl of the bacterial suspensions. The number of colonies has been evaluated after overnight incubation at 30°C.

Resuscitation after stress

Two-ml aliquots of the microcosms have been prepared in sterile tubes. To each one the appropriate volume of hydrogen peroxide/water solution has been added to reach the final concentration of 0.007 mM, 0.02 mM or 0.05 mM. After overnight incubation at 30°C, an aliquot of cell suspension has been plated, while a second one has been treated with catalase (C9322 Sigma-Aldrich; adding to 1 ml aliquot 25 µl of catalase solution -stock 1 mg/ml-)

and incubated at 30°C until plating the following day. Cells were analyzed by flow cytometry at the two stages of the experiment.

Catalase production

The production of catalase by the four *Vibrio* strains has been visually assessed using the straightforward method described by Iwase et al. (2013).

Results

Morphological changes during starvation in cold seawater

To monitor the changes of cells subjected to starvation and temperature stresses, we analysed by flow cytometry the microcosms at different times during a time span of about 50 days; simultaneously we plated the cells to also assess their cultivability. For all the strains we observed the same trends, although with a different timing. First of all, in all the microcosms a cell shrinkage has been observed (Fig. 1A, 2A, 3). Interestingly in case of *V. harveyi* BAA-1117^{TM}, this process has been already completed at day 33, while for all the other strains, i.e. *V. hepatarius* UU21, *V. nereis* M5 and *V. fortis* UU24, we observed at day 50 the appearance of a smaller population, even if bigger cells were still present (Fig 2A; see also day 47 in Fig. 3D, F, H). The flow cytometry data of *V. harveyi* BAA-1117^{TM} are shown at day 50 for comparison with the other strains, considering that after day 33 no changes have been detected (Fig. 1A, 3). Similarly, the green fluorescence signal detected upon staining with Syto9, a parameter that has been proportionally correlated to the DNA abundance, decreased rapidly in *V. harveyi* BAA-1117^{TM}, while for the other strains the process resulted slower (Fig. 1B, 2B, 4B-E).

Concomitantly to the reduction in size and DNA quantity, we observed that a fraction of the cells (the smallest in size) recorded an unexpected upshift of the green fluorescence upon staining with propidium iodide (Fig. 1C, 2C, 3). Experiments with microcosms artificially killed by heat shock (10 min at 100°C) or by adding 50% isopropanol demonstrated that in our experimental setup propidium iodide is not useful to trace dead cells based on red fluorescence. However, in our case it appeared to confer an increased green fluorescence to the dwarf cells. In *V. harveyi* BAA-1117^{TM}, all the cells resulted labelled with an increased value of green fluorescence upon staining with propidium iodide already at day 33, while for the other strains two populations appeared; one identified by a lower size and higher fluorescence, and one that was bigger in size but less fluorescent (Fig. 3 and 4).

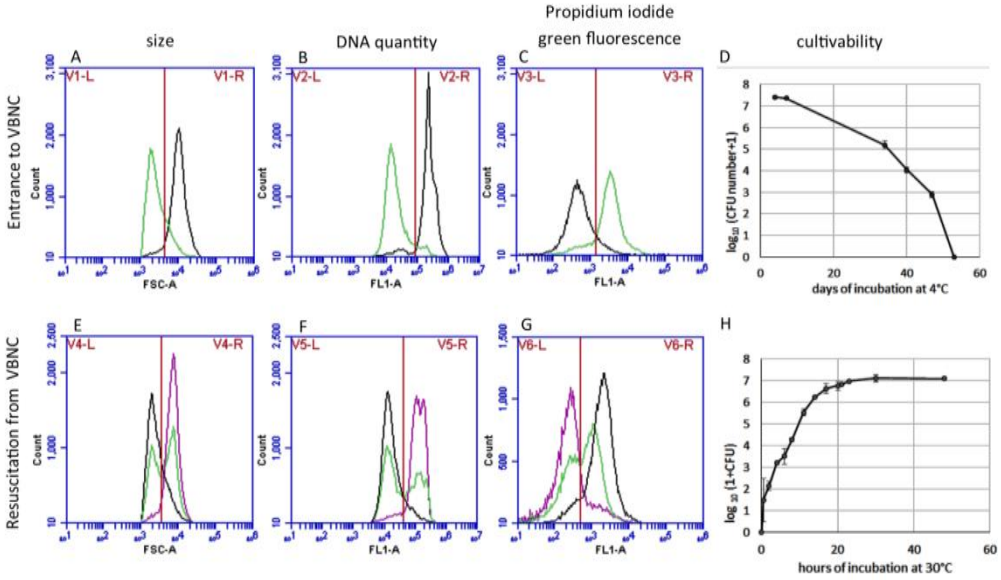

Figure 1. Change in size, fluorescence and cultivability of *V. harveyi* BAA-1117^{TM} cells during the VBNC entrance and resuscitation process. A, B, C: entrance in VBNC by flow cytometry. Black line: microcosm at age 3 days. Green line: microcosm at age 50 days. A: cell size (FSC-A). B: green fluorescence (FL1-A) of cells stained with Syto9. C: green fluorescence (FL1-A) of cells stained with propidium iodide. Along with time the cell size decreases, while the green fluorescence induced by propidium iodide increases. DNA quantity, which is proportional to the green fluorescence of Syto9, decreases. D: decrease in cultivability during incubation at 4°C. E, F, G: resuscitation by flow cytometry. Black line: no incubation at 30°C. Green line: after 17h of incubation at 30°C. Purple: after 21h of incubation at 30°C. E: cell size (FSC-A). F: green fluorescence (FL1-A) of cells stained with Syto9. G: green fluorescence (FL1-A) of cells stained with propidium iodide. Changes detected before for cell size, DNA quantity and propidium iodide fluorescence (A, B, C) are reverted (E, F, G). H: increase in cultivability registered by incubating the VBNC cells at 30°C. The process of resuscitation happens exponentially

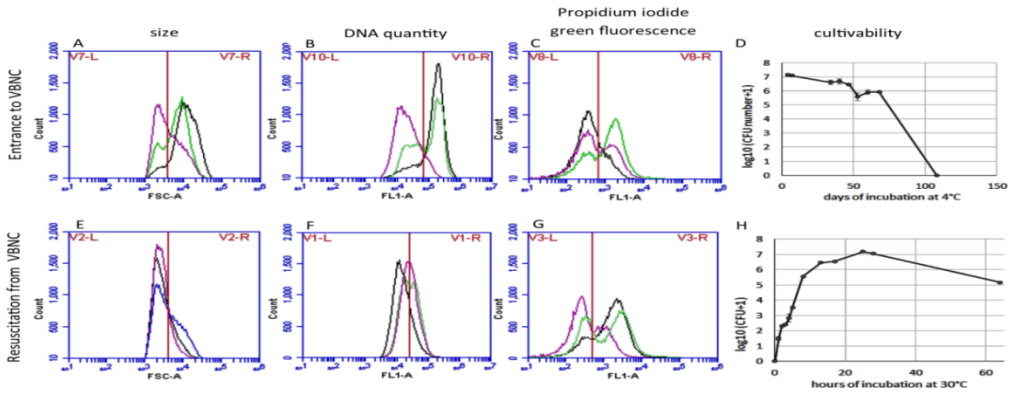

Figure 2. Change in size, fluorescence and cultivability of *V. fortis* UU24 cells during the VBNC entrance and resuscitation process. A, B, C: entrance in VBNC by flow cytometry. Black line: microcosm at age 3 days. Green line: microcosm at age 50 days. Purple line: microcosm at age 127 days. A: cell size (FSC-A). B: green fluorescence (FL1-A) of cells stained with Syto9. C: green fluorescence (FL1-A) of cells stained with propidium iodide. Along with time the cell size decreases, while the green fluorescence induced by propidium iodide increases. DNA quantity, which is proportional to the green fluorescence of Syto9, decreases. D:

decrease in cultivability during incubation at 4°C. E, F, G: resuscitation by flow cytometry. Blue line: no incubation at 30°C. Black line: after 1h of incubation at 30°C. Green line: after 24h of incubation at 30°C. Purple line: after 64h of incubation at 30°C. E: cell size (FSC-A). F: green fluorescence (FL1-A) of cells stained with Syto9. G: green fluorescence (FL1-A) of cells stained with propidium iodide. Changes detected before for propidium iodide fluorescence (C) and to a lesser extent for DNA quantity (B) are reverted (F, G). On the contrary, cell size (A) slightly decreases (E). H: increase in cultivability registered by incubating the VBNC cells at 30°C. The process of resuscitation happens exponentially

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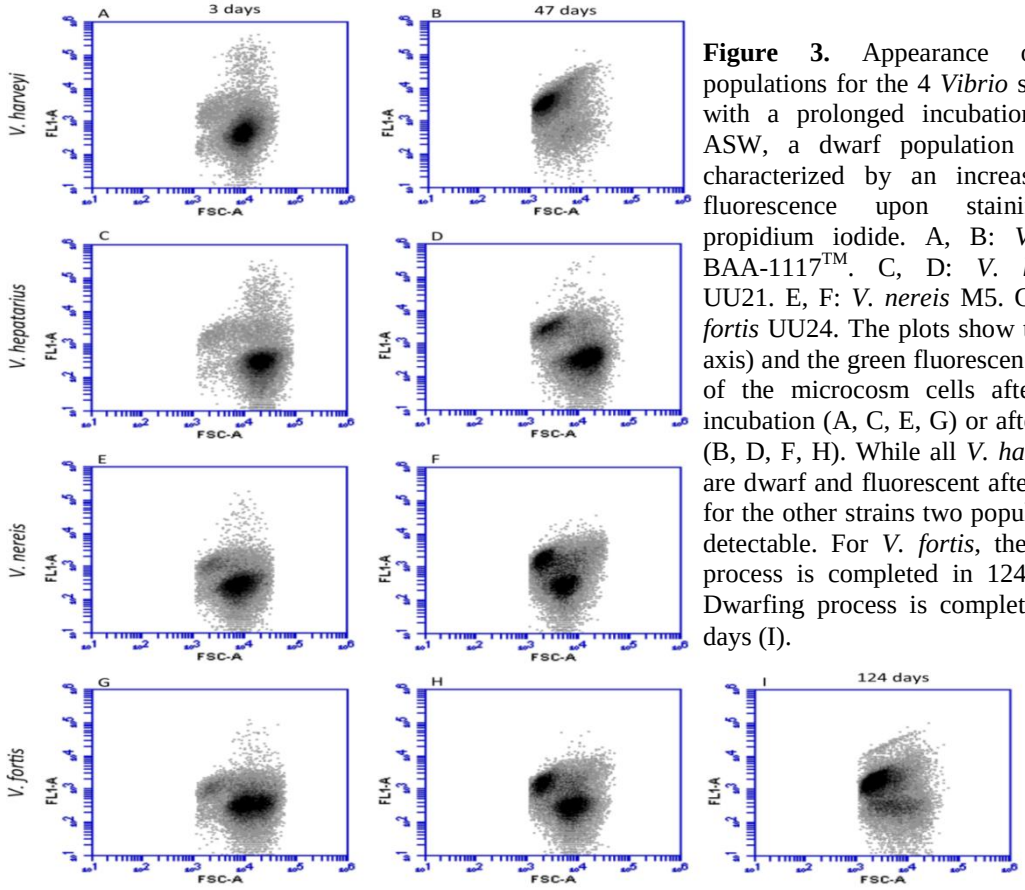

Figure 3. Appearance of dwarf populations for the 4 *Vibrio* spp. Along with a prolonged incubation in cold ASW, a dwarf population appeared, characterized by an increased green fluorescence upon staining with propidium iodide. A, B: *V. harveyi* BAA-1117^{TM}. C, D: *V. hepatarius* UU21. E, F: *V. nereis* M5. G, H, I: *V. fortis* UU24. The plots show the size (x axis) and the green fluorescence (y axis) of the microcosm cells after 3 days incubation (A, C, E, G) or after 47 days (B, D, F, H). While all *V. harveyi* cells are dwarf and fluorescent after 47 days, for the other strains two populations are detectable. For *V. fortis*, the dwarfing process is completed in 124 days (I). Dwarfing process is completed in 124 days (I).

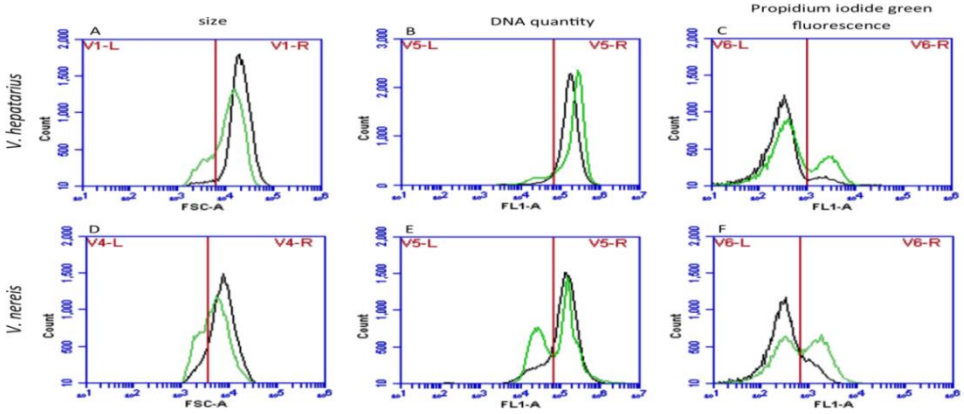

Figure 4. Morfological transitions in *V. hepatarius* UU21 (A, B, C) and *V. nereis* M5 (D, E, F) during long-time incubation in cold ASW by flow cytometry. Black line: microcosm at 3 days of incubation. Green line: microcosm at 50 days of incubation. A and D: cell size shrinkage. In *V. hepatarius* only a small dwarf population appeared. B and E: decrease in DNA quantity (green fluorescence of cells stained with syto9). C and F: increase in green fluorescence upon staining with propidium iodide.

Entrance to VBNC state and resuscitation

Microcosms of *V. harveyi* BAA-1117^{TM} and *V. fortis* UU24 have been monitored until they entered into VBNC state (Fig. 1-2). For *V. harveyi* the capability to generate colonies on TSB-NaCl plates was completely lost at day 53, while for *V. fortis* at day 108 (Fig. 1D, 2D). *V. fortis* VBNC-induced cells at day 127 showed a morphology that was similar to that showed by *V. harveyi* at day 50 (Fig. 3B, 3I): almost all the entire population was smaller in size with a lower DNA content and increase of green fluorescence upon staining with propidium iodide (Fig. 2A-C; Fig. 3G-I).

To restore the cultivability of these cells, we applied the protocol described by Whitesides and Oliver (1997) incubating an aliquot of the microcosm at 30°C overnight: plating the cell suspension after this treatment resulted in colony growth on TSB-NaCl agar medium. Thus, we will refer to this process as "resuscitation"(Whitesides and Oliver, 1997). In order to demonstrate that the observed colonies appeared after resuscitation rather than being related to the growth of a previously undetectable fraction of non-VBNC induced cells (i.e. to verify that the microcosms were really in VBNC state), we serially diluted the VBNC-induced microcosm prior to incubation at 30°C and we verified that cells resuscitate independently in all the dilutions. Dilutions have been plated immediately to verify the absence of CFUs and following the resuscitation process at 30°C. In the case of *V. harveyi* BAA-1117^{TM} microcosm, after 48 hours of resuscitation we counted 9×10^2 cells/ml in the dilution 1:100. Assuming that they can be grown from a single non-VBNC parental cell, there should have been in the original microcosm at least 9×10^4 cultivable cells/ml, which we should have detected in the first plating. Similarly, in the case of *V. fortis* UU24, we obtained a patina of cells plating all the decimal dilutions from 1:10 to 1:10,000 after resuscitation.

Resuscitation of *V. harveyi* BAA-1117^{TM} and *V. fortis* UU24 has been monitored over time. Aliquots of the microcosms have been incubated at 30°C along different times, after which the cells have been plated and observed by flow cytometry (Fig. 1 E-H; Fig. 2E-H). In both strains the increase in CFUs was correlated to the time of incubation at 30°C (resuscitation time) with an exponential relationship, until the number of CFUs reaches a plateau. However, flow cytometer revealed a different behaviour of the two strains. In *V. harveyi* BAA-1117^{TM}, the decrease in size and DNA quantity and the increase in fluorescence resulted completely reverted after resuscitation with the process completed between 17 and 21 hours. After 17 hours, two populations are detectable: one smaller and with an increased fluorescence upon staining with propidium iodide, and one bigger (resuscitated) which showed a decrease of fluorescence (Fig. 1E, 1G, Fig. 5). Concomitantly, the green fluorescence upon staining with Syto9 increased (Fig. 1F). The same reversal has been observed in *V. fortis* UU24, but has been not completed in the monitored time (Fig. 2G, Fig. 5). The green fluorescence gradually decreased, but still after 64 hours cells were distributed in two populations of different fluorescence, (Fig. 5). DNA quantity and cell size did not seem to increase significantly (Fig. 2E-F).

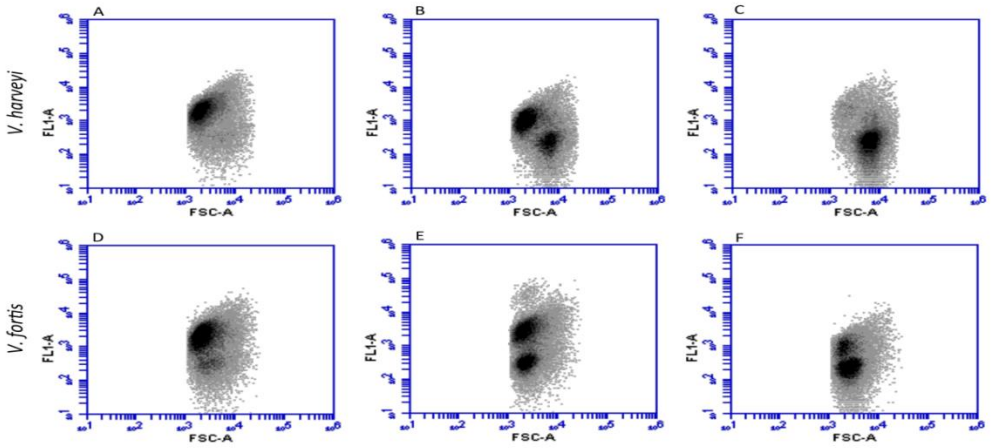

Figure 5. Size and green fluorescence upon staining with propidium iodide of *V. harveyi* BAA-1117^{TM} and *V. fortis* UU24 during the resuscitation process. A, B, C: *V. harveyi* VBNC-induced cells after 0, 17 and 21 hours of incubation at 30°C, respectively. D, E, F: *V. fortis* VBNC-induced cells after 1, 24 and 64 hours of incubation at 30°C, respectively. *V. harveyi* cells that were smaller in size were also the ones exhibiting the increase of green fluorescence upon staining with propidium iodide. During incubation at 30°C in "resuscitation" process, a less fluorescent population appeared, which in *V. harveyi* resulted bigger in size.

