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Isolation and characterization of sea urchin P.lividus microbiota from coelomic fluid

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Isolation and characterization of sea urchin *P.lividus* microbiota from coelimic fluid

Graduate Boris Zuber

Objectives

The identification of the microbiota present in the coelomic fluid of *Paracentrotus lividus* has been highlighted in order to understand the role of bacteria in the physiology, ecology and aquaculture activities of this echinoderm.

Methods | Experiences | Results

The presence of bacteria into the coelomic fluid of the Mediterranean sea urchin *Paracentrotus lividus* has been explored. Two different methods of analysis, the culture-based and culture-independent investigations, are described. The dependent culture method based on the growth of bacteria on marine agar and DNA sequencing after the amplification of the 16S rRNA gene, reported a predominance of taxa belonging to *Proteobacteria* and *Bacteriodetes*. The analysis of the production of extracellular enzymes and antibacterial compounds has also been performed and reports that the isolated strains produced proteases, lipases and esterases. The independent culture method was performed by the sequencing by minION™ technologies of the metagenomic DNA extracted from the coelomic fluid. This approach reported a predominance of taxa belonging to *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, *Thermodesulfobacteria*, *Ignavibacteria*, *Firmicutes* and *Tenericutes*. In addition, studies on interactions with marine microorganisms are providing additional tools to understand phenomena of symbiosis, development and morphogenesis.

Opened sea urchin. Five orange-red gonads bath in the coelomic fluid.

Thesis realised in the Laboratory of Molecular Microbiology and Biotechnology of the STEBICEF Departement, University of Palermo

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Isolation and characterization of sea urchin *P.lividus* microbiota from coelomic fluid

Description / *Beschreibung*

The study of the microbiota is a subject of considerable and growing interest since it is drawing new important perspectives in the life sciences concerning the functional relationships between metazoans and microbial cells. In fact, it has already shown that the endogenous microbial community affects various physiological activities of multicellular organisms.
The coelomic cavity of echinoderms contains a fluid in which coelomocytes are reported to exert immune functions like phagocytosis, opsonization and production of antimicrobial agents against marine bacteria. However, up to day nothing is known about the endogenous bacterial population of coelomic fluid. We focused on this issue, and, to this aim, both bacterial culture-dependent and -independent approaches were adopted. By the former approach, we isolated 8 distinct Gram-negative marine bacterial strains identified for their 16S rDNA sequence. Interestingly, almost all isolated strains show a considerable extracellular hydrolytic activity. Moreover, one of them exerts antimicrobial effect against Gram-negative bacteria, including most of the other strains isolated from the coelomic fluid. Finally, molecular investigation on metagenomic DNA composition confirmed culture-dependent results and give us more information about uncultured bacteria inside coelomic fluid.
This study not only suggests insights on functional interaction between sea urchin and marine microorganisms, but also could provide a novel source of biochemical diversity for the production of bioactive compounds and enzymes that can find biotechnological applications.

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Leiter der Vertiefungsrichtung / Studiengang:

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To answer to the questions about my future from my professors, family and friends I shall use the words of Peter Drucker: “The best way to predict the future is to create it”.

Table of contents

List of abbreviations

APS	:	Ammonium PerSulphate
mA	:	milliAmper
atm	:	atmosphere (unit of pressure)
bp	:	base pairs
CMC	:	CarboxyMethyl Cellulose
°C	:	Celsius degree
conc.	:	Concentration
DNA	:	DesoxyriboNucleic Acid
dNTP	:	desoxyNucleotide Tri-Phosphate
DMSO	:	DiMethyl SulfOxyde
E	:	East
EDTA	:	EthylenDiamineTetraAcetic acid
h/min/s	:	hour/minute/second
µg/mg/g	:	microgram/milligram/gram
G	:	Gravity force
µL/mL/L	:	microLiter/milliLiter/Liter
LB	:	Luria Broth medium
MA	:	Marine Agar
MB	:	Marine Broth medium
MM	:	Master Mix
nm/µm/cm/m	:	nanometer/micrometer/centimetre/meter
M, mM and µM	:	Molecular weight [mol/L], [mmol/L] and [µmol/L]
N	:	North
NFW	:	Nuclease-Free Water
PCR	:	Polymerase Reaction Chain
pH	:	“potential Hydrogene” (-log*[H^+])
rx	:	reaction
rpm	:	revolutions per minute
RNA/rRNA	:	RiboNucleic Acid/ ribosomal RiboNucleic Acid
RT	:	Room Temperature
SDS	:	Sodium Dodecyl Sulphate
TAE	:	buffer Tris-Acetate-EDTA
TEMED	:	TEtraMEthylethyleneDiamine
UV	:	UltraViolet
V	:	Volt
Vol.	:	Volume
(v/v) and (w/v)	:	(volume/volume) and (weight/volume)
W	:	Watt
WIMP	:	What’s In My Pot?

