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Diversity, ecological distribution and biotechnological potential of *Actinobacteria* inhabiting seamounts and non-seamounts in the Tyrrhenian Sea

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ABSTRACT

In the present study, the ecological distribution of marine *Actinobacteria* isolated from seamount and non-seamount stations in the Tyrrhenian Sea was investigated. A collection of 110 isolates was analyzed by Automated Ribosomal Intergenic Spacer Analysis (ARISA) and 16S rRNA gene sequencing of representatives for each ARISA haplotype (n = 49). Phylogenetic analysis of 16S rRNA sequences showed a wide diversity of marine isolates and clustered the strains into 11 different genera, *Janibacter*, *Rhodococcus*, *Arthrobacter*, *Kocuria*, *Dietzia*, *Curtobacterium*, *Micrococcus*, *Citricoccus*, *Brevibacterium*, *Brachybacterium* and *Nocardioides*. Interestingly, *Janibacter limosus* was the most encountered species particularly in seamounts stations, suggesting that it represents an endemic species of this particular ecosystem. The application of BOX-PCR fingerprinting on *J. limosus* sub-collection (n = 22), allowed their separation into seven distinct BOX-genotypes suggesting a high intraspecific microdiversity among the collection. Furthermore, by screening the biotechnological potential of selected actinobacterial strains, *J. limosus* was shown to exhibit the most important biosurfactant activity. Our overall data indicates that *Janibacter* is a major and active component of seamounts in the Tyrrhenian Sea adapted to low nutrient ecological niche.

1. Introduction

Marine *Actinobacteria* have been recovered from various marine habitats such as marine sediments (Maldonado et al., 2009), seawater (Subramani and Aalbersberg, 2013), freshwater ecosystems (Allgaier and Grossart, 2006), Ocean sediments (Solano et al., 2009; Jensen et al., 2005), marine sponges (Sun et al., 2010), hypersaline and saline sediments (Wu et al., 2009). In these environments, *Actinobacteria* contribute to the breakdown and recycling of organic materials and they play a significant role in mineralization of organic matter, immobilization of mineral nutrients and improvement of physical parameters and marine environmental protection (Manivasagan et al., 2014, 2013). In addition to their ecological role, marine *Actinobacteria* have currently received considerable attention due to their capacity to produce novel secondary metabolites (antibiotics, exopolysaccharides, extremozymes, biosurfactants, etc.) with a wide range of biological and pharmaceutical properties such as antitumor, immunosuppressive, anti-inflammatory and antiviral activities (Hong et al., 2009; Yuan et al., 2014; Olano et al., 2009; Dharmaraj, 2010). Besides their wide applications in bioremediation and hydrocarbon degradation, marine biosurfactants were recently investigated in medical and therapeutic applications (Kiran et al., 2010; Rodrigues et al., 2006; De Araujo et al., 2011; Gudina et al., 2013). The antiadhesive potential of marine biosurfactants and their involvement in the inhibition and disruption of

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pathogenic biofilm formation could also present a promising alternative to combat the development of antibiotic resistance caused by bacterial pathogens (Kiran et al., 2014; Padmavathi and Pandian, 2014).

Studying the diversity of marine environments leads to the selection of novel marine *Actinobacteria* with new adaptive strategies and the synthesis of extremophilic secondary metabolites. According to Subramani and Aalbersberg (2013), in the period from 2007 to 2013, a total of 38 new actinobacterial species belonging to 15 different families have been isolated from marine sediments. The emergence of molecular techniques has increased the recovery of many actinobacterial taxa different from their terrestrial counterparts (Subramani and Aalbersberg, 2013). Culture-independent studies such as metagenomic and metaproteomic as well as emerging "omics" technologies enable the investigation of marine environments and the access to metabolic pathways of microbial populations without cultivation (Rath et al., 2011). Although their wide application in the field of microbial ecology, molecular approach alone could not allow the whole microbial diversity of marine *Actinobacteria*. Consequently, cultivation-dependent approaches remains, in combination with cultivation-independent techniques, the most practical method to access bacterial diversity in marine environment and to strengthen results. Moreover, cultivation-dependent studies have demonstrated that *Actinobacteria* were also indigenous to marine habitat after the description of the first obligate marine genus *Salinispora* by Maldonado et al. (2005) and other 14 novel actinobacterial genera discovered later by Goodfellow and Fiedler (2010). In fact, the use of appropriate isolation procedures could promote the growth of marine *Actinobacteria* and provide an important source of novel metabolites (Subramani and Aalbersberg, 2012).