Growth of VBNC-induced cells on catalase-treated plates

According to the protocol described by Kong and co-workers (2004), we verified if the treatment of TSB-NaCl plates with catalase could restore the cultivability of VBNC cells. The trial has been made using aliquots of cells from a microcosm of *V. fortis* UU24 at age 179 days and a microcosm of *V. harveyi* BAA-1117^{TM} at age 31 days. Although the latter was not yet completely in VBNC state, the use of catalase greatly improved the cultivability of the cells in both cases, even though the number of colonies obtained with this procedure was 100 or 1000 fold lower than the number of colonies obtained by resuscitation at 30°C (Fig. 6).

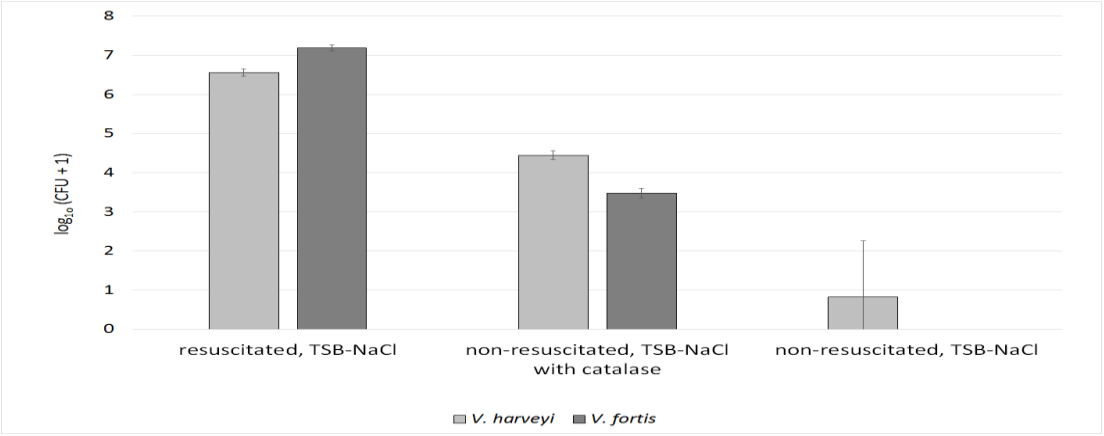

Figure 6. Cultivability of *V. harveyi* BAA-1117^{TM} (light grey) and *V. fortis* UU24 (dark grey) following different treatments i) after resuscitation on TSB-NaCl agar plates; ii) without resuscitation on TSB-NaCl agar plates treated with catalase; iii) without resuscitation on TSB-NaCl agar plates. Cells from *V. harveyi* BAA-1117^{TM} microcosm were not yet completely in VBNC state. Use of catalase greatly improved the cultivability of the cells for both strains.

Growth of resuscitated cells in the presence of H_2O_2

To assess the capability of our strains to grow in the presence of high oxidative stress, we challenged both cultured and VBNC-induced cells with different concentrations of hydrogen peroxide in microtiter plates. We noticed that the response to H_2O_2 was discrete, which means that in some wells we observed growth and in others not, while the growth rate and the final O.D. of the grown wells were similar. Thus, we counted the number of grown wells considering the different conditions. Standard-growing cells of *V. harveyi* BAA-1117^{TM} and *V. fortis* UU24 showed a different sensitivity to hydrogen peroxide. The growth of *V. harveyi* failed in a small proportion of the wells added with 0.05 mM hydrogen peroxide, and it has been consistently inhibited with 0.1 mM. Conversely, *V. fortis* showed a higher resistance: at 0.1 mM H_2O_2 it always grew as good as the control (no added H_2O_2). To prevent the growth of this strain in some of the wells was necessary to increase the hydrogen peroxide concentration to 0.2 mM. The same behaviour has been observed for cells withdrawn from the microcosms at 3 days of age (with cells not yet entered in VBNC state).

When in VBNC condition, the cells of both strains could not grow on any H_2O_2 concentration. Thus, we tried to grow them after different periods of resuscitation at 30°C in TSB-NaCl medium with hydrogen peroxide concentrations from 0.05 to 0.25 mM. According to our data, the capability to cope with oxidative stress is gradually acquired with the resuscitation time. After 7 and 5 hours of resuscitation, *V. harveyi* and *V. fortis* displayed a behavior similar to non-VBNC cells, respectively, while after 20 hours they seemed to be even more resistant to oxidative stress, growing in all the wells containing 0.1 or 0.2 mM of hydrogen peroxide, respectively (Fig. 7).

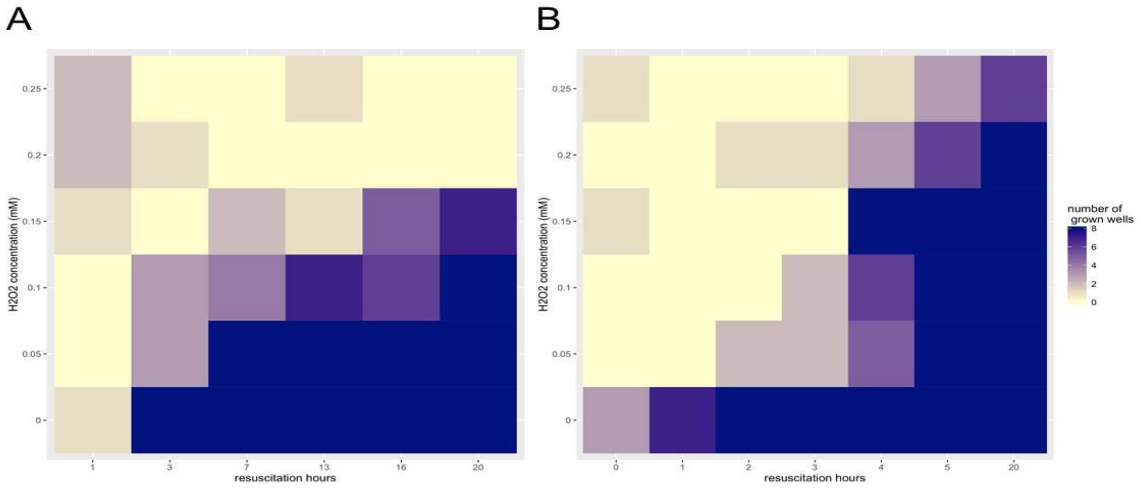

Figure 7. Heat maps showing the growth of *V. harveyi* BAA-1117^{TM} (A) and *V. fortis* UU24 (B) in presence of increasing hydrogen peroxide concentrations after different resuscitation times (incubation at 30°C) in a microtiter plate. X axis: hours of incubation at 30°C; Y axis: H_2O_2 concentrations (mM). Different colours represent number of wells in which growth has been detected based on the increase in optical density. Both the strains acquired the capability to overcome oxidative stress gradually during resuscitation, though with a different timing.

Resuscitation in presence of hydrogen peroxide

To further investigate the relationship between resuscitation and sensitivity to H_2O_2, we added hydrogen peroxide to the seawater prior to the incubation of VBNC cells at 30°C for resuscitation. According to Kong et al. (2004) a H_2O_2 concentration of 0.007 mM, present in the standard cultural medium, is enough to prevent cell growth. We verified that the same concentration (0.007 mM) prevented the resuscitation of VBNC cells of *V. harveyi* BAA-1117^{TM}, while for *V. fortis* UU24 concentrations higher than 0.02 mM were necessary. The cells did not show an increase in size nor anyone of the morphological changes described before for the resuscitation process (Fig. 8). Thus, we wanted to verify if they were still capable to resuscitate after relieving the oxidative stress by the addition of catalase. As expected, the cells can not resuscitate after a further overnight incubation with catalase; probably they are permanently damaged by the prolonged exposure to stress.

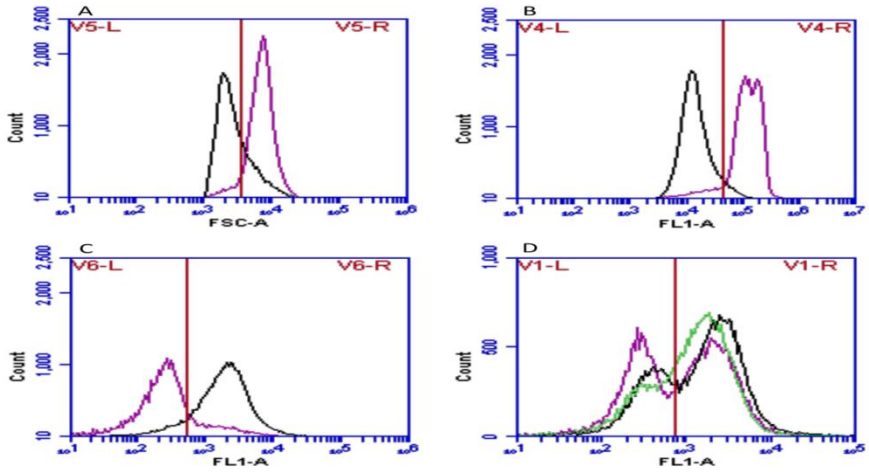

Figure 8. Size and fluorescence of the VBNC-induced cells of *V. harveyi* BAA-1117^{TM} and *V. fortis* UU24 incubated at 30°C to induce resuscitation in the presence of oxidative stress. Purple line: cells "resuscitated" at 30°C. Black line: cells incubated at the same conditions in the presence of 0.007 mM H_2O_2. Green line: cells incubated at the same conditions in the presence of 0.02 mM H_2O_2. A, B, C: *V. harveyi* BAA-1117^{TM}. While the resuscitated cells increased in cell size and DNA quantity and decreased in green fluorescence when stained with propidium iodide (cfr. Fig. 1), stressed cells preserved the morphological features of VBNC cells. A: Size (FSC-A). The size of stressed cells did not increase as that of resuscitated cells. B: Green fluorescence (FL1-A) of cells stained with Syto9. The quantity of DNA in stressed cells did not increase. C: Green fluorescence of cells stained with propidium iodide. The fluorescence of stressed cells did not decrease. D: *V. fortis* UU24; green fluorescence of cells stained with propidium iodide. While in resuscitated cells a big population has lost the fluorescence, in stressed cells this population is much smaller. While cells stressed with 0.007 mM H_2O_2 were still able to resuscitate and grow, cells stressed with higher concentrations of H_2O_2 are not. Size and DNA quantity did not show in this strain any appreciable difference between resuscitated, VBNC-induced and stressed cells.

Catalase production

Interestingly, only *V. harveyi* BAA-1117^{TM} showed a very high catalase production. The method of choice (Iwase et al., 2013) did not allow us to detect differences in the low response of our three environmental strains (*Vibrio hepatarius* UU21, *Vibrio fortis* UU24 and *Vibrio nereis* M5), which catalase production appears negligible if compared to the one of *V. harveyi* BAA-1117^{TM}.

Discussion

The timing of VBNC entrance has been described as highly variable in the genus *Vibrio*. The shorter times are reported for *V. vulnificus*, with 3 (Kong et al., 2004) or 4 days (Whitesides and Oliver, 1997), while in case of *V. cholerae* 60 (Asakura et al., 2007) or 77 days have been reported (Imamura et al., 2015; Senoh et al., 2015). Regarding *V. parahaemolyticus* we found contrasting reports, namely 9 (González-Escalona et al., 2006) or 69 days (Falcioni et al., 2008); the difference may be depending on the strain or on the specific experimental conditions. *V. harveyi* has been studied in the stage immediately preceding VBNC (Kaberdin et al., 2015; Montánchez et al., 2014). Besides these, no other attempts have been done to induce VBNC state in environmental *Vibrio* strains. Our experiments, performed on four *Vibrio* strains isolated from environmental samples and one collection strain (*V. harveyi* BAA-1117^{TM}), suggest that the entrance in VBNC state could be a very slow process, involving only a fraction of the population while another fraction remains unchanged.

In the *V. harveyi* BAA-1117^{TM} microcosm, all the cells rapidly and almost simultaneously lost their cultivability and acquired specific features when observed by flow cytometry (Fig. 1, 3, 5). Consistent with the observations reported in Falcioni et al. (2008), the size of the cells markedly decreased, as well as the DNA content (which can be evaluated assessing the fluorescence level of the cells stained with Syto9, a DNA-binding, green-fluorescent fluorophore). At the same time, when stained with propidium iodide they acquired an uncommon green fluorescence, which in our experiments seem to be a marker of VBNC induction (as discussed later, Fig. 1, 3, 5). In the other three strains, *V. fortis* UU24, *V. nereis* M5 and *V. hepatarius* UU21, we observed the same phenomena, even if at a significantly slower rate (Fig. 2-4). For the first 50 days, the cells were still cultivable, but it has been possible to detect a double population. Indeed, a fraction of the cells was smaller in size and showed a decrease in DNA quantity and an increase of the green fluorescent signal when stained with propidium iodide, while another fraction did not register any change. For one of the strains, *V. fortis* UU24, the observation has been further prolonged and after 108 days almost all the cells acquired the "VBNC features", while the cultivability has been lost. Based on these observations, we hypothesize that the microcosms at day 50, even if still cultivable, harboured a small VBNC-induced population that perhaps was about to increase with time.

The well-known "plate count paradox" states that only a minor fraction of the bacterial species in an environment is cultivable. Our findings pointed out the fact that also for cultivable strains, plate count could underestimate bacterial load. Indeed, if we compare the number of CFU of *V. fortis* at day 50 (4.5×10^5 CFU/ ml) to the number of CFUs of the same microcosm after resuscitation (8×10^6 CFU/ml) we registered a more-than-ten fold increase. The difference is due to a population of cells entering in VBNC state earlier than the others. In summary, while all the cells of *V. harveyi* BAA-1117^{TM} acquired the features of VBNC in a short time span, for our environmental strains the VBNC entrance appears to be a slow process, which eventually involved only a fraction of the total population. Thus, even if we

count a number of colonies of a species from an environmental sample, we can not be sure that it is representative of the entire population.

When VBNC-induced cells of *V. harveyi* BAA-1117^{TM} have been incubated at 30°C, the cultivability has been restored (Fig. 1H). Observed by flow cytometry, the cells showed no changes in the first 16 hours. At time 17h, half of the cells showed simultaneously an increase in size and DNA quantity and a decrease in the green fluorescence caused by propidium iodide. At time 21h, the process has been completed (Fig. 1). Interestingly, during the resuscitation we observed the exact reversion of the process of VBNC transition (Fig. 1). For this strain, that was the quicker to enter into VBNC phase in our experimental conditions, the resuscitation was also a rapid process involving almost all the cells at the same time. On the contrary, the resuscitation of *V. fortis* UU24 was slower and gradual (Fig. 2). Although returning cultivable, the cells only partially reverted the VBNC phenotype, particularly considering cell size and DNA quantity (Fig. 2E-G).

As observed for the entrance into VBNC, the resuscitation of the environmental strains is not a sharp process and can involve only one subpopulation (Fig. 5). This possibly indicates that the mechanisms of this process and the causative factors involved, maybe more than one, vary across species and/or that more time is necessary for a complete resuscitation. This implies that every attempt to improve the cultivation of environmental bacteria based on resuscitation protocol should be aware of the variability among strains. The timing and protocols applied to restore the cultivability could affect each strain differently. The attempt of resuscitating simultaneously a mixed community, for example from an environmental sample prior to a cultivation-based survey, could hence result in different proportions of resuscitated cells from each of the strains.

Besides resuscitating them by incubation at 30°C, *V. harveyi* and *V. fortis* VBNC-induced cells can be also grown by supplementing the plates with catalase to remove the hydrogen peroxide that is naturally present in the medium. However, the numbers of colonies are respectively two and three orders of magnitude less than the ones obtained by resuscitation (Fig. 6). Apparently, the oxidative stress is not the only factor implied in the loss of cultivability of these cells, in particular in the case of *V. fortis*.

To further investigate the role of hydrogen peroxide, we measured the sensitivity to hydrogen peroxide of both strains during the growth in standard conditions and the ability to produce catalase. Interestingly, although more sensitive to H_2O_2, *V. harveyi* BAA-1117^{TM} showed a catalase production far higher than *V. fortis* UU24, *V. hepatarius* UU21 and *V. nereis* M5, suggesting that our environmental strains could have different mechanisms to protect themselves. Next, we verified that during resuscitation in the presence of H_2O_2, the oxidative stress resistance is gradually acquired with the resuscitation time (Fig. 7). However, the addition of small amounts of hydrogen peroxide to the microcosms prior to incubation at 30°C prevented the resuscitation. Kong and colleagues (2004) measured that H_2O_2 concentration in their plates of Heart Infusion Agar was 0.007 mM. We verified that the addition of this hydrogen peroxide concentration to the microcosm aliquots to be resuscitated can prevent the resuscitation and growth of *V. harveyi*, which is more sensitive to hydrogen

peroxide, but not of *V. fortis*, which requires concentrations approximately three times higher. Nevertheless, in both cases the concentration that can prevent the resuscitation is far lower than the one that can inhibit growth, confirming the hypothesis that VBNC cells become more sensitive to oxidative stress. This effect seemed sharper in *V. harveyi*, which possibly relies more on catalase for the protection against oxidative stress. The VBNC cells incubated at 30°C with the addition of H_2O_2 do not show any morphological change as reported in case of resuscitated cells (Fig. 8). Thus, we wanted to verify if they retain the capability to resuscitate or they were dead. To this aim, we added catalase and we incubated them at 30°C for an additional overnight. When we plated them, we observed no colonies, suggesting that the exposure to hydrogen peroxide killed the VBNC cells before they could resuscitate.

According to our data, hydrogen peroxide seemed to be an important factor preventing the growth of VBNC-induced cells in plates, although it was not sufficient to explain this phenomenon in the tested strains. Its toxic effects varied in magnitude for the two strains, which resulted more sensitive as VBNC-induced cells rather than as growing cells.