List of Figures

1 Introduction

1.1 Microbiota

Complex organisms (like plants and animals) cannot live without interaction with their environment and microorganisms. They live in a complex community and need some interactions to growth, produce energy and protection. Moreover, microorganisms are not only a source of contamination and disease but also serve for a correct development. The microbiota is the set of microorganisms (bacteria, yeasts, fungi and virus) that live in symbiosis with other organisms (such as animals or plants). This set can be found inside or outside (like on the skin for animal) to the host organism. The first approach of this term for human was realised by J.Lederberg with the terms microbiome, "to signify the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space" [1]. To determine the population of the human microbiota and to evaluate the capacity of the bacterial microbiota to growth in vivo, an analysis of the relatively well-conserved 16S rRNA gene was performed with a mixture of organisms [2]. Results showed that 40% to 80% of microorganisms are cultivable, others organisms (depending on the body site and complex interactions) are uncultivable. For this reason, the population and the number of microorganisms are probably underestimated [3]. The human genome contains about 20'000 different genes and the bacterial gut metagenome meanwhile contains more than 3 million genes (cells ratio is about 1:10 between eukaryotes and prokaryotes cells) [4]. This difference is not negligible and helps to understand why the microbiota plays an important role during the live and for the health of each host organism [5]. The gastrointestinal track is the most important region of the animal body where this amount of microorganisms is present and can be considered like an independent organ. The intestinal microbiota (Figure 1) can perform trophic, metabolic, digestive and also protective and adaptive processes to the host organism [7]. Nevertheless, the microbiota of the buccal cavity, vagina and skin are important too to regulate and protect the body against external contamination and should not be overlooked [8]. The symbiotic micro-organisms are able to respond to changes in our behaviour but also to the environment and therefore can affect our health: may contribute to the pathogenesis of certain diseases such as arthritis [9], cirrhosis and liver disease [10], pneumonia in HIV-infected individuals [11], type 2 diabetes [12] and obesity [13].

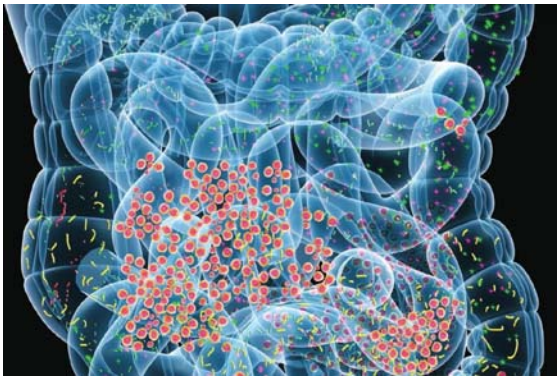


Figure 1: Human gut microbiota [6].

For plants, the microbiota is also important and is involved in vital function such as plant nutrition, nitrogen assimilation, vitamins and hormones production and endurance of these to biotic and abiotic stresses. Leaves (phyllosphere) and roots (rhizosphere) are two different possibilities to be in contact with their environment and contribute in a different way to the symbiosis. Roots are directly in contact with soil, one of the biggest microbial ecosystems on Earth, but not all types of bacteria are good for one plant species. Members of the phyla *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Firmicutes* are mostly represented in soil sample [14].

That is why plants need to do a selection for choosing by molecules secretion, such as salicylic acid or ethylene production, the good species of bacteria to live in symbiosis. The molecular secretion can be realised directly in a specific zone by root secretion or in a large zone by leaves diffusion.

The promotion of animal and plant health by microorganisms has been described for many decades and demand again more studies to understand the complexity of differentiation carried out by the host organism to live in symbiosis and not in pathogen way.

The Earth is also called the blue planet because more than 70% of the surface is covered with water. About 95% of this water is contained between ocean and sea. The seawater has a pH between 7.5 and 8.4, and is a slightly alkaline mixture with solids, gases and dissolved organic matter, the concentration of which varies according to region and temperature. At the beginning of the life on Earth (3.5 billion years ago), microorganisms were the only living form and this for about 3 billion years. So the development of life on Earth and its environment depend on the past and present activities of marine bacteria. During the twentieth century, a lot of experiment (metagenomics studies) were realised with sea samples, for several reasons. As described above, water covers a surface biggest than continents and then microbial water life has an important role in the regulation of Earth ecosystems. It's a natural resource. The environment of marine niches is particular and demands to the microorganisms to be resistant against salts concentration, temperature, sun UV rays. Some new genes and expressed proteins can be found and used for biotechnological processes; this part is fast growing. The change of the marine microbial diversity is directly affected by their environment and can be used as a detector for ecosystem sustainability [15]. The discovery of abundance microbial life in this environment has confirmed the old theories on the vital role of marine microorganisms for the maintenance of life on the Earth [16]. The sea microbiota is studied in coastal regions over the world but the comparison between studies is difficult because the conditions and environment of each location are too different. Furthermore, these studies are mostly concentrated in the photic zone (superficial water) while the aphotic zone (more than -200m) of the ocean is biggest. This second zone contains the life of the ocean, with fishes and mammalian animals, and brings the essential part of nutrients that needs the superficial zone [17]. The first results about the diversity of sea microbiota show that the micro-organism density decreases with depth [18]. However, some results obtained in another study show that aphotic zone contains more micro-organisms than the other part of the ocean and that this community is more productive than earth microorganisms [19, 20]. The nutrient cycle of Deep Ocean bring essential nutrients for the photic and terrestrial zone and then microorganisms' communities of these two zones are dependent on the aphotic zone microbiota [20].

1.2 Marine organisms microbiota

The study of interactions between marine organisms and their microorganisms is providing interesting insights into phenomena of symbiosis and pathogenicity. In fact, it is known that even in the marine environment the bacteria establish relationships with guests and in some cases organisations promoting the acquisition of nutriment, tissue development or immune system [21].

Symbiotic microbial communities are common in marine invertebrates and include several species of bacteria, microalgae virus and archaea [22]. The prokaryotic microbiota of marine invertebrates is constituted, for the most part, by obliged symbionts [23] but there are also optional [24] and many of them produce specific extracellular compounds.

