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Isolation by Miniaturized Culture Chip of an Antarctic bacterium *Aequorivita* sp. with antimicrobial and anthelmintic activity

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ABSTRACT

Microbes are prolific sources of bioactive molecules; however, the cultivability issue has severely hampered access to microbial diversity. Novel secondary metabolites from as-yet-unknown or atypical microorganisms from extreme environments have realistic potential to lead to new drugs with benefits for human health. Here, we used a novel approach that mimics the natural environment by using a Miniaturized Culture Chip allowing the isolation of several bacterial strains from Antarctic shallow water sediments under near natural conditions. A Gram-negative Antarctic bacterium belonging to the genus *Aequorivita* was subjected to further analyses. The *Aequorivita* sp. genome was sequenced and a bioinformatic approach was applied to identify biosynthetic gene clusters. The extract of the *Aequorivita* sp. showed antimicrobial and anthelmintic activity towards Multidrug resistant bacteria and the nematode *Caenorhabditis elegans*. This is the first multi-approach study exploring the genomics and biotechnological potential of the genus *Aequorivita* that is a promising candidate for pharmaceutical applications.

1. Introduction

The total number of bacterial species in the world is estimated to vary from 10^7 to 10^9 [1], but most of them have never been cultivated. Culture-independent methods have demonstrated the enormous richness of biodiversity in the microbial world [2,3], but this wealth remains largely inaccessible. The "challenge of uncultivable microorganisms" is a topic that has been concerning scientists for many years. Solutions to this problem could facilitate the discovery of new strains holding novel and different features, which biotechnology could exploit. The problem, which is also known as the *"great plate count anomaly"* [4,5] is based on the observation, that less than 1% of microbes present in an environmental sample can be grown on an agar plate. Although there is a large number of reasons that marine bacteria cannot be easily cultured in laboratory conditions, the problem can often be the difficulty of replicating the very precise environmental conditions required for the growth. Free-living microbes can have a broad array of environmental requirements, for example many organisms cannot survive in high-nutrient conditions common to many types of culture media. The development of low-nutrient media has greatly increased the number of organisms that have been successfully cultured [6]. An even more serious problem is that many marine microorganisms have adapted to their low resource environment by growing very slowly or entering in a dormant stage, hence it is necessary to find the right conditions to trigger entry into cell replication [6]. Among the strategies for the isolation and cultivation of new or atypical microorganisms, the simulation of the natural environment is probably the most promising. The use of innovative devices that are able to mimic natural conditions enhances the possibility of cultivating different microbial species. One of these systems is the Miniaturized Culture Chip (MCC), which is a disposable array of thousands of

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miniaturized Petri dish on a chip [7]. One of the major advantages of the MCC is that it can be placed directly on natural samples (such as sediments) with nutrients available to the microorganisms via a porous ceramic that forms the base. This device simulates the natural habitat, in which microorganisms live in community, and allows the communication among microorganisms [7–9]. The isolation of new strains also enables exploration of the chemical diversity of natural products they synthesize. Microorganisms are well-known for producing a large variety of antimicrobial agents. Marine microorganisms are no exception and they have also proven to be a rich source of potent natural products with antimicrobial, antiinflammatory, antiviral, and anticancer activity [10]. In particular, the discovery of novel secondary metabolites as antibiotic lead compounds is urgently needed in order to counteract the spread of MultiDrug Resistant (MDR) bacteria. Recently, the World Health Organization [11] published its first ever list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria that pose the greatest threat to human health. The list includes MDR bacteria such as *Acinetobacter baumannii, Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA). They are able to provoke recalcitrant infectious, especially in hospitals where they pose the major challenge to patient safety, resulting in one of the leading causes of death in the USA and Europe with high costs for their public health [12]. The redundancy in the discovery of already known compounds, generally produced by well-known and repeatedly isolated species, increases the demand of searching for novel drugs. In the search of new antibiotics or new molecules of pharmaceutical interest, the exploration of microorganisms from extreme environments may lead to the identification of strains that can provide novel types of compounds for biotechnology [13]. Antarctica is one of the most extreme places on Earth. The isolated and unique Nature of Antarctica, characterized by low temperatures, oligotrophic environment, long periods of light/dark, has drawn the attention of the scientists. Antarctica and the surrounding oceans represent an untapped area for exploring biodiversity and potentially unknown organisms adapted to the extreme living conditions. These extremophiles exhibit physical and chemical adaptations not found elsewhere on the planet [14]. The genus *Aequorivita* (family Flavobacteriaceae) was discovered for the first time in 2002 from Antarctic terrestrial and marine habitats [15]. The members of this genus are Gram-negative, non-motile, psychrotolerant, strictly aerobic bacteria, producing orange or yellow pigments. Thus far, the genus *Aequorivita* has remained rarely isolated and poorly investigated. Previous studies have reported the taxonomy, fatty acid composition and DNA G + C content of *A. lipolytica*, *A. viscosa*, *A. sublithincola*, *A. crocea*, *A. antarctica* and *A. capsosiphonis* [15]. Herein we report the isolation of a poorly investigated strain of *Aequorivita* sp., from Antarctic shallow water sediments by employing the MCC method, as well as its whole genome sequencing, the analysis of the biosynthetic gene clusters and the evaluation of its antimicrobial and anthelmintic potential. To our knowledge, this is the first study exploring the genus *Aequorivita* for its bioactivity.