Propidium iodide is a well-known red-fluorescent stain used to detect cells with damaged membranes. Although it is not clear if cells could actually recover after the damage (Davey and Hexley, 2011), it is commonly used to detect dead cells, often as part of live/dead kits (Falcioni et al., 2008; González-Escalona et al., 2006; Imamura et al., 2015). Unfortunately, we did not detect any difference in the red fluorescence of propidium iodide throughout the whole experiment. For this reason, we killed the cells in the microcosms by heating (10 min, 100°C) or by exposure to 50% isopropanol. Whereas in stationary phase cells the propidium iodide was effective in staining damaged cells, in the microcosm's cells the fluorescence did not change. There are two possible explanations of this phenomenon: one involves the salinity of the 50% ASW solution, which could impair the binding of the fluorophore, while the other is more directly related to the possible changes in the membranes of the cells exposed to starvation. Thus, we were not able to assess the number of damaged cells. However, observing the fluorescence of propidium iodide-stained cells in the green channel (FL1) we observed that the dwarf, low-DNA population that appeared as a consequence of prolonged incubation in cold ASW recorded an increase in green fluorescence. The fluorescence appeared only in the aliquots stained with propidium iodide, and not in the unmarked ones, and the observation was repeated in all the strains. During the resuscitation process the green fluorescence seemed to be linked to the VBNC-induced cells as well. While in *V. harveyi* BAA-1117^{TM} the decrease in fluorescence has been combined with other morphological changes, in *V. fortis* UU24 it was the only phenotypic feature useful to trace the resuscitation process. As described before, *V. fortis* resuscitated cells did not increase their size or DNA quantity, but lose the propidium iodide-induced green fluorescence. This phenomenon is rather difficult to explain, but yet consistent in our data. For this reason, we decided to report it to encourage scientists in this field to further investigate on it and find out if this is an universal phenomenon or rather an uncommon outcome of our experimental setup. Indeed, the discovery of a marker of VBNC *Vibrio* cells would be of great interest both from public health and marine ecology perspectives.

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Chapter III

Effects of cryoprotectants on viability of marine microorganisms

Abstract

Research on long-term preservation of marine bacteria is scarce, despite its essential importance in ordinary laboratory practices. Here, we evaluated the preserving ability of five different cryoprotective agents (CPA) on a set of marine and host-associated bacteria belonging to ten different taxa. Four common cryoprotective molecules, such as glycerol (20% v/v), skim milk (10% v/v), ethylene glycol (10% v/v) and glycine betaine (5% v/v), together with a combination of molecules, not yet reported as a cryoprotectant, i.e. urea and glucose (2.4%) have been used. After six, twelve, eighteen and twenty-four months of storage at -80°C, the viability of the cells has been assessed by plate count method. Among all strains, eight have been cryopreserved without a significant loss in culturability for up to 18 and 24 months by the use of different cryoprotectants. Data suggest that long-term cryopreservation of marine bacteria depends on the microorganism subjected to storage, the preservation time and the CPA used.

Keywords: preservation, glycerol, skim milk, ethylene glycol, glycine betaine, urea/glucose

Introduction

Marine microorganisms produce a large supply of pharmaceutically and biotechnologically useful metabolites, including antimicrobial, anticancer and antioxidant compounds and nutraceuticals (Bhatnagar and Kim, 2010; Anbu et al., 2013; De Vitis et al., 2015). However, most of them are largely uncultured and unexploited due to the lack of efficient isolation and/or cultivation methods (Prakash et al.2013). In this context, researches aimed at developing innovative cultivation methods and new molecular biology tools to disclose the enormous biotechnological potential of marine microorganisms are fundamental, as documented by different project funding e.g. MaCuMBA, www.macumbaproject.eu;PharmaSea, www.pharma-sea.eu; MicroB3, www.microb3.eu and Blugenics, http://www.bluegenics.eu/ (Romano et al., 2016).

Microorganisms are routinely maintained in the laboratory using long-term preservation methods, among which the cryopreservation and lyophilisation (also called freeze-drying) are the most used ones (Desolme and Bernardet, 1996; Vekeman et al., 2013). High efficient methods are important to avoid bacterial death in order to conserve the original biological characteristics of the strains subjected to storage. The preservation efficiency can vary according to the microbial species (Cody et al., 2008) and conditions such as the rate of freezing and thawing (Morgan et.al., 2006),temperature, cryoprotectant (considering its concentration and shelf life), composition of the growth medium and time of preservation (Hoefman et al, 2012; Heylen et al., 2012; Zhi-hui et al., 2009; Terezia et al., 2008).

Moreover, an optimal preservation sustains the accessibility to bacterial collections by the scientific community (Zhi-hui et al., 2009; Hoefman et al., 2013; Prakash et al., 2013).

Microorganisms subjected to freezing are exposed to intracellular ice formation, depending on the cooling rate (Mazur et al., 1972). If the cooling rate is too fast, the cell membranes cannot bring water out of the cell and water likely freezes inside. The formation of ice in the bacterium cytosol leads to DNA and protein damage, resulting in deprivation of culture activity and viability (Mazur et al., 1972). On the other hand, if the cooling rate is slow, the cells lose water rapidly enough to concentrate the intracellular solutes. As a result, the cells dehydrate and do not freeze intracellularly which may lead to cell shrinkage and cell injury (Gao et al., 2000). Some microorganisms are able to tolerate moderate cold and osmotic stresses by secreting substances called "osmoprotectants" that help them to maintain an internal osmotic potential equal to the external surrounding environment without high intracellular salt accumulation (Csonka and Epstein, 1996). Some other organisms, like animals and plants, respond to external stimulations such as freezing in peculiar ways. For example, some freeze-tolerant insects, such as the dipteran *Eurosta solidaginis*, accumulate glycerol and sorbitol in their bodies during autumn to protect themselves from cold conditions of winter (Storey., 1997). The freeze-tolerant frog *Rana sylvatica* accumulates urea during extended dormancy and when exposed to low water potential, a response that aids in maintaining hydration during saline adaptation and estivation. However to overcome freezing conditions it produces glucose as an immediate response to the initiation of ice formation by cleaving glycogen from liver allowing glucose to circulate in core organs as cryoprotectant (Storey., 1997; Sinclair et al., 2012).

Cryoprotective agents (CPA) include a variety of chemical compounds e.g. glycerol, glycine betaine, sucrose, amino acids and carbonic acids. These compounds work increasing the total amount of solute in the system and, therefore, reducing the ice formation. Glycerol is the conventionally used compound in laboratory for conservation at -80°C; it has been used in different concentrations and reported effective for preserving many strains, among which halophilic archaea (Cleland et al., 2014; Terezia et al., 2008; Zhi -hui et al., 2009; Wu et al., 2008). Other compounds have been used as CPA with varying degrees of success, including DMSO, glycine betaine, skim milk, maltose, sucrose, trehalose and ethylene glycol (Hubálek, 2003). Nonetheless, none of them is considered a universal protectant that could work with different microbial genera (Cleland et al., 2004).

CPAs are highly hydrophilic molecules due to the presence of chemical groups forming strong hydrogen bonds with water (hydroxyl, sulfoxide, amide, etc.). They can provide protection by being intracellular or extracellular (Pichugin, 1993), hence they can be classified upon the rate of their penetration in the cell (Meryman, 1971). Mono-, oligo-, and polysaccharides, mannitol, sorbitol, dextran, albumin, gelatin and other proteins do not penetrate inside the cell; they adsorb on the microbial surface and inhibit the growth of ice crystals by increasing solution viscosity, keeping the structure of ice amorphous in the close proximity of the cell (Hubálek., 2003). Glycerol penetrates slowly and ethylene glycol, methanol, ethanol penetrate quickly within 30 min. Penetrating CPA protect cells by accumulating in their cytosols, binding colligatively intracellular water and preventing the

formation of large ice crystals and excessive dehydration. Creating a gel-type glass phase below the eutectic point, they prevent hyperosmotic injury to the cells and surface lesions caused by NaCl (Hubálek., 2003).

To our knowledge, few investigations have been specifically carried out on preservation of marine bacteria. Emerson and his group in 2004 reported that 12% (w/v) glycine betaine is effective in cryoprotection of prokaryotes, including two marine strains *Psychromonas marina* and *Silibacter pomeroyi* by freezing in liquid nitrogen. Heylen and his group (2013) showed that 10% dimethyl sulfoxide (DMSO) is effective for preserving three strains of marine nitrite-oxidizing bacteria at -80°C (Vekeman et al., 2013). Combining 5% DMSO with the carbon compounds 0.3% TSB and 1% trehalose results in a more efficient preservation than using DMSO solely in case of methane-oxidizing bacteria and ammonia-oxidizing bacteria , particularly on the three marine strains, *Candidatus Scalindua*, *Nitrosomonas marina* and *Nitrosococcus oceani* (Heylen et al., 2012; Hoefman et al., 2012; Hoefman et al., 2013).

In this study, we aimed to assess the cryoprotection properties of different selected molecules and the effect of freezing time on the viability of ten marine strains encompassing different phylogenetic taxa. We adopted conventionally used CPA such as glycerol, skim milk, ethylene glycol, glycine betaine and a molecules' combination, not so far reported to be used as CPA, i.e. urea and glucose. The microbial viability has been monitored by plate count method after six, twelve, eighteen and twenty-four months of storage at -80°C.

Materials and methods

Microorganisms and culture conditions

The ten bacterial strains used for this work and belonging to different taxonomic groups are reported in table 1. Strains have been routinely grown on Marine Agar (MA, Conda) plates or in Marine Broth (MB, Conda) tubes/flasks. Strains 1U, 5U, 13U and 24U have been isolated from Urania, a deep hypersaline anoxic basin (DHABs) in the Eastern Mediterranean Sea (De Vitis et al, 2015). Strains PG4 and PG8 have been isolated from the gills of the mangrove crab *Perisesarma guttamum*, whereas strains UU11, UU28, UU98 and UU70 have been obtained from the gills of the mangrove crab *Uca urvillei*, as reported in the next paragraph.

Table 1. List of the bacterial strains and their isolation source.

Bacteria	Taxonomic group	Isolation source
Halomonas aquamarina 13U	Gammaproteobacteria	DHABs
Idiomarina loihiensis 1U	Gammaproteobacteria	DHABs
Salinisphaera shabanensis 24U	Gammaproteobacteria	DHABs
Pseudoalteromonas ganghwensis 5U	Gammaproteobacteria	DHABs
Shewanella algae PG4	Gammaproteobacteria	*Perisesarma guttatum* gill
Pseudomonas putida UU11	Gammaproteobacteria	*Perisesarma guttatum* gill
Micrococcus yunnanensis PG8	Actinobacteria	*Perisesarma guttatum* gill
Vibrio furnissii UU28	Gammaproteobacteria	*Uca urvillei* gill
Bacillus algicola UU98	Firmicutes	*Uca urvillei* gill
Rhodococcus qingshengii UU70	Actinobacteria	*Uca urvillei* gill

Isolation procedures

Crab dissection has been carried out in sterility, separating the gills from the rest of the body. After smashing the organ in sterile physiological solution (0.9% NaCl), serial dilutions have been plated on MA plates added with cycloheximide (100 ppm) to inhibit eukaryotic growth. When the bacterial growth occurred, random colonies have been selected and purified. Identity of the strains has been established by amplification of a fragment of the 16S rRNA gene by the use of primers 27F (5′-TCG ACA TCG TTT ACG GCG TG-3′) and 1495R (5′-CTA CGG CTA CCT TGT TAC GA-3′), as previously described (Mapelli et al., 2013). 16S rRNA gene fragments have been sequenced from Macrogen (Korea) and sequences have been compared to the public databases at the National Centre for Biotechnology Information (NCBI) using BLASTn (Altshul et al., 1990).

Cryoprotectant molecules

CPA used in this work are: 20 % v/v glycerol, 10% v/v skim milk, 10% v/v ethylene glycol, 5% v/v glycine betaine (Hubálek, 2003) and 2.4% urea/glucose glycerol (Sinclair et al., 2012).

Experimental procedure of cryopreservation

Strains have been inoculated in 20 ml of MB. Cultures have been incubated at 30 °C until they reached the late exponential or stationary phase. After that, a fixed number of cells for each strain has been inoculated in triplicate in 96-wells microtiter plates in presence of different CPA. Volume transfers have been performed by the use of EpMotion 5070 liquid handling workstation (Eppendorf, Milan, Italy). For each bacterium five identical microtiter plates have been prepared with the aim to incubate all of them at -80 °C and thaw each one after a fixed time. After 6, 12, 18 and 24 months of storage, 100 µl of cryopreserved cultures have been diluted and plated on MA plates in triplicate. Plates have been incubated at 30 °C overnight and then cell viability has been monitored by colony counting.

Statistical analysis

PERMANOVA analysis has been applied to statistically evaluate the differences in the cell counts along time and using the different CPA. A P value below 0.05 was considered statistically significant.

Results and discussion

Identification of the strains

Ten selected strains, indicated in tab. 1, have been used for long-term preservation experiments by which evaluate if chosen molecules can have different cryoprotection properties on the bacterial cells. Specifically, strains derived from the DHAB Urania in the Eastern Mediterranean sea (De Vitis et al., 2015) and from isolation experiments performed from the gills of the mangrove crabs *P. guttatum* and *U. urvillei*. 16S rRNA gene sequencing indicated that PG4, PG8, UU11, UU28, UU70 and UU98 showed >97 % identity with *Shewanella algae*, *Pseudomonas putida*, *Micrococcus yunnanensis*, *Vibrio furnissi*, *Bacillus algicola* and *Rhodococcus qingshengii* respectively (Tab. 2).

Table 2.Taxonomic identification of the tested bacterial strains

Strain code	Isolation source	Closest described species (acc. no.)	Phylum/Class	% Identity
13U	DHABs	*Halomonas aquamarina (AB681582)*	Gammaproteobacteria	99
1U	DHABs	*Idiomarina loihiensis (AY505529)*	Gammaproteobacteria	99
24U	DHABs	*Salinisphaera shabanensis (JF281734)*	Gammaproteobacteria	99
5U	DHABs	*Pseudoalteromonas ganghwensis (DQ011614)*	Gammaproteobacteria	99
PG4	*Perisesarma guttatum* gill	*Shewanella algae (AB681331)*	Gammaproteobacteria	99
UU11	*Perisesarma guttatum* gill	*Pseudomonas putida (NR074739)*	Gammaproteobacteria	99
PG8	*Perisesarma guttatum* gill	*Micrococcus yunnanensis (NR_116578)*	Actinobacteria	99
UU28	*Uca urvillei* gill	*Vibrio furnissii (FJ906812)*	Gammaproteobacteria	99
UU98	*Uca urvillei* gill	*Bacillus algicola (NR_029077)*	Firmicutes	99
UU70	*Uca urvillei* gill	*Rhodococcus qingshengii (KC355321)*	Actinobacteria	99

Cryopreservation efficiency

Before storage, the initial cell concentration (T0) has been measured by plating 0.1 ml of each overnight culture: values between 10^8 - 10^{11} CFU ml^{-1} have been reported. The cryoprotection efficiency of the different CPA has been thus evaluated following the storage at -80 °C for different periods of time, i.e. 6 (T6), 12 (T12), 18 (T18) and 24 (T24) months. In general, we observed that the survival rate of the cultures depended on the microorganism subjected to storage, the freezing period and the CPA used. Figure 1 (a-j) depicts the survival rate for each strain.

All the strains resulted well preserved with glycerol for up to 24 months, except for *H. aquamarina* 13U, *V. furnissii* UU28 and *S. algae* PG4. The latter two strains (UU28 and PG4) showed a significant loss of culturability after 6 months of storage at -80 °C with all the considered CPA. Glycerol efficacy in cryopreserving microorganisms has been widely reported; indeed it is commonly used at concentrations of 2–55% for freezing diverse viruses, bacteria, algae and protozoa (Hubálek, 2003). The cryoprotective activity of hydroxyl

compounds such as the ones present in glycerol or sugars (e.g. glucose) might be caused by the ability of these agents to prevent injurious eutectic freezing of cell fluids by trapping salts (NaCl) in a highly viscous or glass-like phase (Chen et al., 2000). Since in this work we applied a concentration of 10%, we cannot exclude that the use of different glycerol concentrations could work in preserving the two abovementioned strains for which preservation with glycerol failed.

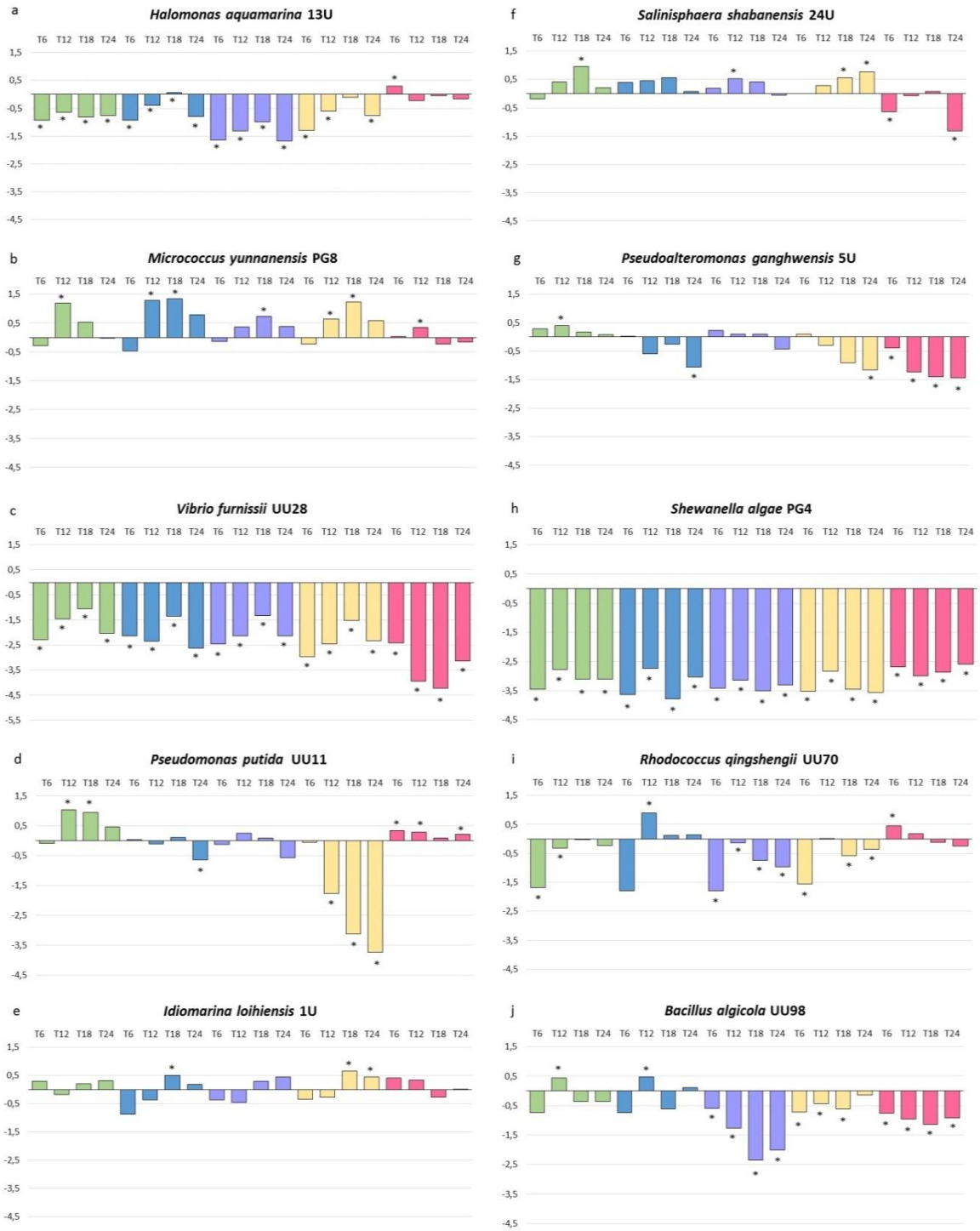

Figure. 1. Bar plots concerning the difference of CFU/ml at T6, T12, T18 and T24 for the following CPA, glycerol (green), skim milk (light blue), ethylene glycol (violet), glycine betaine (yellow) and urea/glucose (purple). Cell counts measured at T0 have been used as comparison parameters, subtracting them to the cell count values measured for the different time points. In the plot measures have been expressed as log Tx-logT0, where Tx is T6, T12, T18 and T24 respectively. Asterisks indicate a statistical difference with T0 (p<0.05).