The microorganism symbiotic also plays a critical role in ensuring the normal growth of the host organism, through the supply of nutrients and the production of secondary metabolites having protective activity against predators. For example sponges, corals and sea squirts, carry out their activities nutrient suppliers, through carbon fixation, that comes from photosynthetic symbionts [25]. The structural and functional diversity of microbial community symbionts, in fact suggests that invertebrates guests create microbial niches that contribute to the biodiversity and to vary the nutrient cycle in the marine ecosystem of the coasts [26]. The specific microorganisms which live in microbial niches include bacteria, archaea and single eukaryotic cells, make up about 40% of the total volume of the sponge [27]; the cyanobacterial symbionts, provide up 50% of energy needed to tropical sponges [28], other strains produce metabolites directly active against predators [29] which are also useful not only for pharmaceutical uses but seems to contribute in determining the development of the sponge [30]. The sponge symbiotic microorganisms are responsible for the synthesis of vitamin B1 that these animals must necessarily obtain from the nutrient [31]. Today there is increasingly focused on the fact that the sponges contain their specific microbiota and through pyrosequencing of different species have been identified, *Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Chloroflexi* are the mostly present [32]. The synthesis of secondary metabolites by symbiotic microorganisms contributes, as we said, for the chemical defences of marine invertebrates [33], the strategy will be useful for organisms who need fixation to a support to growth (sessile organisms) to discourage predators, avoid getting trapped in sly contexts and for the substrate competition [34]. It is to underline the fact that many of these secondary metabolites are useful in the pharmaceutical field and in the biotechnological applications [35].

Many studies on the microbiota of sea squirts focus their attention on cyanobacteria isolated from the cloacal cavity (meeting point of the excretory ducts of the urinary and reproductive apparatus with the end of the intestinal canal) or the host organism tunic (protection layer for the host organism)[36]. Cyanobacteria of the strain *Prochlon* generally provide UV absorbent molecules to their guests. Bacterial symbionts were also isolated from bivalves as *Tyasira flexuosa*. The micro-organisms were isolated from tissue of gills and some of these oxidise sulphates [37]. Another micro-organism named *Calyptogena soyoae* was also isolated from bivalves and help the host organism for the fixation of carbon source [38]. A substantial number of studies report the presence of intestinal micro-organisms in aquatic invertebrates. The most common genra reported as symbionts of the gastrointestinal tract of marine invertebrates are: *Pseudomonas*, *Vibrio*, *Micrococcus*, *Aeromonas*, *Flavobacteria*.

Among the ecologically and commercially important invertabrates, the sea urchin deserves considerable attention, both for its use as a culinary delicacy [39], both as a model organism for the study of biological systems of primary importance such as embryogenesis, development and studies focusing on the dynamics of chromatin [40]. Recently, studies were also made regarding the intestinal microbiota of *Lytechinus variegatus* sea urchin [41] and designated a potential role for the health of the animal, helping its absorption of nutrients activities [42]. However, a little attention has been given to the determination of a possible microbial communities existing in coelomic fluid of the sea urchin, more precisely for the species *Paracentrotus lividus*, which could also provide ecologically important information and sheds light on a possible biological role of these microorganisms in the growth and survival of this species.

The isolation and characterization of the microbiota contained in the coelomic fluid of *P.lividus* is essential to understand how this organism live and share in a sea environment as well as found new enzymes produce by this microbiota usable for biotechnological processes.

1.3 Sea urchin *P.lividus*

The sea urchin *Paracentrotus lividus* is also known as the rock sea urchin or purple sea urchin [43]. However, the colour of this species (Figure 4) can vary between brown (dark and light), olive green and violet (dark and light). It belongs to the phylum Echinodermata (Echinoidea); it's a benthic organism (lives fixed to a solid substrate) and lives principally in the eastern Atlantic Ocean and the Mediterranean Sea. It's subtidal species (species that lives in a zone situated below the variation of the sea's tide because it needs to be always immersing). It lives fixed to a rock pool (boulders) or in meadows of seagrasses at a depth up to 20 meters but can be found until 80 meters when the temperature of the upper water exceeds 29°C (living temperature between 4°C to 29°C). This organism is principally herbivore and eats different kinds of algae like *R.verruculosa, C.nodosa and P.pavonica* [44]. It moves outsides rock cavity during the night to eat and still hide the rest of the time.

Figure 2: Sampling of sea urchin *P.lividus* from Mediterranean Sea [45].

Figure 3: Upper side (aboral pole) of dark violet *Paracentrotus lividus* [45].

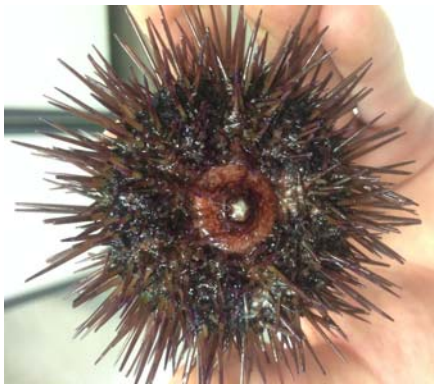


Figure 4: bottom side (oral pole) of light violet *Paracentrotus lividus* [45].

The reproduction period for *P.lividus* is between February and July; during this period, which corresponds to the algal production, the gonads are biggest and contain gametes (sperms and eggs) [46]. The fecundation of eggs is external. Eggs and sperms are ejected in the sea in the same moment, during the night; the fusion of the gametes is realised with some adhesive protein in sperm and specific receptors on eggs surface [47]. It is not possible to determine if the sea urchin is a female or a male before to open it.

P.lividus is comestible and is particularly appreciate in Europe (principally in Italy, Spain and France) and in Japan for its gonads. During these last few years, the fishery has grown too fast and becomes dangerous for the population of this species (also for fish species), despite it is the most widespread species of the Mediterranean Sea [48]. For example between 1996 and 2000, a mean of 500,000 tonnes per year were fished in Spain Atlantic Ocean. This quantity has been reduced to a mean of 100,000 tonnes per year between 2001 and 2011 [49]. This reduction is a good beginning but not sufficient to recover the initial world population of sea urchin.