2. Material and Methods

2.1. Collection of Antarctic sediments

Shallow water (50 cm depth) sediments were collected by using sterile 50 mL Falcon tubes, (Sarstedt) in January 2014, from 3 different sites during an expedition in the framework of National Program for Antarctic Research of Italy (PNRA) in the area of Edmonson Point, Antarctica, 74° 20′ (74.3333°) South, 165° 8′ (165.1333°) East. The samples were transported to the laboratory in dry ice and stored at −80 °C until initializing the experiments.

2.2. Design, fabrication and preparation of culture chips for the isolation of microorganisms

MCC was used to isolate microorganisms grown as microcolonies [9]. MCC contained arrays of microwells with a Porous Aluminum Oxide (PAO) base (8 × 36 mm, 60 μm thick, 40% porosity, 20 to 200 μm pore diameter) acting as a sterile filter. MCC was fabricated by patterning the wall material (Ordyl 300 film, Elga, Italy) using photolithography and then applying it to 8 × 36 mm strips of PAO arrayed on a silicon wafer. Walls were patterned by photolithography of 10 μm thick Ordyl 300 film according to the manufacturer's protocols. The resulting perforated and processed material was heat/pressure applied to the PAO. Platinum (10 nm) was used to sputter coat the upper surface of the MCC. Wells had 180 μm diameter, spaced 160 μm in a hexagonal patterning with 4500 microwells per chip. MCC was sterilized using a high-intensity UV ozone cleaner (PSD, Novascan, USA) for 15 min. It is basically a high number of miniaturized Petri dishes on a chip containing wells which can host one or more microbes. On the bottom of the chip there is a filter of 0.22 μm which circumvents the passage of the microbes through the chip. So, it can be placed directly on sediments or on a flat surface and inoculated with the sample [7]. For this experiment, two different growth conditions were used in order to evaluate the effect of different nutrients on the microbial growth. Condition A, minimal: sediment was packed into a Petri dish and overlaid with a thin layer of agarose 1% (w/v) mixed with a filtered solution of 0.001% (w/v) $FeSO_4 \cdot 7H_2O$. Condition B, richer: sediment was packed into a Petri dish and overlaid with a thin layer of agarose 1% (w/v) mixed with a filtered solution of 0.001% (w/v) FeSO4 · 7H2O, 3% (w/v) Sea Salt, 1 g/L Peptone, 0.5 g/L Yeast Extract. Sterile cultivation chips were placed on the solidified mixture composed by sediments, nutrients and agarose. Then, 0.5 g of Antarctic sediments (same sediments within the chip) were dissolved in 1 mL of sterile water, shaken by using a vortex and subjected to 10-fold serial dilutions until the dilution 10^{-5}. Three μL of each dilution was gently spread-plated on the chip by using a 1 μL sterile plastic loop. Finally, not all the wells will contain microorganisms. After 10 and 45 days of incubation at 7 °C the recovery of microcolonies from wells was carried out using a fine sterile toothpick using a dissection microscope to visualize the target microcolony (see Fig. 1). Finally, picked microcolonies were dissolved in sterile water. Half of the sample was used to perform the identification, while the other half was stored at −80 °C with 20% (v/v) glycerol.

2.3. Molecular identification and phylogenetic analysis of isolated strains

The phylogenetic affiliation of bacterial isolates was performed through the 16S rRNA genes amplification and analysis. The freeze and thaw method was used to obtain bacterial genomic DNA. In particular, a colony of each isolate was picked, dissolved in 100 μL of sterile water, transferred in 0.5 mL Eppendorf-tube, mixed with a shaker and frozen at -20 °C for minimum 1 h. Thereafter, the frozen tubes were incubated for 15 min at 99 °C in a thermocycler, centrifuged for 10 min at 8900 rpm and then 50 μL of supernatant was transferred in a new 0.5 mL Eppendorf-tube and used as template for the amplification via PCR of 16S rDNA genes. PCR was carried out in a total volume of 50 μL containing DreamTaq PCR Master Mix (a ready-to-use solution containing DreamTaq DNA Polymerase, optimized DreamTaq buffer, $MgCl_2$, and dNTPs) and 1 μM of primer Eub27 F (Forward, seq: 5′-AGAGTTTGATCCTGGCTCAG-3′)