In case of *M. yunnanensis* PG8 and *I. loihiensis* 1U no significant loss of culturability compared to T0 has been retrieved using all the CPA, thus indicating that these strains were perfectly preserved up to 24 months at -80°C. The only exceptions are represented by T18 and T24 for strain 1U using glycine betaine For all the other strains (*B. algicola* UU98, *P. putida* UU11, *P. ganghwensis* 5U, *S. shabanensis* 24U and *R. qingshengii* UU70) intermediate situations have been reported: UU98, UU11 and 5U were cryopreserved with either skim milk or ethylene glycol, beyond glycerol, whereas 24U and UU70 strains resulted cryopreserved with either ethylene glycol or urea/glucose, beyond glycerol and skim milk.

Interestingly, *H. aquamarina* 13U, *R. qingshengii* UU70, *I. loihiensis* 1U and *M. yunnanensis* PG8 were perfectly preserved using urea/glucose, a combination never used so far for cryopreservation at -80 °C. Moreover, this novel combination of molecules showed a better efficacy than the other tested CPA in case of *H. aquamarina* 13U (Tab. 2). Finally, we documented an unexpected behavior in few cases, i.e. for *P. putida* UU11 with skim milk and *P. ganghwensis* 5U with skim milk and glycine betaine: in these cases, just the last time point (T24) resulted statistically different from T0. Figure 2 illustrates a summary of the cryopreserving efficacy with the different strains and molecules.

Skim milk is the common CPA used to preserve bacteria during lyophilisation (Hubálek, 2003) and sometimes it is used in combination with other CPAs (Cody et al., 2008). It has been also reported as an efficient molecule for preserving strains subjected to repeated freezing/thawing cycles after freezing at -80°C, such as in the case of *Pseudomonas aeruginosa* (Cody et al., 2008). Therefore, the use of 10% skim milk for storage is recommended by Schurr and his group (2008). Here, we used the same suggested concentration of skim milk to preserve our strains: half of our strains resulted well preserved using this CPA.

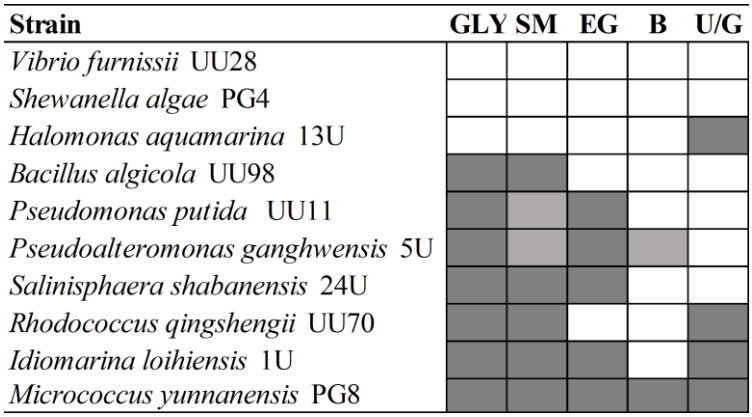

Figure.2. Cryoprotection efficacy of the different molecules used. GLY: glycerol; SM: skim milk; EG: ethylene glycol; B: glycine betaine and U/G: urea/glucose. Grey cells indicate no significant difference from T0; white cells indicate a significant difference with T0, while light grey cells indicate no clear situations (in these cases just T24 resulted statistically different from T0).

In the present study ethylene glycol failed to preserve half of the tested strains. Previous works have reported its use as CPA in freezing of specific microorganisms, such as yeasts, actinomycetes, fungi, algae and the myxomycete *Physarella oblonga* (Hubálek et al., 2003). However, its toxic effect has been reported on protozoans since, as a diol, it has been found to act as a solvent for some microbial polysaccharides (Hubálek et al., 2003).

Glycine betaine is a very common osmoprotectant (Csonka and Epstein 1996). Usually taken by the cell from the extracellular environment, it can be also produced by bacteria: for example, *Escherichia coli* synthetizes it from exogenous choline, which is converted to betaine by choline dehydrogenase and betaine aldehyde dehydrogenase (Caldas et al., 1999). In addition, in *E. coli* cells it works as thermal protectant, by protecting citrate synthase and *β*-galactosidase against thermal denaturation (Caldas et al., 1999). Glycine betaine was also not efficient in preserving the strains of this study, except in case of PG8. It has been indicated as efficient as glycerol in preserving halophilic archaeal and Fe-oxidizing bacterial strains (Cleland et al., 2004).

Conclusions

In this work, we evaluated the preserving ability of five different CPA on a series of marine and crab-associated bacteria belonging to different taxa when stored at -80 °C. Cryopreservation of bacteria is a fundamental step in the routinely laboratory duties and the selection and addition of suitable CPA to the sample culture is essential. The five cryoprotectants we used resulted relatively effective in preserving the different cultures under the same conditions of storage. Glycerol showed the best performance in cryopreserving our strains, whereas glycine betaine resulted the less suitable molecule, at least in our experimental conditions. Skim milk and ethylene glycol displayed a good behavior and, surprisingly, also the combination of urea and glucose, inspired from the *R. sylvatica* physiological response to freezing condition (Sinclair et al., 2013), worked in cryopreserving several of our strains (two strains in the class Gammaproteobacteria and two in the class Actinobacteria). In conclusion, data demonstrated that the efficiency of the preservation depended on the microorganism used, the CPA used and the time of preservation.

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Chapter IV

Genetic manipulation of marine bacteria

Part-I: Methods for the genetic manipulation of marine bacteria

Abstract

Genetic manipulation of bacteria generally aims to obtain new strains expressing peculiar and defined genetic determinants or to introduce genetic variants responsible for phenotypic modifications. It can be applied to explore the biotechnological potential associated to environmental bacteria and to exploit the functional properties of specific genes when inserted in an appropriate host. In the last years, marine bacteria have received an increasing attention, since they represent a fascinating reservoir of genetic and functional diversity useful for the industry. However, there is an urgent need to deeply investigate genetic manipulation tools applicable to marine strains, since the paucity of the knowledge in this regard. This review aims to describe genetic manipulation methods hitherto used in marine bacteria, highlighting the limiting factors of different techniques with the aim to increase genetic manipulation efficiency. In particular, we focus on natural and artificial transformation (especially electroporation) and conjugation, since they have been successfully applied to several marine strains. Finally, we emphasize that future work should be done to establish tailored methodologies for marine bacteria to avoid failures

Introduction

In the endless microbial diversity present on our planet, marine microorganisms are a fascinating reservoir of genetic and functional diversity, the products of which could be exploited in many different biotechnological sectors. As instance, they can produce bioactive natural compounds, enzymes useful in industrial applications or pharmaceuticals agents with anti-cancer, anti-microbial, or anti-inflammation properties (Dionisi et al., 2012; Tziveleka et al., 2003; Yasuhara-Bell and Lu, 2010; Xiong et al., 2013; Zhang et al., 2009; Anbu et al., 2013; De Vitis et al., 2015). However, many marine microorganisms remain largely uncultured, and thus unexploited, due to the lack of current efficient isolation and/or cultivation methods (Joint et al., 2010; Prakash et al., 2013). The recent awareness that marine microorganisms represent a promising source of intriguing compounds is prompting the research and the interest from the scientific community. In this scenario, a better knowledge of genetic manipulation systems to be applied to marine microorganisms is required too. Heterologous expression of genes directly from environmental metagenomes can be seen as an approach able in principle to overcome the limitation given by the poor culturability of marine strains. It is not nevertheless free from drawbacks, as reviewed by Lam et al. (2015), and cannot

entirely substitute the biotechnological application of novel and manipulated marine microbial isolates.

Genetic manipulation (or genetic engineering) of microorganisms relies on different molecular biology tools; they are generally applied to obtain new strains expressing additional genetic properties or to introduce genetic variants with phenotypic alterations (Thomas, 2009). Particularly, one of the most interesting applications in the field of environmental microbiology is to exploit genetic manipulation methods to explore the enormous undiscovered source of genetic information associated to the environmental samples (e.g. through metagenomics libraries expressed in suitable culturable marine hosts; Aakvik et al., 2009) and to study the biotechnological potential of cultured microorganisms (e.g. to investigate gene functions).

DNA can naturally enter into a bacterial cell following different pathways, including transduction through bacteriophage infection (Lederberg, 2000), conjugation mediated by cell-cell contact (Chen et al., 2005) or directly from the environment, through a natural physiological state of competence developed by the bacterium (Johnsborg et al., 2007). Once internalized, DNA can be then maintained in the host cell if integrated in the genome, following homologous recombination, or if internalized genes are recirculated on a plasmid replicative in the host. Otherwise, DNA is lost and hence not transmitted to daughter cells. Generally, studies on horizontal gene transfer by transformation and conjugation have been more considered than those by transduction, because of the lytic effect of phage infection (Jiang and Paul, 1998). Gene transfer via transduction in marine environment has been reported in a study by Jiang and Paul (1998) who showed the transduction of plasmid DNA to marine bacterial isolates and concentrated marine bacterial communities at frequencies ranging from 10^{-7} to 10^{-9} transductants/PFU (plaque-forming unit). By using a mathematical model the authors estimated that approximately 1.3×10^{14} transduction events occur each year in the Tampa Bay Estuary on the coast of Florida (Jiang and Paul, 1998). A particular genetic exchange active in natural environments, including for instance subtropical ocean, is mediated by virus-like Gene Transfer Agents (GTAs), which can package their own DNA with random fragments of the host chromosome and transduced it to a recipient cell (McDaniel et al., 2012), showing differences with the process mediated by transducing phages (Lang et al. 2012).

In the last decades a special attention has been directed to the gene transfer by bacterial natural competence state (Stewart and Carlson, 1986; Meibom et al., 2005). Natural competence is a time-limited physiological state specifically developed in some species in response to different external factors. Heretofore, over 87 bacterial species have been reported as naturally competent to uptake extracellular genetic material (De Vries and Wackernagel, 2005; Johnsborg *et al.*, 2007). The environment in which bacteria thrive plays an important role for their adaptation and evolution and, consequently, it influences gene transfer features. As instance, natural transformation occurs more likely in the marine environment sediments than water column, due to the capability of sands to protect DNA from

DNase degradation (Stewart and Sinigalliano, 1990). Moreover, Meibom et al. (2005) showed that chitin, which is an abundant biopolymer in aquatic environment, promotes the development of natural competence in *Vibrio* strains, including the two pathogens *V. cholerae* and *V. vulnificus*. DNA preservation with transforming potential has been, moreover, demonstrated in extreme marine environments like the brines collected from the deep anoxic hypersaline basins (DHABs) of the Mediterranean Sea (van der Wielen et al. 2005). DHABs brines constitute an extremely aggressive environment for cells and macromolecules, being hypersaline, anoxic and strongly reduced. Plasmid DNA incubated in brines of different DHABs up to 32 days was able to transform natural competent *Acinetobacter baylii* BD413 cells (Borin et al., 2008).

In order to insert DNA into bacteria with high efficiency, different artificial methods have been developed, including chemical and physical procedures (Mandel and Higa, 1970; Neumann et al., 1982). Since homologous recombination of exogenous DNA in a host cell is limited to sequences with high DNA similarity, shuttle vectors are preferred to compel DNA entrance into bacterial cells (Thomas and Nielsen, 2005). Plasmids are extra chromosomal DNA elements that are usually transferred in nature to both closely and distantly-related bacterial species, according to their replication origin (Wang et al., 2007; Mazodier and Davies 1991). Although non-essential for microorganisms, plasmids may harbor important genes that assist bacteria to adapt to changing environmental conditions, such as genes conferring heavy metal resistance and antibiotic resistance (Alippi et al., 2014; Tazzyman and Bonhoeffer, 2015) or codifying for new catabolic properties (Dennis, 2005). They have been generally used as shuttle vectors in electroporation and conjugation-based procedures, due to their relative manipulation ease. Nevertheless, certain bacteria are recalcitrant to internalize and express exogenous genes (Aune and Aachmann, 2010): for example, electroporation is known as an easy and rapid tool to successfully transform a large range of bacteria, but it is still impractical for certain marine species (Wang et al., 2015). In this review, we aim to summarize the genetic manipulation methods currently used for marine bacteria and to emphasize the limits that could prevent their success. Specifically, we considered natural competence, transformation by electroporation and conjugation-base procedures hitherto used to genetically manipulate marine bacteria.

Natural competence of marine bacteria

The ability of bacteria to acquire and express new features conferring improved capacity to adapt to new environmental conditions by internalization of genetic material from surroundings, is an important element of bacterial evolution (Griffith, 1928; Antonova et al., 2015b). Internalized DNA could be used as nutrient (Palchevskiy and Finkel, 2006) or it could to be incorporated into the cell's genome by homologous recombination (Hamilton and Dillard, 2006) or maintained in a re-circularized replicative plasmid.

Mechanisms underpinning natural genetic transformation have been described for several bacterial strains, i.e. the human pathogenic strains *Haemophilus influenzae* and *Streptococcus pneumoniae* and the model organism of Gram-positive bacteria *Bacillus subtilis* (Claverys et al., 2006). Moreover, natural transformation has been studied in relation to evolution of genomes in the environmental natural competent species *Acinetobacter baylii* (Utnes et al., 2015). In aquatic environments natural transformation is considered favored to occur since the wide presence of free DNA (DeFlaun et al., 1987) and the existence of competent cells (Linberg, 1994). Nevertheless, only a limited number of studies have investigated this process in marine isolates, considering only a few species (Stewart et al., 1989; Meibom et al., 2005). For the first time, in 1989, *Stewart and colleagues showed that natural transformation could occur in a marine bacterium, depicting the strain Pseudomonas stutzeri* ZoBell as the first transformed marine model (Stewart et al., 1989). Frischer et al. (1993) focused on chemical and physical factors affecting competence in the marine strain wrongly classified as *Vibrio* WJT-1C (after identified as *Pseudomonas* sp.; Frischer et al., 1996). In this strain, natural competence arises at early exponential phase and lasts almost 10 days. The authors verified that its transformation efficiency is not susceptible to the environmental variations that could occur in the estuarine environment, such as temperature, nutrient and salinity shifts, suggesting that for the considered strain the estuarine environment represents a good niche for natural transformation (Frischer et al., 1993).

Natural transformation studies have been then focused on *Vibrio* genus (Meibom et al., 2005; Wang et al., 2009), particularly on *V. cholerae*. Besides being a human pathogen, *V. cholerae* is also an inhabitant of aquatic environment (Kirchberger et al., 2016), where it has been described as a good colonizer of chitin-based surfaces (Blokesch et al., 2015). Chitin is the most abundant biopolymer in aquatic environment and the major constituent of copepod exoskeletons, crab shells and diatoms (Keyhani and Roseman, 1999; Hunt *et al.*, 2008). It represents an important nutrient source for chitinolytic bacteria including those belonging to the Vibrionaceae family, which can break it down into soluble subunits of N-acetylglucosamine (GlcNAc) and chitobiose (GlcNAc2) and use it as a sole source of carbon (Keyhani & Roseman, 1999; Hunt et al., 2008; Pruzzo et al., 2008). Meibom and co-authors (2005) discovered that chitin plays another intriguing role for *Vibrio* members by making them competent to uptake exogenous DNA. Consequently, the competence state in *V. cholerae* started to be examined in detail (Lo Scrudato and Blokesch, 2012; Altermark et al., 2007; Antonova et al., 2011; Antonova et al., 2012; Antonova et al., 2015a; Antonova et al., 2015b). In this strain, the bacterial regulatory network harmonizes the response of cells to starvation through three extracellular systems. First, a chitin system activates the transcription of a regulatory gene tfoX (Yamamoto et al., 2010), which in turn regulates the activation of chitinase enzymes responsible to hydrolyze chitin in its hydrolyzed forms. Then, TfoX-induced genes, as well as other components, regulate the production of the DNA-uptake machinery,

including a central type IV pilus structure (Meibom et al., 2005). Secondarily, at high cellular density a quorum sensing regulator promotes the expression of hapR gene (Meibom et al., 2005; Borgeaud et al., 2015; Antonova et al., 2015b; Suckow et al., 2011) which represses the expression of the extracellular nuclease Dns and regulates the activation of genes necessary for natural transformation (Blokesch and Schoolnik, 2008; Lo Scrudato et al., 2013). Finally, an extracellular nucleoside system allows the expression of CytR that functions as an additional positive regulator for competence regulation (Antonova *et al.*, 2012; Watve et al., 2015). Natural competence state has been identified also among isolates belonging to other *Vibrio* species, as *V. parahaemolyticus* (Chen *et al*., 2010), *V. vulnificus* (Gulig *et al.*, 2009) and *V. fischeri* (Pollack-Berti *et al*., 2010).

Although chitin is able to induce competence in different *Vibrio* species, the competence apparatus does not seem conserved. Comparison of genomic sequences of *Vibrio* strains showed that in *V. fischeri* and *V. cholerae* an additional TfoX-like protein, designated as TfoY, is present. In *V. fischeri* TfoY contributes with TfoX to the natural transformation but with a different function, whereas in *V. cholerae* it does not contribute directly to the natural competence (Pollack-Berti *et al.*, 2010). Indeed, in *V. cholerae* TfoY is more involved in the activation of type VI secretion system (T6SS), responsible for bacterial killing and eukaryotic intoxication and therefore it enhances the horizontal gene transfer, since it frees genomic DNA from prey cells (Borgeaud et al., 2015). Moreover, the form of chitin able to induce cell competence differs among species: for example in *V. fischeri* only the oligosaccharidic chitin can induce competence, whereas the chitin induces competence in *V. cholerae, V. vulnifivus* (Meibom et al., 2005; Udden et al., 2008; Gulig et al., 2009) and *V. parahaemolyticus* (Chen et al., 2010). In *V. vulnificus* the disaccharide GlcNAc2 also induces competence, but not the monomer GlcNAc (Neiman et al., 2011).