So it's important to understand how this organism lives and shares with its environment in order to control the aquaculture conditions; which correspond only to 0.9% of the world production, and not lose this species definitively in natural habitat. The organic pollution has a little effect on the growth of *P.lividus* and seems in contrast to increasing the density population of the species near the discharge point [50].

However, pollution from oil spills has caused 100% mortality of sea urchin (*P.lividus* and *P.*miliaris) population, three weeks after "Ericka" incident at Piriac-sur-Mer (France). It took about three years to recover the initial density [51].

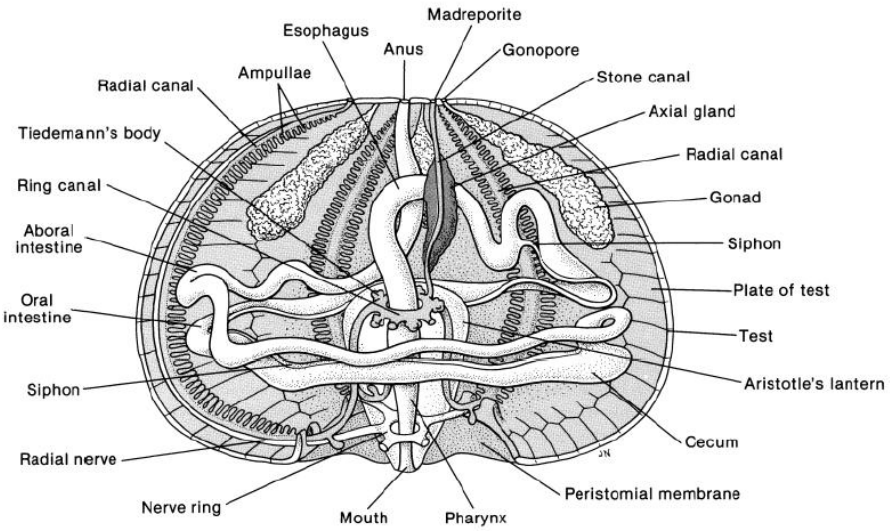


Figure 5: Internal anatomoy of sea urchin *P.lividus*. The coelomic fluid is not represented but all of the internal organs bathed in this liquid [49].

The body structure (named test, Figure 5) of *P.lividus* is composed of five symmetric calcareous plates and the epidermis. The form of this animal is spherical for the top and flattened on the bottom and the maximum test diameter is about 7 centimetres [52]. It is covered by movable spines and podia with which it can move vertically and horizontally. The spines are fixed on the shell by muscles and serve also as protection against the predator and to catch and hold the nutrients. The weight of spines on healthy sea urchin corresponds to 50% of the total weight. The podia serve principally for oxygen absorption, body cleaning or covered the body with pieces of algae, shell and rocks as a camouflage (named spine canopy). The mouth is situated on the bottom surface (oral pole, Figure 2) and is called Aristotle's lantern, it is composed of five triangular plates with the tooth at the end. The sea urchin *P.lividus* also uses this strong mouth to create some protection cavities in the rock against predators and Sea/Ocean current. The mouth is connected to the test by a peristomial membrane; this membrane allows the mouth to move up, down and on the sides. The mouth is following by the pharynx, the oesophagus, two intestines (oral and aboral) and finish by the anus on the aboral surface (on the top, Figure 3).

The digestive system is directly associated to the gonads by hemal and mesenterial strands. The gametes and excrements are released into the sea by the anal pole. Inside the coelomic cavity (cavity forms by the test) you can also find five gonads (reproductive organs, Figure 6) disposed radially on the test. The colour of desirable gonads can be varied between yellow-orange and orange-red due to carotenoid pigments if the gonads present different colours (black, brown, green or white) it is a sign of healthy problem.

Figure 6: Opened sea urchin. Five orange gonads from adult.

The weight of healthy gonads corresponds to 10-14% of the adult's body but can be smallest [53]. Two major cell types are present in the gonads: germinal cells and nutritive phagocytes, which give the energy and nutrients necessary for the development of the gametes [54-55]. The most important circulatory medium of sea urchins is then the coelomic fluid enclosed in the body cavity (test). This internal fluid contains a mixture of organic molecules and living cells that control essential functions (as nutrient transport or immune activity) [56-57]. The coelomic fluid is not in contact with the surrounding seawater environment and in direct contact with internal tissues. This is why this liquid is composed by immunocytes that respond to pathogen challenge and producing antibacterial molecules [58-59]. Therefore, the coelomic fluid could be considered as a complex tissue that mediates responses against injury and microbial infections by different approaches such as coagulation, opsonization, phagocytosis and encapsulation [60]. In a second case, the coelomic fluid of *P.lividus* has been supposed to be a microorganism-free compartment. Only recently, a report describing the identification of a bacterial microbiota associated with the coelomic fluid of *P.lividus* [61], was carried out in the Laboratory of Molecular Microbiology and Biotechnology (where the practical part of this thesis was realised).

In the present study, the presence of bacteria into the coelomic fluid of the sea urchin *Paracentrotus lividus* from a geographical area different from that considered in the first report has been explored. Two different methods of analysis, the culture-based and culture-independent investigations, are described. The dependent culture method based on the growth of bacteria on marine agar (MA) and DNA sequencing after the amplification of the 16S rRNA gene, reported a predominance of taxa belonging to *Proteobacteria* and *Bacteriodetes*. The analysis of the production of extracellular enzymes has also been performed and reports that the isolated strains produced proteases, lipases and esterases. This kind of biomolecules can be useful for biotechnological processes but needs to be characterised because only qualitative approaches have been done. The independent culture method was performed by the sequencing by minION™ technologies of the metagenomics DNA extracted from the coelomic fluid. This approach reported a predominance of taxa belonging to *Proteobacteria*, *Actinobacteria*, *Cyanobacteria*, *Fusobacteria*, *Thermodesulfobacteria*, *Ignavibacteria*, *Firmicutes* and *Tenericutes*. The identification of the microbiota present in the coelomic fluid of sea urchin *Paracentrotus lividus* have been highlighted in order to understand the role of bacteria in the physiology and ecology and aquaculture activities of this echinoderm. Moreover, this research could allow the discovery of new bioactive compound useful for biotechnological processes.