The Mediterranean Sea is a complex semi-enclosed deep environment including two interacting basins characterized by strong energetic gradients and low nutrient concentrations, in which the eastern basin is more oligotrophic than the western (Barsanti et al., 2011; Pusceddu et al., 2009). The biological production decreases from north to south and west to east and is inversely related to the increase in temperature and salinity (Coll et al., 2010). The topographic complexity of the Mediterranean Sea is responsible for the formation of eddies and circular currents affecting the primary productivity and the flux of organic matter settling to the seafloor (Danovaro et al., 1999, 2009). In the Mediterranean Sea, the deep Tyrrhenian Sea is spotted by at least 14 large and intermediate seamounts separated by great depths (Bo et al., 2011). These underwater mountains have been well investigated from geographic point of view, but little is known about the biodiversity of their benthic prokaryotes (Danovaro et al., 2010). This biodiversity remains largely unexplored, and much work is needed to discover the contribution of seamounts to the biodiversity. In this study, a culture-dependent approach was applied to investigate the diversity of marine *Actinobacteria* by Automated Ribosomal Intergenic Spacer Analysis (ARISA), 16S rRNA gene sequencing and BOX-PCR typing. Moreover, we compared the bacterial distribution between seamounts and non-seamounts stations of the Tyrrhenian sediments. Finally, the biotechnological potential of marine isolates was evaluated by screening of efficient biosurfactant producing *Actinobacteria*.

2. Material and methods

2.1. Sampling sites and bacterial isolation

Sediment sampling was carried out during the oceanographic campaign CIESM-SUB1 (R/V, Universitatis, Naples from 21 to 30 July 2005) in the southern part of the Mediterranean Sea, called the southern Tyrrhenian area up to the Sardinia-Sicily channel (Fig. 1). A total of 13 sediment samples were collected at different depths (from 3430 to 3581 m) from the seamounts (Station 6: Palinuro, station 2: Marsili) and non-seamounts stations (Station 4: non-seamount 1, station 8: non-seamount 2) (Fig. 1) (Danovaro et al., 2009, 2010; Ettoumi et al., 2010) using multiple and box corers. Different levels of sampling were aseptically performed from the surface layer 0 cm, 10 cm, 20 cm and the 30 cm horizons. Strains were isolated from marine sediments by the dilution of 1 g of each sample in sterile seawater, plating on marine agar (Supplementary data Table S1 in the online version at DOI: 10.1016/j.micres.2016.03.006) and incubated at 25 °C for more than 7 days. Colonies with different morphological characteristics were purified by repeating streaking and cryopreserved at −80 °C in marine broth supplemented with 25% glycerol.

2.2. DNA extraction and PCR amplification

The DNA was extracted from marine collection following the method of Murray et al. (1998) adapted to pure isolates. As previously published ARISA was adapted to analyze intergenic spacer of pure strains (Ettoumi et al., 2010; Cardinale et al., 2004). Following primers were used to amplify bacterial intergenic transcribed spacers: ITSF (5′GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′). ITSReub was labelled at its 5′ end with HEX fluorchrome (6-carboxy-1,4 dichloro-20,40,50,70-tetra-chlorofluorescein). ITS-PCR were migrated on 2% agarose gel and were prepared for ARISA analysis according to Cardinale et al. (2004) prior to their automated separation by capillary electrophoresis on an ABI Prism 310 Genetic analyser. ARISA electrophoregrams were analyzed using the GeneScan 3.1 software program (Applied Biosystems). BOX-PCR was performed using the BOXA1R primer as already described (Ettoumi et al., 2013). Total DNA was extracted directly from marine sediments using the Fast DNA Spin Kit for Soil (Bio 101) according to the manufacturer procedure and Denaturing Gradient Gel Electrophoresis (DGGE) was performed as described by Ettoumi et al. (2010).

2.3. 16S rRNA sequencing and phylogenetic analysis

The 16S rRNA gene from pure cultures was amplified using the following universal primers: S-D-Bact-0008-a-S-20/S-D-Bact-1495-a-A-20 according to the procedure described previously by Cherif et al. (2003). PCR products were subjected to electrophoresis in 1.5% agarose gel in 0.5× tris-borate - EDTA buffer, stained for 10 min in a 0.5 mg L^{-1} solution of ethidium bromide, visualized by exposure to UV light and photographed with a digital capture system (Gel Doc, Bio-Rad).

The sequencing of 16S rRNA gene of representatives strains (n = 49) was performed at Primm Biotech (Milano, Italy). Obtained sequences were initially compared to those available in GenBank database using BLAST (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1990). Generated sequences and their homologous were aligned using ClustalW version 1.8 (Thompson et al., 1994). The method of Jukes and Cantor, was used to calculate evolutionary distances. Phylogenetic tree was constructed by the neighbour joining method and tree topology was evaluated by bootstrap analysis of 1000 data sets using MEGA 4.1 (Tamura et al., 2007).

2.4. Screening of biosurfactant producing marine Actinobacteria

The screening of biosurfactant production was carried out by the drop collapse test as described by Tugrul and Cansunar (2005). To induce biosurfactant production, bacterial isolates (n = 42) were cultivated in marine broth supplemented with glucose (2%). Seven