Endonuclease restriction systems are used by bacteria to protect their DNA from bacteriophage infection, and in general from the effects of exogenous DNA once entered in the cell. These systems are peculiar of each species; for instance, *V. cholerae* has two extracellular nucleases Dns and Xtr, which can degrade the exogenous DNA preventing its internalization. Specifically, at low cellular density Dns, activated by the quorum sensing regulator HapR, degrades the extracellular DNA, whereas at high cell density, HapR regulator represses Dns expression and the external DNA is not degraded (Blokesch and Schoolnik, 2008). The authors suggested that the nuclease, induced by low cell density, favors the bacterial rapid growth supplying the cells with nucleotides to sustain their growth; conversely, when the population reaches a high cell density, the nuclease is not more produced, the uptake of DNA is induced and this would allow a possible genome diversification (Blokesch and Schoolnik, 2008).

Exploiting chitin induction, natural acquisition of plasmid DNA has been reported in *Vibrio* by cell-mediated contact between bacteria (Paul et al., 1992). The authors showed that *Vibrio* strains were able to uptake non-conjugative plasmids after a contact with *Escherichia coli* as donor cells (whether live or dead),

whereas in their absence or when they are separated from recipient cells by a 0.2 µm filter DNA uptake did not occur (Paul et al., 1992). The same system has been also verified in soil bacteria by Wang et al. (2007) who demonstrated that transformation succeeded between *E. coli* and *B. subtilis*, exclusively in the presence of high cellular density of the donors, an important factor for efficient transformations.

Transferred genetic material may also encode for virulence genes. It is likely that the virulence of *V. cholerae* resulted after several horizontal gene transfers, which could explain the evolution of this marine bacterium into a major human pathogen (Waldor and Mekalanos, 1996). Likewise, the transfer of the genes encoding for the capsular polysaccharide (CPS), an important factor of virulence, occurred in *V. vulnificus* via horizontal gene transfer (HGT), providing an explanation of the diversity and evolution of CPS loci in marine bacteria (Nakhamchik et al., 2010).

Despite the number of naturally competent marine strains described for their natural capability to take up and express exogenous DNA, few studies have exploited natural transformation as laboratory method for their genetic manipulation (Frischer et al., 1993; Pollack-Berti et al., 2010). As instance, Pollack-Berti *and his colleagues* (2010) used a simple and efficient protocol to naturally transform *V. fischeri* with DNA. Specifically, the strain has been cultured in minimal medium (MM) supplemented with chitin as carbon source (chitohexaose); 1 ml of culture has been then mixed with 48 µg of DNA and incubated at 25°C for 30 min, followed by a dilution into Luria–Bertani salt medium (LBS) for a recovery period between 4 and 14 h, before selecting putative transformants on selective medium. Natural transformation of this strain has been achieved with high frequencies providing a useful tool for experimental genetic manipulation of the species (Pollack-Berti et al., 2010). Interestingly, *V. fischeri* cells could be genetically modified using other genetic transfer methods, i.e. conjugation of small and large plasmids (Dunn et al., 2005) and transduction, as suggested from the genome inspection of two *V. fischeri* strains (Ruby et al., 2005; Mandel, 2008).

Artificial competence of marine bacteria

Chemical transformation of marine bacteria

Artificial transformation has been applied for the first time by Mandel and Higa (1970): treating *E. coli* cells with a solution of $CaCl_2$, they made the cells competent to uptake exogenous DNA. Subsequently, Cohen and others (1972) demonstrated that the exposure of cold mixture of bacterial cells to DNA and the subsequent application of a thermal shock at 42°C created pores in the cellular membrane promoting DNA uptake and thus bacterial transformation. Also for *Roseobacter* sp. chemical transformation approaches seemed not efficient (Piekarski et al., 2009), whereas electroporation methods resulted successful.

Recently, *V. natriegens* chemical transformation showed efficiencies of 10^5-10^6 CFU/µg plasmid DNA (Weinstock et al., 2016).

Electroporation of marine bacteria

The breakthrough of transformation procedures has been made with the discovery that an electric pulse applied to cells at around 5–10 kV/cm could induce cell membrane permeability by transient pore formation, promoting cell uptake of DNA and allowing transformation of a large range of bacteria (Neumann et al., 1982; Potter et al., 1984). Nowadays, exogenous DNA is broadly inserted into bacterial cells by electroporation using a simple method that includes few steps. First, cells are made competent to acquire exogenous molecules by applying several washings with appropriate buffers. This allows to eliminate remaining ions and substrates/metabolites from growth cultures in order to stabilize cell membranes and facilitate DNA binding. Subsequently, cells are exposed to an electric field in presence of DNA and after the pulse the mix is incubated in the appropriate conditions for bacterial repair (Fig. 1A). This incubation step has the aim to allow cell repair but not growth, since they are not under the pressure of the selective markers present in the acquired DNA. Conventional protocols for *E. coli* electroporation apply 1-hour incubation, whereas longer times would be required for strains having longer duplication time (Favia et al., 2007; Liu et al., 2014). Finally, putative transformant colonies are selected on agarized media taking advantage of proper selection markers. The review by Aune and Aachmann (2010) depicts the different procedures used in artificial transformation and the range of bacteria that can be transformed, showing limited references to marine bacteria.

Mechanisms at the basis of electroporation are not clearly understood (Teissie et al., 2005; Chen et al., 2006). Certainly, the fragility of the cell membrane is one of the major biases of the procedure (Aune and Aachmann, 2010). The application of a proper electric pulse forms instantly pores in membranes, favoring the entrance of charged DNA. Electroporation efficiency is strain-dependent, but additional factors can influence the experimental success, e.g. growth conditions, applied pulse and type of exogenous DNA (Sheng et al., 1995). A range of marine strains belonging to different genera has been transformed by electroporation, e.g. *Roseobacter*, *Vibrio*, *Pseudoalteromonas*, *Caulobacter*, *Cyanobacteria* and *Halomonas* (Piekarski et al., 2009; Wang et al., 2009; Kurusu et al., 2001; Gilchrist et., 1991; Chen et al., 2013; Liu et al., 2014; Harris et al., 2016; Weinstock et al., 2016). However, failings of transformation have been registered and in the next paragraphs we emphasized some factors that could affect electroporation efficiency.

Effect of growth medium and buffer composition

The composition of the medium used to propagate cells before electroporation can affect the physiological state of the membrane (Aune and Aachmann, 2010). It

has been, in fact, reported that addition of membrane-weakening agents to the bacterial growth medium, such as glycine –that is the most used one–, can enhance the efficiency of bacterial transformation, since they act as destabilizers of the peptidoglycan crosslinking (Kim et al., 2005; Gerber and Solioz, 2007; Liu et al., 2014). Noteworthy, a high concentration of these agents might be toxic, hampering the cells viability (Aune and Aachmann, 2010). Conversely, addition of cations during the washing steps has been reported to have positive effects for transformation success: on one side, cations work as stabilizers of the membrane, on the other, they can bind to DNA, minimizing the charge repulsion effect between membrane and DNA itself (Weston et al., 1981). The salinity of the medium is particularly relevant for marine bacteria, adapted to a high salt environment. Marine halophilic bacteria require indeed a growth medium supplemented with NaCl, to avoid cell lysis due to a low salt concentration (Gilchrist et al., 1991). However, presence of salts could impede the electroporation process, like the case of *Pseudoalteromonas* strains (Wang et al., 2015). In *E. coli* the presence of cations, such as Mg^{2+}, in growth medium and buffer solution has been reported to weak the bacterial membrane and to increase the transformation efficiency (Hanahan et al., 1983; Inoue et al., 1990). Nevertheless, the use of a buffer that lacked Mg^{2+} increased the transformation efficiency in *V. parahaemolyticus* for the reason that Mg^{2+} ions are required by DNases to digest DNA, thus affecting transformation efficiency by putatively decreasing the amount of donor DNA in the cell (Wang et al., 2009). For this reason, buffers lacking Mg^{2+} are recommended for bacteria with a functional nuclease restriction system. Furthermore, the use of high concentration of Mg^{2+} (10 mM) can result in arcing when electrical field is applied. The concentration of salt does also affect the activity efficiency of restriction enzymes; as instance, Dns endonuclease of *V. cholerae* is most active in presence of salt (at 175 mM NaCl at pH 7.5-8.0) (Altermark et al., 2007). A protocol based on the evaluation of different parameters has been developed for the marine strain *B. marinus* B-9987 that showed an increased transformation efficiency using glycine betaine (7.5%) as osmoprotectant, 1 mmol L^{-1} HEPES and 2 mmol L^{-1} $MgCl_2$ in the electroporation medium, unmethylated plasmid (see paragraph 3.2.4), and applying 20 kV cm^{-1} of field strength (Liu et al., 2014). The developed protocol has been then used to successfully transform the marine-derived *B. licheniforms* EI-34-6 (Liu et al., 2014).

Other organic compounds have been demonstrated to have a beneficial effect increasing transformation efficiency. Gilchrist and colleagues (1991) reported that treating bacterial cells of cyanobacteria with EDTA-containing buffer disrupts the integrity of the paracrystalline surface (S) layer surrounding the cell of these bacteria and improves the transformation up to 50% than without EDTA. In addition, among a range of different tested buffers (phosphate salts, HEPES, Tris-HCl and glycerol), sucrose has been identified as the best stabilizer for *Vibrio* cell membrane (Wang et al., 2009). Moreover, it has been demonstrated that the addition of 10–15% of ethanol to the electroporation buffer allowed the

transformation of *Oenococcus oeni*, which has been previously reported as a not transformable strain (Assad-Garcia et al., 2008), probably by affecting membrane fluidity (Chu-Ky et al., 2005). The addition of mercaptoethanol during the bacterial growth and in the transformation mixture before electroporation has been reported efficient in increasing transformation of *E. coli* strains (Janjua et al., 2014).

Effect of strength field

High pulse strengths applied during electroporation might reduce cell survival rates and thus influence transformation efficiency (Gilchrist and Smit, 1991). Due to differences in cell envelope structures among species, some strains require nevertheless high voltage to be efficiently transformed; this is the case of *Caulobacter* CB2A strain, for which the transformation optimum voltage has been achieved with field of 12.5 kV cm^{-1} (25 µF capacitance, 4.2 ms time constant, 400 ohms resistance). However, field strengths of 6.25 kV cm^{-1} but with higher capacitance settings (125 to 960 µF) reduced significantly the cells survival rates (Gilchrist and Smit, 1991). *Also the marine fish pathogens V. anguillarum* and *Pasteurella piscicida* required optimal voltage strength of 12.5 kV cm^{-1} and time constant of 5 ms to be properly transformed with the three plasmids pSU2718, pCML, and pEV3 of molecular sizes of 2.6, 5 and 13.7 kb, respectively (Cutrín et al., 1995).

A high voltage has been nevertheless reported deleterious in case of other marine strains. In a recent study, Harris and colleagues (2016) showed a linear increase in transformation efficiency of *Halomonas* sp. O1 from 10^3 CFUs μg^{-1} DNA at 7.5 kV cm^{-1}, to 10^4 CFUs μg^{-1} DNA at 10.5 kV cm^{-1} (with 0.2 cm cuvettes), followed by a slight decrease in efficiency at voltages above 10.5 kV cm^{-1}. Furthermore, 2 kV cm^{-1} (4000 µF) was the best field strength in case of *Synechococcus* sp. CC9311 transformation, tested under field strengths ranging from 1 to 4 kV cm^{-1} (Chen et al., 2013). Lower transformation efficiency has been obtained when field strengths were lower or higher than 2 kV cm^{-1} (Chen et al., 2013). In addition, Piekarski et al. (2009) reported that, in order to transform a range of strains belonging to *Roseobacter* clade, 12.5 kV cm^{-1} (25 µF, 200 ohms, 0.2 cm cuvettes) is the optimum voltage among the different pulse intensities tested (7.5-15 kV cm^{-1}). The voltage strength has been also reported as a crucial value for the transformation of *Fischerella muscicola* PCC 7414, which has been transformed with plasmid DNA at 1.6 kV cm^{-1}, 600 ohms and 15 ms, among a range of values varying from 0.6 to 1.9 kV cm^{-1}, from 5 to 15 ms and from 200 to 600 ohms (Stucken et al., 2012).

Effect of plasmid size and concentration

In general, transformation efficiency increases in proportion to DNA concentrations until a saturation level has been reached. This saturation level seems to be specific to each species, without negative effect applying higher

concentrations (Dower et al., 1988; Wells et al., 1993). Gilchrist and Smit (1991) reported that the number of transformant cells obtained is proportional to the amount of plasmid DNA added during the electroporation of *Caulobacter* strains. Nevertheless, some exceptions have been reported in the thermophilic cyanobacterium *Thermosynechococcus elongates*, 1 µg of exogenous DNA showed the optimal transformation efficiency, while a larger amount (up to 10 µg) reduced the number of transformants obtained (Onai et al., 2003). The reason behind this reduction has not been discussed by the authors.

Interestingly, it has been also reported that the efficiency of transformation decreases with the increase of plasmid size (Leonardo and Sedivy, 1990, Sheng et al., 1995, Szostková and Horáková, 1998). As well, DNA topology is an important determinant that affects electroporation. According to Xie and co-workers (1992) transformation efficiency of *E. coli* is extremely lower with linear DNA than with circular one. Similar results have been obtained with *B. subtilis* (Ohse et al., 1997).

Effect of restriction enzyme systems

The majority of bacterial cells owns specific restriction modification systems to prevent excess of variability introduced into genomes by recombination with exogenous DNA, or lytic phage infection. These systems defend bacteria from the entrance of DNA mediated by viruses or contacts with other cells, degrading phage-derived or exogenous DNA at specific sites. Therefore, if the exogenous DNA is modified in these specific sites, it cannot be recognized by the restriction systems, which selectively digest exogenous DNA by differentiating it from host-endogenous DNA based on host-specific DNA methylation pattern (Suzuki, 2012). Consequently, defense systems of bacteria are responsible for preventing the transformation occurrence. However, an exogenous DNA that imitates the methylation pattern of the host bacterium (host-mimicking DNA) could be incorporated into the recipient (Suzuki and Yoshida, 2012). Yasui and colleagues (2009) developed an efficient method to increase transformation efficiency of genome-sequenced bacteria using Plasmid Artificial Modification (PAM), in which the plasmid vector to be inserted in the bacterial host is pre-methylated in *E. coli* according to the host-specific restriction system. Wallace and Breaker (2011) adopted this method, constructing a specific PAM for a soil-inhabiting strain of *B. halodurans*, increasing the transformation efficiency by 10 to 1000 fold in the recipient bacterium.

Also in cyanobacteria the presence of endonuclease restriction enzymes putatively impairs their transformation efficiency. These bacteria are considered easy to be manipulated since a range of strains belonging to this phylum has been successfully transformed by exploiting their natural competence and by electroporation (Ravindran et al., 2006; Mühlenhoff and Chauvat., 1996). Some cyanobacteria remain nevertheless recalcitrant to incorporate exogenous DNA and their transformation have been achieved only using pre-methylated DNA (Stuchen et al., 2012, Suzuki et al., 2012). Another strategy to bypass the restriction enzyme

barrier has been developed in marine *Vibrio* strains: Kawagishi (1994) showed that exposing *Vibrio* cells to an osmotic shock before electroporation enhanced the membrane permeability, allowing periplasmic DNase excretion.

Many other factors affect electroporation efficiency among bacteria, such as cell concentration, which displays considerable impact on transformation (Wu et al., 2010), or temperature, which could affect the physical properties of the cell membrane by changing lipid composition and membrane fluidity (Yamanaka, 1999). However, transformation, whether natural or artificial, resulted either inoperative or inefficient in other marine species such in *V. splendidus* attempts to transfer plasmids using electroporation have been ineffective, the reason of failure was not discussed. (Le roux et al., 2007) and electroporation does not work on members of *Pseudoalteromonas* genus, which growth is usually salt-dependent, and hence the salt impedes the entrance of DNA into the cell (Wang et al., 2015).

Conjugation in marine strains

Horizontal transfer of genetic material among marine bacteria occurs naturally by conjugation, which is mediated by cell contact between phylogenetically close- and distant-related strains, involving the transfer of mobile elements such as plasmids, transposons and integrons (Gogarten et al., 2009; Wozniak and Waldor, 2010; Weinstock et al., 2016). Bacterial conjugation machinery relies on the origin of transfer, also known as oriT sequence, and *tra* genes, which encode for relaxase proteins, mating pair formation (MPF) complex and type IV coupling protein. The mating process requires two steps: i) inside the cytoplasm of the donor bacterium, plasmid DNA is nicked by the relaxase (which, together with ancillary proteins, forms the relaxosome) at the level of oriT; thereby a single strand DNA is obtained and it is ready to be transferred in unidirectional way to the recipient membrane (Grohmann et al., 2003); ii) the transfer of genetic material from a donor bacterium to a recipient one must bypass the membrane barriers of the two cells. This obviously requires a transfer apparatus which is particularly developed in Gram-negative bacteria (Grohmann et al., 2003).

Conjugation transfer functions can be provided on one individual plasmid, that corresponds to the donor strain, or be present on more plasmids, thus involving besides donor(s) also a helper strain. If one plasmid encodes the complete conjugation machinery, biparental mating occurs (Schweizer, 2008). Otherwise, if it is necessary a helper strain, with a helper plasmid encoding the transfer functions to move the mobilizable plasmid from the donor to the recipient strain, triparental mating happens (Schweizer, 2008). In fourparental mating (a variation of the triparental one) a helper strain allows the transfer of two mobilizable plasmids from two donor strains into the recipient one (Schweizer et al., 2008; Lambertsen et al., 2004). The application of conjugation protocols in laboratory is a time-consuming procedure that also requires a specific step to select the donor strains from the recipient ones. However, it is the most efficient way to genetically

manipulate strains who are recalcitrant to be transformed by electroporation and by chemical methods.

A main issue is how to separate donor cells from transconjugant ones after mating, since the transfer of the mobile element is conferring to donor and recipient cells the same encoded trait (Fig. 1B). Many selection systems have been constructed with this aim. As instance, Sawabe and colleagues (2006) tagged 39 *Vibrio* sp. strains with a Green Fluorescent Protein (GFP) cassette by biparental mating with *E. coli*; the conjugation mix has been incubated on ZoBell2216E agar, containing 0.5% sodium alginate medium at 15°C, allowing the growth of both strains. Then, transconjugant selection has been achieved on a different medium specific for the selective growth of *Vibrio* (Sawabe et al., 2006). Travers and co-workers (2008) developed another system to tag the fish pathogen *V. harveyi* with GFP cassettes; in this way it could be easily followed by flow cytometry and epifluorescence microscopy. The conjugative process has been done by triparental mating, involving the donor strain *E. coli* DH5α carrying GFP gene on plasmid pVSV102, the helper strain *E. coli* CC118 and *V. harveyi* strain ORM4. Green fluorescence of the recipient cells has been verified by epifluorescence microscopy: *Vibrio* cells have been distinguished from *E. coli* donor under microscopy by the presence of flagella and the ability to swim (Travers et al., 2008).