2 Materials and Methods

2.1 Dependent culture method

2.1.1 Marine Agar (MA) and Marine Broth medium (MB)

40.20 g (balance BC1500, ORMA, n°33888) of marine broth (Conda pronadisa, n°1217.00) was suspended in 1.0 L of distilled water. Well mixed and dissolved by heating in a water bath with frequent agitation. The preparation was boiled one minute in the microwave (Electrolux M8, n°EMS21400S) until complete dissolution. After the temperature was below than 30°C, 15.00 g of BactoAgar (BD, n°214010) was added and the medium was sterilised at 121°C, 1 atm during 20 minutes (autoclave Carlo Erba, n°5991). The prepared medium may present a light precipitation. Plates were prepared before the solidification of the medium and under biological laminar flow with UV lamp. The plates were conserved at 4°C until the use.

The marine broth medium (MB) was prepared following the same instruction without the addition of bactoAgar. After the sterilisation, the bottle of the medium was conserved at RT.

2.1.2 Sea urchin and coelomic fluid harvesting

The sea urchin samples used for this thesis were collected from the coastal region of Palermo, from the coast of women's island (Isola delle Femmine, 38°12'00''N and 13°15'00''E), during the months of June and July 2016. The *P.lividus* samples were taken during the early hours of the morning and immediately transported to the Laboratory of Molecular Microbiology and Biotechnology of the University of Palermo, in optimal conditions of temperature and humidity, such as to ensure an analysis as accurate as possible and corresponding with natural conditions. It was then externally sterilised by heat (burned with a Bunsen the external calcareous shell urchin). The harvesting was realised under a biological laminar flow. Sterile syringe (BD plastipak, n°300865) with a needle were used for the harvesting of coelomic fluid. The needle was inserted through the animal's peristomial membrane around the mouth (Aristotle's lantern) and the liquid is pumped off.

The coelomic liquid extracted was mixed with 100 µL of 0.5 M EDTA (Sigma, n° E6758) to prevent coagulation of protein, placed into sterile tubes of 10 mL and kept on ice. The coelomic fluid was plated containing MA as described below:

- 20 µL of native sample
- 20 µL of 10x diluted sample
- 20 µL of 100x diluted sample

The MAs were incubated overnight at 30°C. All the different strains were then selected and plated on fresh MA. The new plates were incubated overnight at 30°C. If there was one strain on the plate, this one was stored at 4°C until the use. If there was more than one colony, one fresh MA was used for each colony and incubated overnight at 30°C. This final step was realised until to obtain a pure clone.

2.1.3 Colony PCR

Cells of the strains isolated from pure cultures were directly taken from their culture medium under sterile conditions with a toothpick and suspended in 25 µL of TE buffer (10 mM Trizma (Sigma, n°T1503), 1 mM EDTA (Sigma, n° E6758), pH adjusted at 8).

The bacterial suspensions were boiled in water bath for 5 minutes, immediately after placed on ice for 5 minutes and finally centrifuged (minispin, Eppendorf, n°5452) at 15000 G for 5 minutes (centrifuge mini spin Eppendorf, n°5452). 1 µL of the supernatant containing the DNA of the selected strain was used as a template for the PCR reaction.

The primers used for the PCR reaction of the 16S rRNA gene are listed in the Table 1.

Table 1: Oligonucleotide used for the amplification of 16S rRNA gene.

Name	Sequences	Lenght	Information
16S_27F	5'- AGAGTTTGATCMTGGCTCAG -3'	20	PCR primer
16S_1492R	5'- TACGGYTACCTTGTTACGACTT-3'	22	PCR primer

Primer 16S_27F (Invitrogen, Q8181A07); Primer 16S_1492R (Invitrogen, n°Q8181A08) [62].

The region amplified by the primers used is visualised on the Figure 7. This PCR reaction can be named as "16S long" amplification [63].

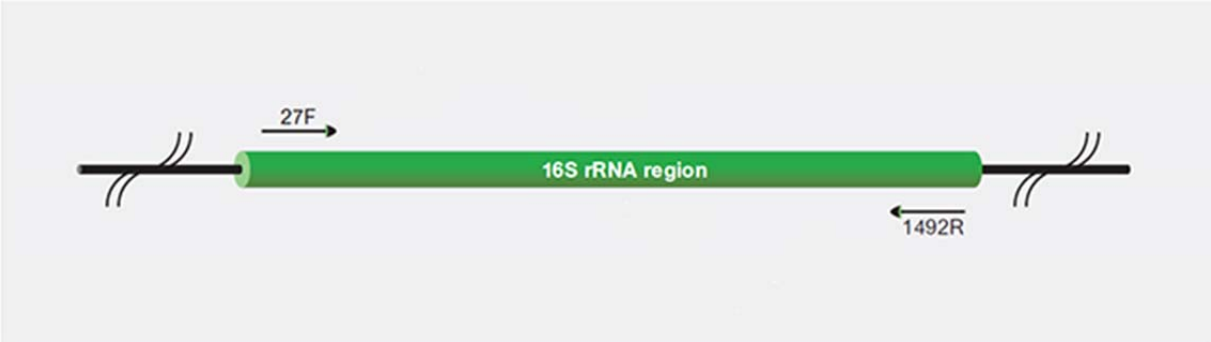


Figure 7: Amplified region on the 16S rDNA [64].

The amplification reaction was carried out in a total reaction volume of 50 µL, using the following quantities of reagents (Table 2):

Table 2: Master Mix used for the colony PCR.

	Final Conc.	Vol. for 1 rx [µL]	Vol. for 5 rx [µL]	Vol. for 15 rx [µL]
5x PCR reaction buffer	1x	10	50	150
10 mM dNTP mix	200 µM each	1	5	15
50 mM $MgCl_2$	1.5 µM	1.5	7.5	22.5
Primer 16S_27F	0.2 µM	1	5	15
Primer 16S_1492R	0.2 µM	1	5	15
Template DNA	-	1	-	-
***Tfi* DNA polymerase (5U/µL)**	5 units	1	5	15
H_2O sterile	-	33.5	167.5	502.5
Total	-	50	245	735

Tfi DNA polymerase, 5x PCR reaction buffer and 50 mM $MgCl_2$ (Invitrogen, n°30342-052); dNTP set (Invitrogen, 10297-018). NFW was used as negative control and genomic DNA from *S.coelicolor* as positive control (University sample gave by A.Vassallo).

The programme used for the amplification (Master cycler personal Eppendrof, n°5332 01037) is the following (Table 3):

Table 3: Program used for the colony PCR.

	Temps	Temperature [°C]	
Initial Denaturation	3 min	94	
Denaturation	45s	94	
Annealing	1 min	50	30 cycles
Extension	1.5 min	72	
Final extension	10 min	72	

The duration of this program is about 2.2 hours.

2.1.4 Gel electrophoresis

To observe the result of the amplification, a 1% (w/v) agarose gel was prepared. 0.5 grams (balance JJ100, FALC, n°630201001) of agarose (Eurx, n°E0301-500) were added in 50 ml of TAE buffer 1x (4.84 g Trizma (Sigma, n°T1503), 1.14 ml acetic acid 100% (Sigma, n°33209), 2 ml 0.5 M EDTA (Sigma, n° E6758) completed until 1000 ml with demineralised water, pH adjusted at 8). The solution was boiled with a microwave (Electrolux M8, n°EMS21400S) until complete dissolution of the agarose. 1 µL of ethidium bromide (Sigma, n°E1510) was added to the solution before the pouring of the gel. Let the gel solidified before the use.

5 µL of ladder (MassRuler DNA ladder mix, Thermo Scientific, n°SM0403 or DNA ladder, Roche, n°14833221) was loaded for each run. 2 µL of sample mixed with 1 µL of loading buffer (DNA gel loading dye 6x, Thermo Scientific, n°R0611) was loaded per well. The run conditions were 100 mA and 100 V (electrophoresis power supply Elvi tipo18, n°877) and the run duration was about 1 hour. Results acquisition was performed with the program AlphaDigiDoc RT2 connected to a trans-illuminator UV (Alpha innotech corporation UV lamp, n°0610193).

2.1.5 Purification of amplified DNA

The following method originates from the NucleoSpin® gel and PCR clean-up kit by Macherey-Nagel (n°740609.250).

The purification step was realised from a 1% agarose gel. 1.2 grams (balance JJ100, FALC, n°630201001) of agarose (Eurx, n°E0301-500) were added in 120 mL of TAE buffer 1x (4.84 g Trizma (Sigma, n°T1503), 1.14 ml acetic acid 100% (Sigma, n°33209), 2 mL 0.5 M EDTA (Sigma, n° E6758) completed until 1000 mL with demineralised water, pH adjusted at 8). The solution was boiled with a microwave (Electrolux M8, n°EMS21400S) until complete dissolution of the agarose. 2.5 µL of ethidium bromide (Sigma, n°E1510) was added to the solution before the pouring of the gel. Let the gel solidified before the use. 5 µL of ladder (MassRuler DNA ladder mix, Thermo Scientific, n°SM0403) was loaded for each run. 48 µL of sample mixed with 3 µL of loading buffer (DNA gel loading dye 6x, Thermo Scientific, n°R0611) was loaded per well. The run conditions were 100 mA and 100 V (electrophoresis power supply Elvi tipo18, n°877) and the run duration is about 2.75 hours.

After the run, the gel was placed on a trans-illuminator UV 312 nm (Vilber Lourmat, model TFX 20M) and the expected band (1500 bp) was excised with a clean scalpel. The weight of the cut band was determined (balance JJ100, FALC, n°630201001) and transferred to a tube of 1.5 ml. 200 µL of buffer NT1 (Macherey-Nagel, n°740609.250) was added for each 100 mg of agarose gel. The sample was incubated 10 min at 50°C (thermobloc BBD2, Grant Boekel, n°7W9906001) with vortexing every 3 minutes until the gel slice was completely dissolved. A column from the kit was placed into a collection tube (2 mL) and a maximum of 700 µL of sample was charged. The whole was centrifuged (minispin, Eppendorf, n°5452) for 30 seconds at 11,000 G. The flow-through was discarded and the column was placed back into the collection tube. If the sample volume was more than 700 µL, the centrifugation step was repeated. 700 µL of buffer NT3 (Macherey-Nagel, n°740609.250) was added to the column and centrifuged for 30 seconds at 11,000 G. The flow-through was discarded and the column was placed back into the collection tube. This step was realised twice. The silica membrane of the column was dried by centrifugation for 1 minute at 11,000 G and incubation at 70°C for 5 minutes. The column was placed into a new tube of 1.5 mL and 50 µL of demineralised water is added for the elution. The whole was incubated at RT for 1 minute and centrifuged for 1 min at 11,000 G. The elution step was realised twice.

After the elution and to confirm the purification step, 1 µL of the sample was charged on a 1% (w/v) agarose gel as described in the section 2.1.4.

2.1.6 Sequencing

The sequencing of amplicons was performed by the company Macrogen EU (Meibergdreef, Amsterdam, The Nederlands), according to the Sanger method.