Genetic manipulation systems for *Pseudoalteromonas* strains have been developed by Yu et al. (2014) and Wang et al. (2015). *Pseudoalteromonas* sp. are ubiquitous bacteria in marine environment and play important biological and ecological roles in deep-sea sediment ecosystem. (Yu et al., 2014). Up to now, more than 50 *Pseudoalteromonas* genomes have been sequenced. *Pseudoalteromonas* cannot be efficiently transformed by electroporation because of their restriction modification systems that prevent the electroporation of double strand DNA (Wang et al., 2015). Thus, Yu and co-workers (2014) constructed a conjugation system to tag the deep sea psychrophilic bacterium *Pseudoalteromonas* SM9913, using *E. coli* ET12567 as donor of the vector pOrit-4Em and obtaining an efficiency of 1.8×10^{-3}. Transconjugants have been selected from *E. coli* donor on the base of the growth temperature at 20°C, which resulted restrictive to *E. coli* (Yu et al., 2014). Moreover, a suicide vector has been successfully constructed for the same bacterium, using pOrit-4Em vector and *sacB* gene as the counterselectable marker to knock-out the *epsT* gene encoding the UDP-glucose lipid carrier transferase. On the other side, Wang et al. (2015) selected *P. haloplanktis* TAC125 conjugants from *E. coli* donors by growing the conjugation mix at 4°C, which is not suitable for *E. coli* growth. With this system, nevertheless, knock-out mutants of genes involved in the bacterial cold adaptation cannot be obtained because their absence affects the bacterial growth at this temperature (Wang et al., 2015).

The use of spontaneous antibiotic-resistant mutants of the recipient strains can allow the counter-selection of transconjugants against *E. coli* donor (Espinosa-Urgel and Ramos, 2004). Actually, such mutations may also cause indirect

pleiotropic effects that might influence the general physiology of the target strain, which consequently affects the mutant growth behavior (Jin and Gross, 1989). For this reason, auxotrophic donor strains are often adopted. Wang et al. (2015) developed a universal efficient conjugation system for *Pseudoalteromonas* strains, by using shuttle vectors and suicide vectors. The system is based on RP4 conjugation machinery in *E. coli* WM3064, which is auxotrophic for diaminopimelic acid (DAP). Conjugation efficiency in 9 strains of *Pseudoalteromonas* has been measured as 10^{-6}-10^{-8} transconjugants per cells; selection of recipient transconjugants has been performed on modified LB mating medium (MLB) without DAP (Wang et al., 2015). Furthermore, a conjugative system has been constructed for *Roseobacter* clade using a plasmid encoding, as reporter gene, the flavin mononucleotide-based fluorescent protein (FbFP protein) and a donor *E.coli* strain, auxotrophic for aminolevulinic acid (ALA) (Piekarski et al., 2009). A genetic system has been also constructed for *Marinobacter adhaerens* HP15 using two plasmids pBBR1MCS and pSUP106 using biparental and triparental mating, with *E. coli* donor ST18 and helper *E. coli* HB101. Selection of the transconjugants has been done by marker selection and by exploiting the donor ALA-auxotrophic feature (Sonnenschein et al., 2011).

In case of cyanobacteria, genetic manipulation system has been achieved with *Prochlorococcus* strain MIT9313 by transferring RSF1010-derived plasmids i.e. RL153, pRL153-GFP and RL27, in conjugation experiments with *E. coli* donors (Tolonen et al., 2006). Particularly, plasmid RL27 contained mini-Tn5 transposon, showing the possibility to randomly inactivate genes by transposon mutagenesis. Selection of transconjugants has been done by growing the conjugation mix on poor medium with appropriate antibiotic. Since the medium was not efficient to prohibit the growth of all *E. coli* donors, remained donors have been eliminated by using *E. coli* phage T7. Importantly, transconjugant colonies did not appear on the selective medium after mating; they required at least one step of culture in liquid medium before isolation (Tolonen et al., 2006). An improvement step for conjugation in cyanobacteria has been mentioned by Stucken et al. (2012) who showed that washing cells with NaCl (1M) before conjugation contributes to increase the efficiency by weakening the thick cell wall, rich in exopolysaccharides.

Efficiency of the conjugation could be affected by ratio of donor and recipient cells, which varies upon strains. The best conjugation efficiency for *Roseobacter* has been obtained at ratio 5:1 and 10:1 of *E. coli* donors: *Roseobacter* recipients (Piekarski et al., 2009). In case of *Pseudoalteromonas* SM9913, The transfer efficiency has been affected by the proportion of donor cells (*E. coli* ET12567) and recipient cells (*Pseudoalteromonas* SM9913) in which the optimal proportion was donor: recipient = 100: 1 (Yu et al., 2014)

The use of antibiotics as selection markers could affect conjugation efficiency, particularly in the case of marine bacteria owe to their requirements of salty media, since several antibiotics, including tetracycline and gentamicin, could be negatively influenced by high salt concentrations (Lambs et al., 1988).

Furthermore, in conjugation experiments of *Pseudoalteromonas* strains, which are mostly sensitive to chloramphenicol and erythromycin, the latter resulted more recommended than chloramphenicol for unclear reasons (Wang et al., 2015).

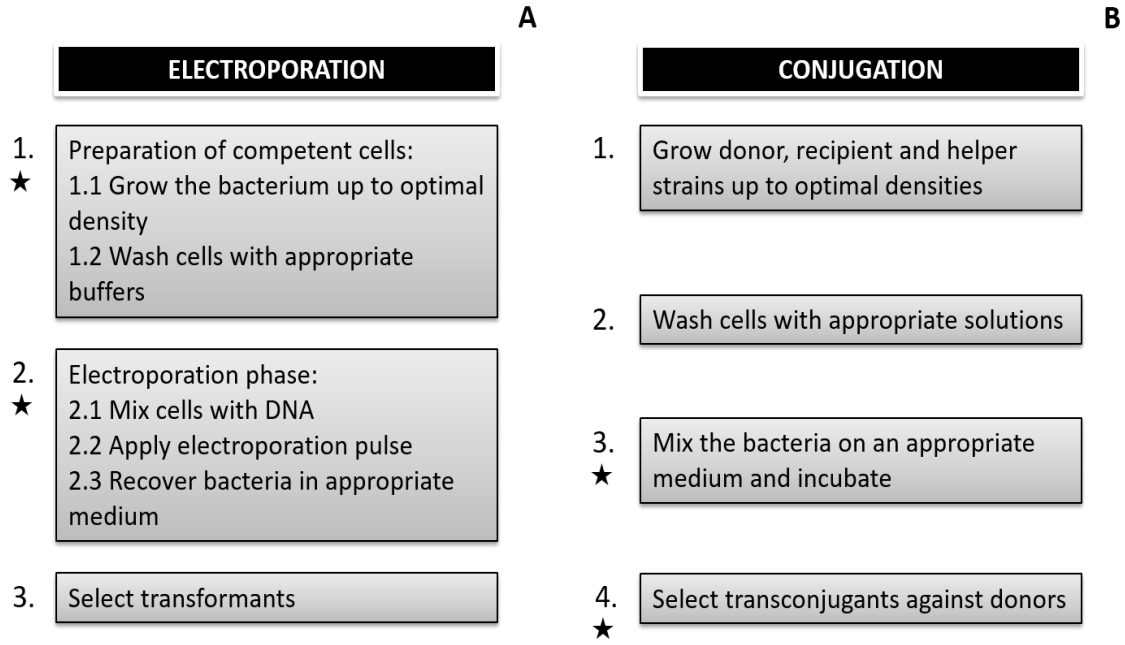

Figure 1. Schematic representation of the workflow for the genetic manipulation of bacterial strains (A) by electroporation and (B) by conjugation. Stars indicate critical steps in electroporation (preparation of competent cells and pulse application) and conjugation procedures (selection of suitable media for growth of bacterial mixes and selection of transconjugants)

Conclusions

In the last decades lateral gene transfer processes among bacteria (Mandel and Higa 1970; Meibom et al., 2005) and between bacteria and their hosts, such as plants (Kay et al., 2002; Gebhard and Smalla, 1999) and animals (Griffith, 1928), have been a focal point in microbial molecular biology and genetic studies. Genetic manipulation methods have been also applied to marine bacteria at a certain, but limited, extent (Wang et al., 2015, Yu et al.2014). Nowadays this has a special impact in the wake of the improvements of isolation and cultivation procedures and since the possibility to exploit their biotechnological potential. With this aim, different genetic manipulation systems, including chemical transformation, electroporation, and conjugation, are known to be useful to manipulate marine bacteria (Shoeb et al., 2012, Piekarski et al., 2009, Tolonen et al., 2006). Nevertheless, the success of genetic manipulation relies on different variables that must be examined in depth and that are peculiar of different strains/species, including both technical characteristics of the methodologies and bacterial features. For instance, strategies that combine different methods to debilitate cell membrane, without affecting cell viability, have been suggested (Aune and Aachmann, 2010), e.g. combining different shock treatments, like heat-shock transformation followed by electroporation. In case of transformation of

marine bacteria, which generally failed to be manipulated by electroporation, it is noteworthy to discover growth media with a low content in salts (still capable to sustain the bacterial growth) and to apply multiple chemical agents and tools to weaken the cell membrane, such as sonication and heat, cold and osmotic shocks (Hamashima et al., 1990; Yamanaka et al., 1999; Shoeb et al., 2012; Song et al., 2007).

Further researches are required in the field of genetic manipulation of marine bacteria. The procedures to obtain optimized protocols by testing different parameters, such as DNA concentration and size, electric field voltage, buffers and their composition, bacterial cell density, temperature and incubation time of the transformation/conjugation mixture, will be time-consuming, but they will allow to investigate in depth both physiological properties and biotechnological potential of these bacteria, which have been recognized as a untapped source of genetic and functional diversity (Bhatnagar and Kim, 2010).

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Part II: Genetic manipulation of marine and Crab-associated strains

Introduction

Marine microorganisms are a source of various bioactive compounds of industrial and pharmaceutical values (Bhatnagar et al., 2010). Among them, extremophiles are thoroughly adapted to thrive in inaccessible conditions (e.g. high salinity and extreme pressure and temperature) due to their peculiar metabolism and enzymatic components or since they produce particular molecules (Singh et al., 2012). Deep-sea hypersaline anoxic basins (DHABs) are one of the extreme marine environments that have attracted the interest and attention of the scientific community since long (Van der Wielen et al., 2005). Particularly, in the Eastern Mediterranean Sea DHABs are characterized by high salinity, absence of light and oxygen, high hydrostatic pressure and sharp chemoclines between brines and seawater. These features selected the presence of a peculiar and stratified prokaryotic community, which is able to thrive in the harsh environmental conditions (Van der Wielen et al., 2005; Daffonchio et al., 2006; Yakimov et al., 2007; Borin et al., 2009). Mangrove ecosystems, including the animals associated to this habitat, e.g. crabs, represent another interesting extreme environment (Nagelkerken et al., 2008). Mangroves show specialized characteristics for growing in presence of high amounts of salt and in soils with low availability of oxygen. Since the presence of peculiar enzymatic sets, extreme environment-associated microorganisms could be an explorable source of potentially interesting compounds and molecules for industrial and pharmaceutical application (De Vitis et al., 2015; Ferrer et al., 2005; Ferrer et al 2007).

Bacterial genetic engineering aims to introduce DNA into bacterial cells in order to express new traits or characterize genes of interest in a biotechnological perspective or for a basic study (Luo et al., 2015). By the use of insertional mutagenesis the function of genetic determinants can be unraveled and likely studied in relation to host and environment (Yu et al., 2014; Travers et al., 2008; Le Roux et al., 2007). Genetic manipulation procedures foresee the introduction of genes into a host cell, after being inserted in cloning vectors -plasmids are the mostly used ones- through different transfer systems. Vectors containing optical markers are useful to easily verify the success of the genetic manipulation. The marker commonly used in microbial genetic engineering to monitor gene expression or to localize particular cells or proteins are fluorescent proteins (e.g. green fluorescent protein, GFP) (Southward & Surette, 2002).

One of the principal artificial methods developed to insert foreign DNA into bacterial cells is transformation, which includes chemotransformation and electroporation. Another adopted method to transfer genetic material is through conjugation of bacterial cells.

Aim of this work was to assess the capability of selected strains with biotechnological interest to be genetically manipulated. In particular, artificial transformation by electroporation and conjugation have been employed. Specifically, we focused our attention on the following strains:

- *Halomonas aquamarina* 9B, isolated from the seawater-brine interface of the deep hypersaline anoxic basin Bannock (De Vitis et al., 2015);
- *Halomonas boliviensis* M2, isolated from mangrove crabs;
- *Pseudomonas plecoglossicida* PG21, isolated from mangrove crabs;
- *Halomonas axialensis* M10, isolated from mangrove crabs.

The first strain has been selected since its enantioselective esterase activity on a key intermediate for prostaglandin synthesis, respectively (De Vitis et al., 2015). M2, PG21 and M10 have been selected for their ω-transaminase (ω-TA) activity (see Fig. 1).

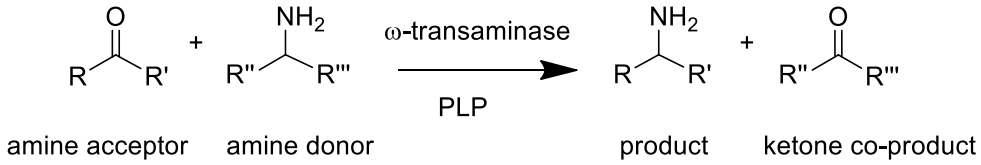

Figure 1. ω-TA-catalyzed reaction. PLP: pyridoxal-5'-phosphate. R, R',R" and R'" denote alkyl groups. R-R'": H, alkyl, aryl, arylalkyl.

Materials and methods

Electrotransformation of *Pseudomonas plecoglossicida* PG21

Preparation of competent cells of *P. plecoglossicida* PG21 has been performed accordingly to Mostafa *et al.* (2002). After an overnight growth in Tryptic Soy Broth (TSB) medium added with 3% NaCl (TSB-NaCl), the culture has been diluted 5:100 into 100 ml of the same medium and incubated in aeration at 30°C for 4 hours, until the cells reached the early log phase (OD =0.57/600 nm). Then, 80 ml of the culture have been centrifuged at 3000 rpm for 10 min at 4°C. Cells have been kept cold through the rest of the experiment. Cells have been then washed twice with 50 ml of cold 1 mM Hepes, resuspended in 10 ml of 10% (vol/vol) glycerol, centrifuged (3000 rpm, 10 min, 4°C) and finally resuspended in 0.1 ml of 10% (vol/vol) glycerol. Aliquots of 60 µl have been conserved at -80°C.

P. plecloglossida PG21 has been transformed by electroporating the plasmid pHM2-Gfp (Favia et al., 2007) as described by Mostafa *et al.* (2002): 60 µl of competent cells have been mixed with 175 ng of pHM2-Gfp. The cell-plasmid mixture has been transferred to a cold 0.2-cm-diameter cuvette: pulses have been set at 1.7kV and 2.1kV with the gene pulser (Electroporator 2510, Eppendorf). Cells have been immediately diluted in 1 mL of TSB added with 3% NaCl, transferred to a tube, and incubated at 30°C for 5 h. Thereafter, to select putative transformants, cells have been plated on TSB-NaCl agar plates added with 50 µg

ml^{-1} kanamycin, 40 $mg \cdot ml^{-1}$ 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) and 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG) and incubated at 30°C. Blue colonies appeared on the plates have been checked by fluorescent microscopy. Intergenic Transcribed Spacer (ITS)-PCR has been performed to confirm the identity of putative transformants.

Manipulation of Halomonas axialensis M10 and Halomonas aquamarina 9B by conjugation

The two strains have been genetically manipulated following the protocol described elsewhere (Lambertsen et al., 2004) with the aim to insert into the bacterial chromosome a mini-Tn7 transposition system. The procedure consists in the site-specific insertion on the recipient chromosome of a mini-Tn7 gene cassette, containing the Gfp cassette and the antibiotic markers. *E. coli* donor and helper strains have been grown overnight on Luria Bertani (LB) medium with the following antibiotics: i) *E. coli* AKN68 (pUXBF13, helper plasmid) on LB added with 100 µg ml^{-1} ampicillin, ii) *E. coli* AKN67 (Gfp donor) on LB added with 100 µg. ml^{-1} ampicillin, iii) *E. coli* #331 (pRK600, plasmid mobilizator) on LB added with 6 µg ml^{-1} chloramphenicol. After overnight incubation, cultures have been diluted 1:100 into the same medium and incubated at 37°C, until the cells reached OD: 0.96 , 0.613 , 0.724 (at 600 nm), respectively.

Simultaneously, cultures of *H. axialensis* M10 and *H. aquamarina* 9B have been diluted 5:100 into 100 ml of TSB from overnight cultures and grown until the cells reached the early log phase OD 0.48 and 0.57 (at 600 nm), respectively. Cells of each culture have been counted taking advantage of Thoma chamber: 10^9 cells ml^{-1} for *E. coli* strains and 10^{10} cells/ml of each recipient strain have been centrifuged in 50-ml tubes at 3200 rpm at 4°C for 8 minutes, and washed once with 0.9% NaCl. Therefore, the conjugation mix has been spotted in a big drop on a sterile nitrocellulose filter (0.2 µm) placed on a LB plate without any antibiotic selection and incubated overnight. The day after, the filter has been removed from the plate and placed in a 50-ml tube with 1-2 mL of 0.9% NaCl, kept for 15-20 min in order to rehydrate the pellet and cells have been detached. Cells have been plated on the selected medium Reasoner and Goldrich agar (R2A), supplemented with 10% NaCl, 50 µg ml^{-1}kanamycin, 50 µg ml^{-1} spectinomycin and 6 µg ml^{-1}chloramphenicol, and incubated at 30°C. Colonies appeared on the selected plates after 5-6 days of incubation. Fluorescence of putative transconjugants have been checked by fluorescent microscopy. ITS-PCR have been performed to confirm the identity of the transconjugants.

Results and discussion

Electrotransformation of P. plecoglossicida PG21

P. plecoglossicida PG21 has been first tested for kanamycin sensitivity (showing to be sensitive to 50 µg ml^{-1} kanamycin), and then used in electroporation trials with plasmid pHM2-GFP, which contains kanamycin resistance and lacZY genes (Favia et al., 2007). PG21 has been successfully transformed applying 1.7 kV and 2.1 kV: blue colonies appeared the following day after incubation at 30°C. The identity of putative transformants has been confirmed by ITS-PCR fingerprinting profiles as *P. plecoglossicida*. In Fig.2 cells of *P. plecoglossicida* PG21 expressing GFP, namely *P. plecoglossicida* PG21(Gfp), are showed

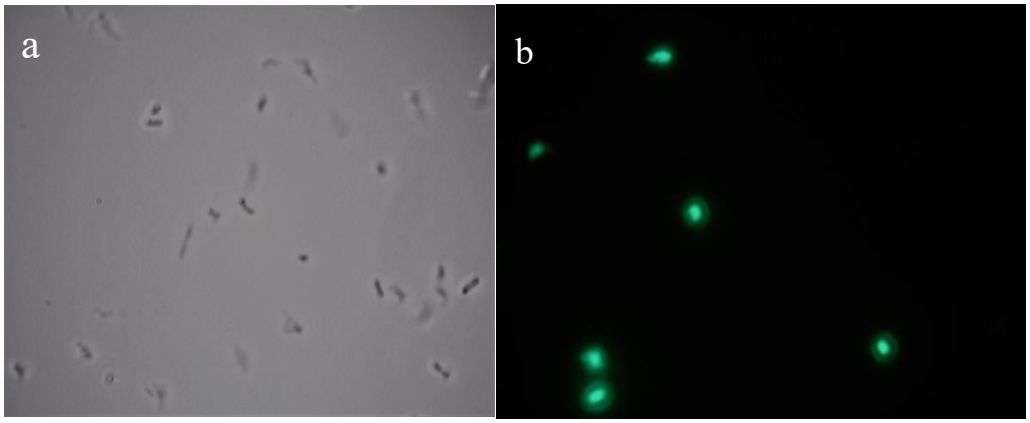

Figure 2. Green fluorescent protein (GFP)-tagged *P. plecoglossicida* PG21 under (a) contrast microscopy, (b) fluorescent microscopy.

Manipulation of H. axialensis M10 and H. aquamarina 9B by conjugation

Transfer of genes between bacteria through conjugation bypasses the barriers induced by the restriction enzyme system (DNase) in the recipient cells, because the transferred single-stranded DNA is not a substrate for most restriction enzymes. Currently, the common method to genetically manipulate *Halomonas* spp. strains is conjugation, while electroporation has been successfully reported only in a limited number of papers (Azachi et al., 1996; Burch et al., 2013; Harris et al., 2016).

In order to use the mini-Tn7 system described in Lambertsen et al. (2004), the sensitivity of *Halomonas* strains to the following antibiotics have been firstly checked: 50 µg ml^{-1} kanamycin, 50µg ml^{-1} spectinomycin, and 6 µg ml^{-1} chloramphenicol. Both M10 and 9B resulted sensitive to the abovementioned antibiotics. In order to find conditions to select marine *Halomonas* strains against *E. coli* donor after the mating, the growth of *Halomonas* and *E. coli* strains has been tested on R2A agar plates, added with NaCl in a percentage varying from 0%

to 10%, to favor the growth of marine strains. Indeed, *E. coli* strains were not capable to grow on R2A medium added with 10% NaCl, making this medium suitable for the counterselection of transconjugants, after the addition of appropriate antibiotics.

Conjugation mixture has been prepared by mixing the donor *E. coli* AKN67 with the helper strains, namely *E. coli* AKN68 (pUXBF13) and *E. coli* #331 (pRK600), and the recipient ones. Since both *E. coli* and *Halomonas* strains grow properly on LB agar plates, these have been chosen to support the conjugation mating to guarantee the growth of both donor and recipient strains.In this way, we successfully tagged with the GFP the two *Halomonas* strains M10 and 9B. Colonies of strain 9B appeared after 2 days of incubation at 30 °C, while M10 colonies appeared after 5 days of incubation (Fig. 3).

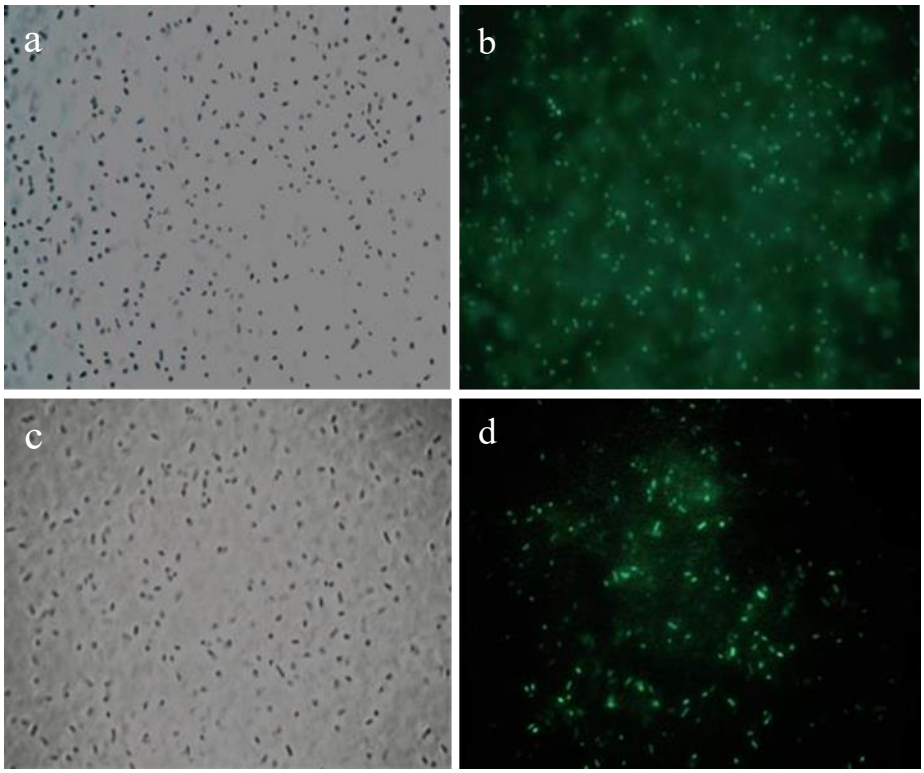

Figure 3. Images of recombinant *Halomonas axialensis*-GFP under (a) phase contrast microscope and (b) an epifluorescence microscope, and recombinant *Halomonas aquamarina*-GFP; (c) phase contrast and (d) fluorescence.

Conclusions

GFP has been successfully expressed into the chromosome of *H. axialensis* M10 and *H. aquamarina* 9B taking advantage of a mini Tn7 transposition system, while *P. plecoglossicida* PG21 has been transformed by electroporation, inserting the plasmid pHM2-GFP. To our knowledge this is the first time that a crab-associated *Pseudomonas* species has been transformed through electroporation. In conclusion, these systems could be used in further studies that foresee the genetic manipulation of the strains.

Acknowledgments

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Chapter V

Complete genome sequence of Virgibacillus sp. strain 21D, a halophilic bacterium isolated from the deep hypersaline anoxic basin Discovery

Abstract

We report the genome sequence of the bacterium *Virgibacillus* sp. strain 21D, isolated from the seawater-brine interface of the deep hypersaline anoxic basin Discovery in the Eastern Mediterranean Sea. The isolate grows in the presence of NaCl and $MgCl_2$ at concentrations up to 9% and 9.52%, respectively, with optimum growth at 6-9% NaCl and 0.95-4.76% $MgCl_2$; pH optimum (8-9) and temperature optimum (30-37°C) have been also determined. By the use of PacBio platform, a total number of 117,330 reads have been retrieved and 1 circular contig obtained after assembly. Automatic annotation of the genome showed that it contains genes that could help the bacterium to thrive under osmotic stresses typical of extreme environments, e.g. osmotically activated L-carnitine/choline ABC transporters, glycine betaine transporters, choline uptake and an ectoine synthase. Genes involved in iron acquisition and metabolism have been also found in the genome, suggesting adaptation of the strain to marine conditions characterized by iron depletion.

Introduction

Microorganisms inhabit different and heterogeneous habitats, including environments indicated as "extreme" and characterized by severe and inhospitable physico-chemical conditions e.g. polar sea, cold and hot deserts and hydrothermal vents. Particularly, extreme environments represent a fascinating source of bacterial diversity and metabolic activities with interesting biotechnological potential (Raddadi et al., 2015; De Vitis et al. 2015). Microorganisms living in such habitats, namely "extremophiles", show a metabolic and physiological adaptation to the harsh environmental condition: they possess peculiar enzymes adapted to the extreme conditions which could be usefully employed in different industrial applications (Mapelli et al., 2016).

Among the different extreme habitats present on Earth, deep-sea hypersaline anoxic basins (DHABs) have attracted the attention of researchers, especially microbiologists. In the Eastern Mediterranean Sea different brine pools have been discovered, e.g. L'Atalante, Bannock, Discovery, Urania, Thetis and Kryos (Mapelli et al., 2016): they are highly saline lakes located on the seafloor, at around 3000 m below sea level, characterized by anoxia, high hydrostatic pressure and the presence of a sharp chemocline at the seawater-brine interface (Van der Wielen et al., 2005; Borin et al., 2009). Moreover, each brine pool is characterized by peculiar chemical compositions which select a specialized prokaryotic community (Van der Wielen et al., 2005)

With the perspective to exploit the biotechnological potential associated with deep-sea microorganisms, we have recently explored the bacterial diversity of DHABs located in the Eastern Mediterranean Sea, investigating the capability of the strains to resolve a racemic mixture of propyl ester of anti-2-oxotricyclo[2.2.1.0]heptan-7-carboxylic acid (R,S), a key intermediate for the synthesis of D-cloprostenol (De Vitis et al., 2015). Interestingly, one of the strains, *Virgibacillus* sp. strain 21D, showed reduction of the substrate with medium/high enantioselectivity based on NaCl percentage (0, 3, 6 and 9%) used in the biotransformation, exhibiting optimum growth at 6-9% NaCl. The strain was isolated from the seawater-brine interface of the deep hypersaline anoxic basin Discovery (De Vitis et al., 2015). a basin characterized by the presence of a high concentration of $MgCl_2$ (5M) and indicated as one of the most extreme environments on Earth (Wallmann et al., 2002). In the past, due to the chaotropicity of $MgCl_2$ in the absence of other compensating ions, the high concentration of this molecule has been suggested to create conditions unsuitable for life (Hallsworth et al., 2007) although the recent study of microbial community thriving at Kryos brine pool extended the chaotropicity limit of life (Yakimov et al., 2015).

In this study we present the complete genome sequence of *Virgibacillus* sp. strain 21D. We report the presence of genes likely involved in osmoadaptation of the microorganism to the stressful conditions occurring in the Discovery interface, together with genes involved in iron acquisition and metabolism that suggest 21D adaptation to marine conditions which are characterized by iron depletion. To date, few *Virgibacillus* spp. genomes are available in the public databases, including the draft genomes of 21D-closely related species, namely *V. pantothenticus* DSM 26^T, isolated from soil (Wang et al., 2015) and *V. chiguensis* CGMCC 1.6496^T (=NTU-101^T), isolated from a disused saltern in Taiwan (Wang et al., 2008).

Organism Information

Classification and features

Virgibacillus sp. strain 21D was isolated on 246 DSM medium from the brine pool Discovery located in the Eastern Mediterranean Sea (De Vitis et al., 2015) It belongs to the order Bacillales and the class Bacilli. The genus *Virgibacillus* was created in 1998 (Heyndrickx et al., 1998), and the emended description appeared in 2003 (Heyrman et al., 2003). A phylogenic tree was constructed by aligning the 16S rRNA gene of 21D with 16S rRNA genes from selected strains and species belonging to the same genus by the use of Maximum-Likelihood algorithm incorporated in MEGA6 (Tamura et al., 2013). The phylogenetic tree resulted to be congruent with the taxonomic affiliation of the strain (Fig. 1; Tab. 1). The previous assignment of the strain to *V. pantothenticus* species based on a 800 bp-fragment of the 16S rRNA gene is not supported (De Vitis et al., 2015). Through analysis of an approximately 1500 bp-fragment of the 16S rRNA gene, 21D was found to share 99% sequence identity with both the strains *V. dokdonensis* DSW-10^T and *V. chiguensis* NTU-101^T, isolated from an island in the East Sea (Korea) and from a saltern in southern Taiwan, respectively (Wang et al., 2008; Wang et al., 2015)

Virgibacillus sp. strain 21D is a Gram-variable, motile and spore-forming bacterium with rod shaped cells (Fig. 2). It grows on Marine Broth (MB, Conda) agar plates at 30 °C forming 1–2 mm colonies within 48 h. The cells show an average width of 0.4 μm and length of 1.5 μm, as confirmed by scanning electron microscopy performed using a Quanta 600 FEG (FEI) (Fig. 2) [14]. We observed the capability of cells to form long filamentous chains and the presence of peritrichous flagella (Fig. 2), consistent with previous observations of *Virgibacillus* spp. (Wang et al., 2008; Wang et al., 2015).

Phenotypic features of 21D have been characterized, i.e. temperature optimum, pH optimum and optimal growth in presence of NaCl and $MgCl_2$, as reported in Tab. 1. The isolate is a mesophilic bacterium with a temperature optimum of 30-37°C and a pH optimum between 8 and 9. Growth is observed in concentrations ranging between 3 and 9% NaCl, with optimum growth at 6-9% [2]. Finally, 21D growth has been tested for $MgCl_2$concentrations ranging between 0 to 23.80% (0, 0.95, 4.76, 9.52, 19.04 and 23.80%) and found to occur from 0 to 9.52% with optimum at 0.95-4.76% $MgCl_2$. The interface of Discovery is characterized by a particular $MgCl_2$ gradient ranging from the value of seawater, i.e. 0.48% (0.05M) to the one of the brine, i.e. 48.08% (5.05M) (Hallsworth et al., 2007). Characteristics of *Virgibacillus* sp. strain 21D are summarized in Table 1, together with information about the isolation origin.

Table 1. Classification and general features of the strain *Virgibacillus* sp. strain 21D (Field et al., 2008)

MIGS ID	Property	Term	Evidence code[a]
	Classification	Domain *Bacteria*	TAS (Woese et al., 1990)
		Phylum *Firmicutes*	TAS (Gibbons et al., 1978; Schleifer et al.,, 2009)
		Class *Bacilli*	TAS (Ludwig et al., 2009)
		Order *Bacillales*	TAS (Prévot et al.,1953; Skemann et al., 1980)
		Family *Bacillaceae*	TAS (Skeman et al., 1980)
		Genus *Virgibacillus*	TAS (Heyndrickx et al.,1998; Heyman et al., 2003)
		Species *Virgibacillus* sp.	IDA
		Strain: 21D (CP018622)	IDA
	Gram stain	Gram-variable	IDA
	Cell shape	Rod	IDA
	Motility	Motile	
	Sporulation	Spore forming	IDA
	Temperature range	Mesophile	IDA
	Optimum temperature	30-37°C	IDA
	pH range; Optimum	7-10; 8-9	IDA
	Carbon source	Not reported	
MIGS-6	Habitat	DHAB	TAS (De Vitis et al., 2015)
MIGS-6.3	Salinity	3-9% NaCl (w/v)	IDA
MIGS-22	Oxygen requirement	Aerobic	IDA
MIGS-15	Biotic relationship	Free-living	IDA
MIGS-14	Pathogenicity	Non-pathogen	NAS
MIGS-4	Geographic location	Eastern Mediterranean Sea	TAS (De Vitis et al., 2015)
MIGS-5	Sample collection	2003	TAS (De Vitis et al., 2015)
MIGS-4.1	Latitude	35° 17′ N	TAS (De Vitis et al., 2015)
MIGS-4.2	Longitude	21° 41′ E	TAS (De Vitis et al., 2015)
MIGS-4.4	Altitude	-	

[a] Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project (Ashburner et al., 2000).