2.1.7 Bioinformatics analysis

2.1.7.1 FinchTV

The chromatograms obtained for the sequencing of amplicons were analysed by bioinformatics software FinchTV 1.4.0. This application is free and was developed by Geospiza Inc. (2004-2006). FinchTV allows determining the correct allocation of the positions of the nucleotide bases, and then manually modifying the sequence to then be exported in FASTA format, so it can be used in the subsequent analyses.

2.1.7.2 BLAST

The Basic Local Alignment Search Tool (BLAST) is an online software who finds regions of local similarity between sequences. The program compares nucleotide sequences to sequence databases and calculates the statistical significance of matches using a heuristic algorithm. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families [65].

The sample sequences were compared with the 16S ribosomal RNA sequences (Bacteria and Archaea) database of the program to find the most similar strains. The 10 first similar sequences found in this database were used to build the phylogenetic tree.

2.1.7.3 MEGA 7

Molecular Evolutionary Genetics Analysis (MEGA) is an integrated tool for managing automatic and manual sequence alignment, mining web-based databases, deducting phylogenetic trees, evaluating rates of molecular evolution, and testing evolutionary hypotheses. To access this software connect to the Windows systems [66].

2.1.8 Strain glycerol stock (SGS)

The strains isolated from pure cultures were directly taken under sterile conditions with a platinum loop and added in 5 mL of marine broth medium. The cultures were stored overnight at 30°C and 220 rpm. 0.5 mL of the culture is mixed with 0.5 mL of sterile 40% (v/v) glycerol (Sigma, n°G5516) to arrive at a final concentration of 20% (v/v) of glycerol. The SGS was conserved at -20°C.

2.1.9 Protease analysis

5 µL of SGS was added in 5 mL of MB and incubated overnight at 30°C and 220 rpm. The culture was centrifuged (Thermo Scientific SL16R, n°75004030) at 1800 G during 20 minutes at 4°C. 10 µL of the supernatant was mixed with 10 µL of native buffer 2x (100mM Tris-HCl (Sigma, n°T5941), 4% (w/v) SDS (Sigma, n°62862), 20% (v/v) glycerol (Sigma, n°G5516) and 0.2 % (w/v) bromophenol blue (Bio-Rad, n°133538A)). 15 µL of the obtained solution was charged per well in the stacking gel. 8 µL of the pre-stained ladder (SeeBlue® Plus2, novex, n°LC5925) was charged in the well. The conditions for the samples migration were the following: 100 W, 160 V and 15 mA until the samples were entered in the resolving gel after the current was raised to 25 mA (electrophoresis power supply EPS601, n°56327481).

For one analysis the gel was prepared using the following reagents (Table 4):

Table 4: Preparation of the entire gel for the protease analysis.

	Resolving gel [mL]	Stacking gel [mL]
H_2O demineralised	1.15	0.68
Gelatine stock solution	0.45	-
30% Acrylamide mix	2	0.17
1.5 M Trizma pH 8.8	1.3	-
1 M Trizma pH 6.8	-	0.13
10% SDS	0.05	0.01
10% APS	0.05	0.01
TEMED	0.002	0.001

Gelatine (Sigma, n°G7765); 30% Acrylamide mix (Sigma, n°A3574); Trizma (Sigma, n°T1503); SDS (Sigma, n°62862); APS (Sigma, n°A3678); TEMED (Sigma, n°T9281).

The concentration of the resolving gel was usually 12% of acrylamide. The gelatine stock solution had a concentration of 6 g/L. The two last components must be added just prior to casting for the both gel. The resolving gel corresponded to the lower part of the entire gel and the stacking gel to the upper part. The migration of the samples was realised inside the running buffer (25 mM Trizma (Sigma, n°T1503), 192 mM glycine (Sigma, n°G8898) and 0.1% SDS (Sigma, n°62862)) [67].

After the migration of the samples, the gel was washed with the washing buffer (2.5% Triton X-100 (Sigma, n°T9284) and 0.02% NaN_3 (Serva, n°30175)) during 10 minutes at RT. The gel was then treated for the staining with the activation buffer (1.5% Triton X-100 (Sigma, n°T9284), 0.02% NaN_3 (Serva, n°30175), 2 mM $CaCl_2$ (Carlo Erba, n°433381) and 50 mM Trizma (Sigma, n°T1503), pH was adjusted at 7.5) for 1 hour at 50°C. The staining was carried out overnight with the colorant R250 (0.25% (w/v) Coomassie blue (Merck, n°12553), 45.5% (v/v) methanol (Sigma, n°32213) and 9% (v/v) acetic acid (Sigma, n°33209)).

The destaining was then realised in three steps of 1 hour with fresh destaining buffer (5% (v/v) methanol (Sigma, n°32213) and 7.5% (v/v) acetic acid (Sigma, n°33209)). The destained gel was conserved in demineralised water.

2.1.10 SDS-Page

5 µL of SGS was added in 5 mL of MB and incubated overnight at 30°C and 220 rpm. The culture was centrifuged (Thermo Scientific SL16R, n°75004030) at 1800 G for 20 minutes at 4°C. 2 mL of the supernatant was recovered and lyophilised (lyphilisator VaCo 2-E, Zirbus technologie, n°2366) overnight. The lyophilisate was dissolved with 150 µL of demineralised water and vortexed gently. 10 µL of the obtained solution was mixed with 10 µL of native buffer 2x (same preparation as section 2.1.9). 15 µL of the obtained solution was charged per well in the stacking gel. The ladder used and the condition of the migration has been carried out as the protease analysis (section 2.1.9).

For one analysis the gel was prepared using the following reagents (Table 5):

Table 5: Preparation of the entire gel for the SDS-Page analysis.