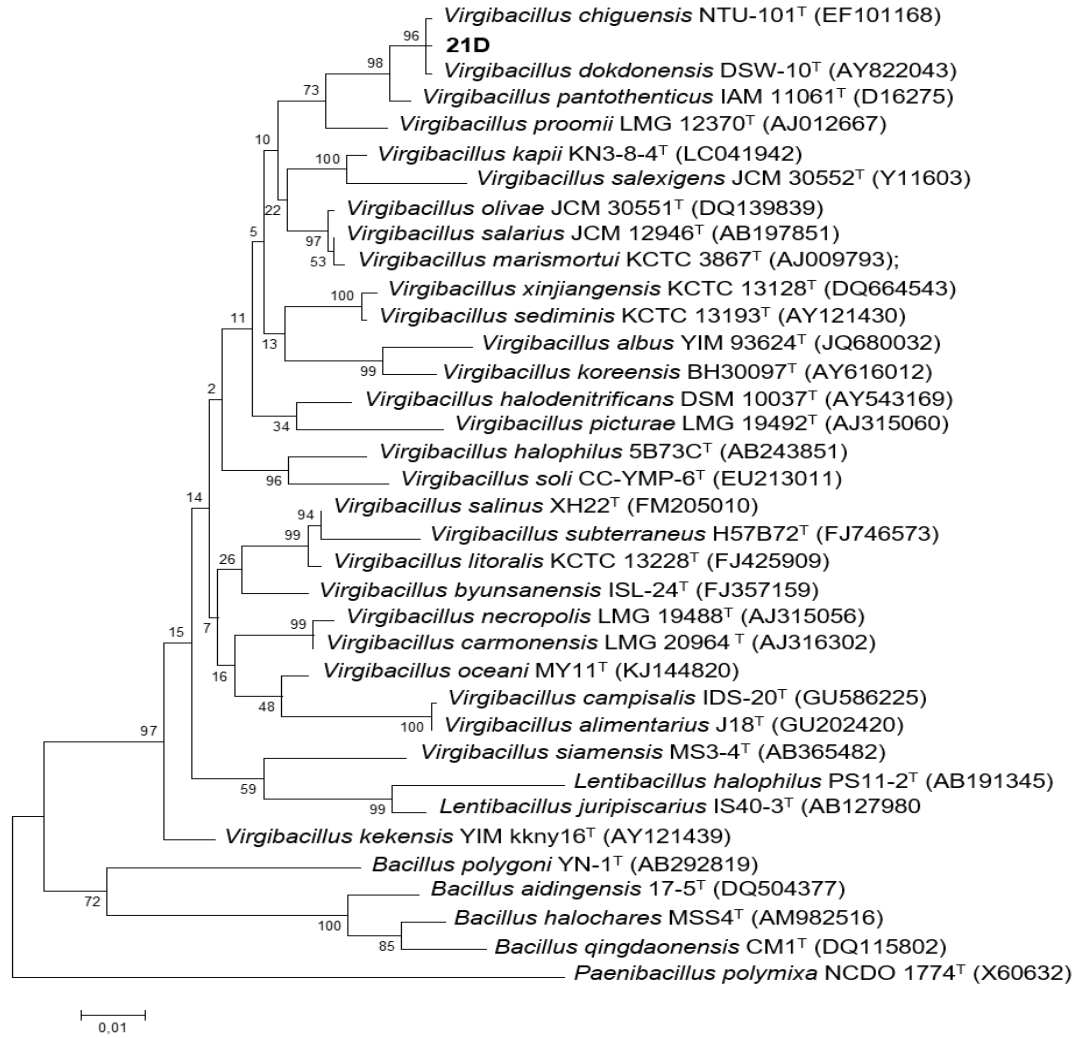

Figure 1. Phylogenetic tree highlighting the relative position of *Virgibacillus* sp. 21D within other *Virgibacillus* species. The strains and their corresponding GenBank accession numbers for 16S rRNA genes are (type = T): *Lentibacillus halophilus* PS11-2^T (AB191345); *Lentibacillus juripiscarius* IS40-3^T (AB127980); *Virgibacillus siamensis* MS3-4^T (AB365482); *Virgibacillus halophilus* 5B73C^T (AB243851); *Virgibacillus soli* CC-YMP-6^T (EU213011); *Virgibacillus campisalis* IDS-20^T (GU586225); *Virgibacillus alimentarius* J18^T (GU202420); *Virgibacillus oceani* MY11^T (KJ144820); *Virgibacillus necropolis* LMG 19488^T (AJ315056); *Virgibacillus carmonensis* LMG 20964 ^{T} (AJ316302); *Virgibacillus byunsanensis* ISL-24^T (FJ357159); *Virgibacillus litoralis* KCTC 13228^T (FJ425909); *Virgibacillus subterraneus* H57B72^T (FJ746573); *Virgibacillus salinus* XH22^T (FM205010); *Virgibacillus kekensis* YIM kkny16^T (AY121439); *Virgibacillus halodenitrificans* DSM 10037^T (AY543169); *Virgibacillus picturae* LMG 19492^T (AJ315060); *Virgibacillus dokdonensis* DSW-10^T (AY822043); *Virgibacillus chiguensis* NTU-101^T (EF101168); *Virgibacillus pantothenticus* IAM 11061^T (D16275); *Virgibacillus proomii* LMG 12370^T (AJ012667); *Virgibacillus xinjiangensis* KCTC 13128^T (DQ664543); *Virgibacillus sediminis* KCTC 13193^T (AY121430); *Virgibacillus kapii* KN3-8-4^T (LC041942); *Virgibacillus salexigens* JCM 30552^T (Y11603); *Virgibacillus olivae* JCM 30551^T (DQ139839); *Virgibacillus salarius* JCM 12946^T (AB197851); *Virgibacillus marismortui* KCTC 3867^T (AJ009793); *Virgibacillus albus* YIM 93624^T (JQ680032); *Virgibacillus koreensis* BH30097^T (AY616012); *Bacillus polygoni* YN-1^T (AB292819); *Bacillus aidingensis* 17-5^T (DQ504377); *Bacillus halochares* MSS4^T (AM982516);*Bacillus qingdaonensis* CM1^T (DQ115802); *Paenibacillus polymyxa* NCDO 1774^T (X60632

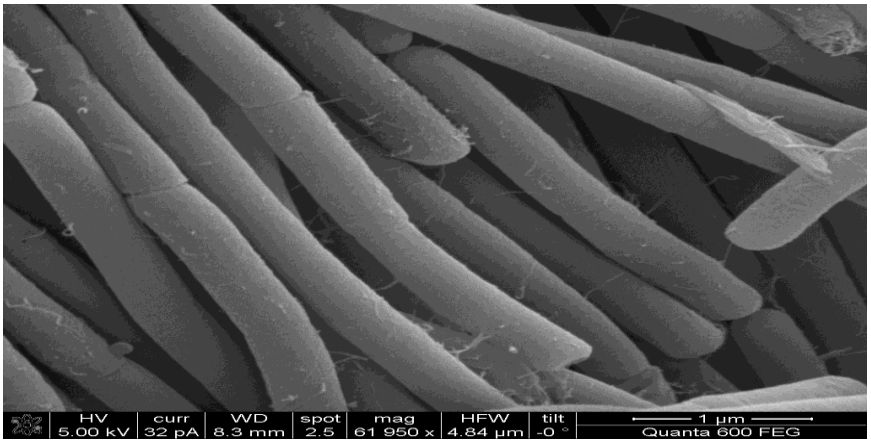

Figure 2. Scanning electron microscopy of Virgibacillus sp. Strain 21D

Genome sequencing information

Genome project history

The genome of *Virgibacillus* sp. strain 21D has been sequenced using PacBio technology. This organism was selected based on its ability to thrive in the presence of NaCl and $MgCl_2$ concentrations up to 9% and 9.52%, respectively, and its enantioselective ketoreductase activity on a key intermediate for prostaglandin synthesis (De Vitis et al., 2015) The complete genome sequence is deposited in GenBank under the accession number CP018622, BioProject PRJNA354837, BioSample. SAMN06052362. In Table 2, we provide a summary of the project information and its association with MIGS (Field et al., 2008).

Table 2. Project information

MIGS ID	Property	Term
MIGS 31	Finishing quality	Complete genome
MIGS-28	Libraries used	10-kb libraries
MIGS 29	Sequencing platforms	PACBIO
MIGS 31.2	Fold coverage	167X
MIGS 30	Assemblers	FALCON (January 2016)
MIGS 32	Gene calling method	-
	Locus Tag	A21D
	Genbank ID	CP018622
	GenBank Date of Release	12 December 2016
	GOLD ID	-
	BIOPROJECT	PRJNA354837
MIGS 13	Source Material Identifier	21D
	Project relevance	Marine microbiology

Growth conditions and genomic DNA preparation

Virgibacillus sp. strain 21D was grown in MB and incubated overnight in aerobic condition at 30°C, 150 rpm. Genomic DNA was prepared from an overnight culture using

DNeasy Blood & Tissue Kit (Qiagen, Italy) following the manufacturer's protocol for Gram-positive bacteria. Quantification and quality control of the DNA was performed by spectrophotometry and agarose (0.8%) gel electrophoresis. DNA concentration of 129 ng/µl has been determined.

Genome sequencing and assembly

Third generation sequencing was performed using PacBio (Pacific Biosciences, CA, USA) at Macrogen (Korea). Specifically, a library with size of 10 kbp was prepared and 1 SMRT cell used for sequencing. A total number of 117,330 reads were retrieved with a mean subread length of 8,461 bp and N50 of 11,849 bp. Raw reads were filtered and *de novo* assembled using FALCON Assembler software (v0.2.2 release, January 2016) (Koren et al., 2008). The genome showed a GC% of 36.6, with 3,915 CDS, 63 tRNA and 18 rRNA.

Genome annotation

After whole genome assembly, the complete genome of *Virgibacillus* sp. strain 21D was annotated using the pipeline Prokka (http://www.vicbioinformatics.com/software.prokka.shtml) (Seemann et al., 2014).

Genome Properties

Detailed information related to the complete genome of *Virgibacillus* sp. strain 21D is provided in Table 3. One circular chromosome of 4,263,520bp in size was obtained after assembly. The annotation predicted 3,915 CDS and 81 RNA genes, considering 63 tRNA and 18 rRNA (Table 4). The G + C reached 36.6%. The graphical genome map is provided in Fig. 3

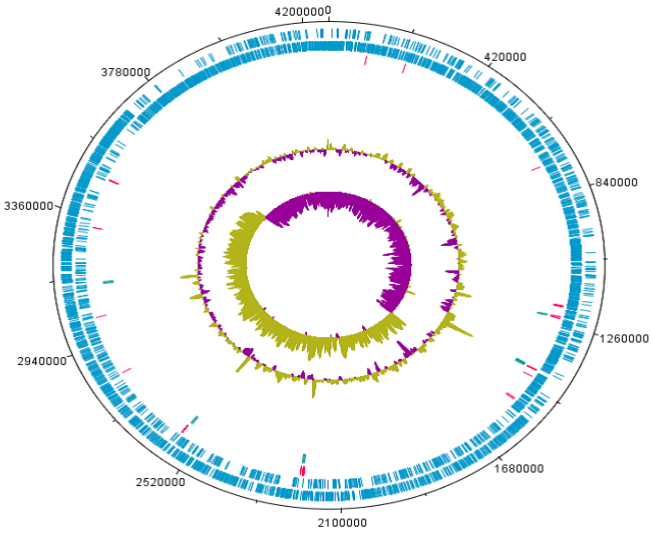

Figure 3. Graphical map of *Virgibacillus* sp. strain 21D chromosome: Marked characteristics are shown from outside to the center: CDS on forward strand, CDS on reverse strand, tRNA, rRNA, GC content and GC skew.

Table 3. Genome statistics, listed in base pairs and percent of total

Attribute	Value	% of Total
Genome size (bp)	4,263,520	
DNA coding (bp)	3,425,954	
DNA G+C (bp)	36.6	
DNA scaffolds	1	
Total genes	3,996	
Protein coding genes	3,915	
RNA genes	81	
Pseudo genes	0	
Genes in internal clusters	1069	
Genes with function prediction	3037	
Genes assigned to COGs	3398	
Genes with Pfam domains	441	
Genes with signal peptides	179	
Genes with transmembrane helices	1077	
CRISPR repeats	2	

Table 4. Number of genes associated with general COG functional categories.

Code	Value	%age	Description
J	168	4.29	Translation, ribosomal structure and biogenesis
A	0	0	RNA processing and modification
K	288	7.36	Transcription
L	210	5.36	Replication, recombination and repair
B	1	0.03	Chromatin structure and dynamics
D	40	1.02	Cell cycle control, Cell division, chromosome partitioning
V	63	1.61	Defense mechanisms
T	184	4.70	Signal transduction mechanisms
M	163	4.16	Cell wall/membrane biogenesis
N	62	1.58	Cell motility
U	49	1.25	Intracellular trafficking and secretion
O	109	2.78	Posttranslational modification, protein turnover, chaperones
C	183	4.67	Energy production and conversion
G	289	7.38	Carbohydrate transport and metabolism
E	288	7.36	Amino acid transport and metabolism
F	89	2.27	Nucleotide transport and metabolism
H	138	3.52	Coenzyme transport and metabolism
I	107	2.73	Lipid transport and metabolism
P	211	5.39	Inorganic ion transport and metabolism
Q	63	1.61	Secondary metabolites biosynthesis, transport and catabolism
R	386	9.86	General function prediction only
S	307	7.84	Function unknown
-	517	13.21	Not in COGs

Insights from the genome sequence

Automatic annotation using "Rapid Annotation using Subsystem Technology" (RAST) platform [http://rast.nmpdr.org/; 19-21] showed that the 21D genome contains genes that could help the strain to thrive under osmotic stresses typical of extreme environments. For

example, we observed the presence of proteins involved in osmotically activated L-carnitine/choline ABC transporter systems (CDS 1589, 1590 and 1591), glycine betaine transporter systems (CDS 70, 2144, 2603 and 2602), choline uptake (CDS 743), L-proline glycine betaine ABC transport systems (CDS 1592 and 3385) and an ectoine synthase (CDS 3837).

Taking advantage of RAST function-based comparison tool, we compared the genome of strain 21D with those of the closely-related species *V. pantothenticus* DSM 26^T and *V. chiguensis* CGMCC 1.6496^T (Wang et al., 2008; Wang et al., 2015). We observed that basic metabolic functions are conserved between the three genomes. All of them showed the presence of the above-mentioned genes involved in osmotic stress response. Interestingly, some differences were found in the category "Iron acquisition and metabolism". In the 21D genome we found the presence of genes in the subcategories "siderophore metabolism" and "heme, hemin uptake and utilization systems in Gram-positives bacteria" that have not been found in *V. pantothenticus* DSM 26^T and *V. chiguensis* CGMCC 1.6496^T genomes. In particular, genes related to iron uptake mediated by the siderophore petrobactin were not found in the other two genomes (in 21D: CDS 1208, 1209, 1210 and 1211, encoding for petrobactin ABC transporter components). Likely, genes of the iron-regulated surface determinant system Lsd have been found only in 21D and not in the other two genomes (in 21D: CDS 1173, 1174, 1175, 1176, 1177, 1179, 1183). The presence of these genes suggests the strain's adaptation to marine conditions characterized by low concentrations of iron (Boyd et al., 2010).

Conclusions

This work represents an important resource in terms of knowledge of the bacterial genetic material of the strain 21D adapted to thrive in extreme marine environments. It may help to identify the genetic determinants, the products of which might be useful in industrial applications. The complete genome sequence of *Virgibacillus* sp. 21D has been deposited in GenBank database under accession number CP018622.

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Chapter VI

General discussion and conclusions

Marine microorganisms are a huge reservoir of bioactive molecules with interesting biotechnological potential and a source of genetic and functional diversity that should be explored in depth (Dalmaso et al., 2015). As instance, enzymes that act in high salt concentrations have been found in anoxic hypersaline environments (Siglioccolo et al., 2011), or enzymes working in high pressure conditions have been found associated to several deep-sea polyextremophile microorganisms, because they evolved mechanisms of adaptation to counteract high pressures, including modification of membrane and transport proteins and accumulation of osmolytes, which stabilize the proteins (Lauro and Bartlett, 2008).

Developing innovative approaches, such as new cultivation methods, efficient preservation protocols and genetic manipulation systems, together with increasing the knowledge on marine strains by sequencing their genomes are essential requirements for the exploitation of the marine microbial potential (Romano et al., 2016). In the first part of the thesis, devoted to the study of the mechanisms of entrance of *Vibrio* strains in the "viable but non culturable" (VBNC) state, I characterized in microcosms the morphological changes on four *Vibrio* strains (i.e. *V. harveyi*, *V. fortis*, *V. hepatarius* and *V. nereis*) during the incubation in cold seawater (4 °C) for 50 days by means of flow cytometry. Then, I focused on *V. harveyi* and *V. fortis* strains, monitoring the microcosms until the cells completely entered in VBNC state, to further characterize their resuscitation to the cultivable state. The relation between resuscitation and oxidative stress has been investigated: cultivability of VBNC cells was rescued, at least in part, by plating the VBNC cells with the addition of the stress-protective enzyme catalase. Moreover, I observed that during the process of resuscitation cells gradually increased their resistance to H_2O_2. Substantially in this work I monitored the entrance in and the exit from a VBNC state by vibrio strains. The entrance of *Vibrio* strains into this state is likely a natural response to adverse environmental factors (in this case, low temperature, starvation and oxidative stress) and represents a survival strategy of the strains. Cells of *V. harveyi* and *V. fortis* in this state resuscitate acquiring plate cultivability after being able to react to removal of the stress. This work showed, moreover, that the VBNC phenomenon may be attributable to different pathways even in the context of a single genus.

The second part of this thesis has been focused on the cryopreservation ability of different cryoprotective agents (CPA) on ten marine strains belonging to different taxa. The work has been performed by evaluating the vitality of the strains during 24 months of storage at -80°C. Cryopreservation of microbial strains is a fundamental step in the routine laboratory practices and the selection and addition of suitable CPA to the sample culture is a basic procedure to allow a long term maintenance and archiving of the strains for future exploitation. In this work, I used five CPA (glycerol, skim milk, ethylene glycol, glycine betaine and urea/glucose) and I found that they are relatively effective in preserving the different cultures. The best cryopreservation performance has been showed by glycerol, a widely used

molecule in cryopreservation, whereas glycine betaine resulted the less suitable one, at least in the specific experimental conditions adopted. In case of skim milk and ethylene glycol, I demonstrated a good level of cryopreservation, since they resulted effective in half of the tested strains, likely being strain-dependent. Interestingly, the combined use of urea and glucose, inspired from the physiological response of the wood frog *Rana sylvatica* to freezing condition (Sinclair et al., 2013), cryopreserved efficiently several of the strains; however, this should be confirmed by future investigations specifically directed to the evaluation of this combination of molecules. In conclusion, data showed that the efficiency of the preservation is dependent upon the microorganism subjected to storage, the cryoprotectant used and the lenght of preservation.

A further part of this thesis has been dedicated to the evaluation of the different genetic manipulation systems effective in case of marine bacteria. The investigation of the genetic manipulation tools on these microorganisms is an urgent need, since the paucity of the knowledge in this regard, as I reviewed in chapter IV, Part I. In this thesis work, I assessed the capability of some selected strains, with biotechnological interest, to be genetically manipulated through electroporation and conjugation, two methods widely used in for model strains (e.g. E. coli). Specifically, I succeeded in the manipulation of *Halomonas axialensis* M10 and *Halomonas aquamarina* 9B, taking advantage of a mini Tn7 transposition system (Lambertsen et al., 2007) through an approach that included both conjugation and transposition of DNA sequences of interest, obtaining the insertion of a green fluorescent protein (GFP) into the chromosome of the recipient bacteria. Then, I have successfully transformed *Pseudomonas plecoglossicida* PG21 by electroporation, inserting the plasmid pHM2-GFP in the cell (Favia et al., 2006). These systems are important for a detailed molecular investigation of the strains.

The last part of the thesis has been devoted to the genome sequencing of the marine bacterium *Virgibacillus sp.* 21D, previously demonstrated of biotechnological interest due to stereoselective esterases and ketoreductases activity (De Vitis et al. 2015). From the automatic annotation of the genome, genes coding for proteins related to the production and secretion of compatible solutes and molecules that could help the bacterium to thrive osmotic stresses typically of extreme environments have been found. Indeed I identified genes coding for osmotically activated L-carnitine/choline ABC transporters, glycine betaine transporters, choline uptake and an ectoine synthase. Moreover, genes involved in iron acquisition and metabolism have been retrieved: the presence of these genes in 21D suggested its strain adaptation to marine conditions characterized by iron depletion.

In conclusion, this thesis represents an important contribution to study those aspects that could affect the biotechnological exploitation of marine bacteria, i.e. cultivation of novel strains, cryopreservation of isolated ones and their genetic manipulation. Further studies on the uncultivable bacteria and on identifying the most suitable culture media and other conditions for optimal marine microbial growth is needed in order to enable their accessibility to the scientific community. Moreover investigation of a variety of metabolic pathways involved in the production of bioactive agents is important in order to investigate marine complex natural products and to discover new drugs such as novel antibiotics and

anticancer compounds. Comprehension of mechanisms driving the entrance and in the VBNC state and the resuscitation from it, would allow to set up more effective cultivation approaches, also by means of better suited sample manipulation procedures. Finally, a better understanding of basic mechanisms in microbial physiology, metabolism and genetics, which are at present still limited in the marine microbiota, would contribute to improve the different stages of the marine biodiscovery pipeline.

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