	Resolving gel [mL]	Stacking gel [mL]
H_2O demineralised	1.15	0.68
30% Acrylamide mix	2	0.17
1.5 M Trizma pH 8.8	1.3	-
1 M Trizma pH 6.8	-	0.13
10% SDS	0.05	0.01
10% APS	0.05	0.01
TEMED	0.002	0.001

30% Acrylamide mix (Sigma, n°A3574); Trizma (Sigma, n°T1503); SDS (Sigma, n°62862); APS (Sigma, n°A3678); TEMED (Sigma, n°T9281).

The concentration of the resolving gel was usually 12% of acrylamide. The two last components must be added just prior to casting for the both gel. The resolving gel corresponded to the lower part of the entire gel and the stacking gel to the upper part. The running buffer used is the same than in section 2.1.9.

After the migration of the samples, the gel was washed with milliQ water during 5 min at RT. The gel was then fix with the destaining solution (40% (v/v) methanol (Sigma, n°32213), 10% (v/v) acetic acid (Sigma, n°33209), 50% (v/v) demineralised water) for 25 min at RT. To eliminate the residue of acid acetic the gel was rinsed 3 times during 45 min at RT and overnight with demineralised water. The gel was sensitised with the solution S (0.08% (w/v) sodium thiosulfate (Sigma, n°S6672)) for 5 min at RT and then rinsed two times with demineralised water for 1 min (each).

The gel was incubated in chilled silver solution (0.4% (w/v) silver nitrate (Sigma, n°31630)) at 4°C for 60 min and then rinsed two times with demineralised water during 1 min (each). The gel was developed with the developing solution (0.04% (v/v) formaldehyde (Sigma, n°F8775), 2% (w/v) sodium carbonate (Sigma, n°31432)) until to see the coloration expected (grew-brown colour) and then the staining was stopped immediately with the quench solution (1% (v/v) acetic acid (Sigma, n°33209). The gel was stored in the quench solution at 4°C.

2.1.11 Cellulase analysis

The preparation of the samples, the ladder used and the condition of the migration has been carried out as the SDS-Page analysis (section 2.1.10). For this analysis, as positive control, a cellulase from *Aspergillus niger* (Sigma, n°22178) had added to a final quantity of 8 µg per lane.

For one analysis the gel was prepared using the following reagents (Table 6):

Table 6: Preparation of the entire gel for the cellulase analysis.

	Resolving gel	Stacking gel [ml]
H_2O demineralised	1.15 mL	0.68
CMC	0.01 g	-
30% Acrylamide mix	2 mL	0.17
1.5 M Trizma pH 8.8	1.3 mL	-
1 M Trizma pH 6.8	-	0.13
10% SDS	0.05 mL	0.01
10% APS	0.05 mL	0.01
TEMED	0.002 mL	0.001

CMC (Sigma, n°C5678); 30% Acrylamide mix (Sigma, n°A3574); Trizma (Sigma, n°T1503); SDS (Sigma, n°62862); APS (Sigma, n°A3678); TEMED (Sigma, n°T9281).

The concentration of the resolving gel was usually 12% of acrylamide. The CMC added had a concentration of 0.2 % (w/v). The two last components must be added just prior to casting for the both gel. The resolving gel corresponded to the lower part of the entire gel and the stacking gel to the upper part. The running buffer used is the same than in section 2.1.9.

After the migration of the samples, the gel was washed with the washing/activation solution (0.1M sodium succinate (Sigma, n°S2378), pH adjusted at 5.8) twice for 6 minutes at RT. The gel was then treated for the staining with the washing/activation solution for 40 minutes at 50°C. The staining was carried out with the colorant Congo red (0.1% (w/v) in water solution, (Sigma, n°C6277)) for 20 minutes at RT. The destaining was then realised in three steps of 1 hour with fresh destaining solution (1 M NaCl (Sigma, n°S7653)). To enhance the visualization of the band, 0.1% (v/v) of acid acetic (Sigma, n°33209) was added in the destaining solution for the last step. The gel destained was conserved in demineralised water.

2.1.12 Esterase analysis

The esterase activity assay was tested qualitatively on ester myristate agar plate (0.14% (w/v) 4-nitrophenyl myristate (Sigma, n°BCBP9506V); 1.5 % (w/v) BactoAgar (BD, n°214010), pH adjusted at 7.0). The myristate was dissolved in DMSO, filtrated with a 0.20 µm filter and then added after the autoclaving of the medium and before the pouring of the plate. The sample was plated as a drop and the agar plate was incubated at 37°C during 24 hours. The esterase reaction was observed through the formation of a clear halo after incubation. A 15 µL drop of MB has been used as negative control.

2.1.13 Lipase analysis

The determination of the lipase activity was assessed qualitatively by the growth of bacterial cultures for 10 days at 30°C on plate containing marine broth, 0.001% rhodamine-B (gave by the department of chemistry), 1% olive oil (Fratelli Madonia, n°L.267/15/extra c) and 1.5% Bactoagar (BD, n°214010). The solution of rhodamine-B and olive oil were filtrated at 0.20 µm, and then added after the autoclaving of the medium and before the pouring of the plate. 5µL of SGS was deposed as a drop on the plate. The protocol performed allows to evaluate, through the use of rhodamine-B dye, such bacterial strains are able to activate the enzyme for the use of olive oil. In fact, the bacteria that possess the lipase is capable to hydrolysed fats, animal and vegetable oils (such as olive oil) and free fatty acids (saturated and unsaturated such as oleic acid). The liberation of fatty acids due to the decrease of pH is visible by the change in colour of rhodamine-B. The positivity of the test is given by the presence of an orange-pink fluorescence exposure under UV light following the incubation.

2.1.14 Antimicrobial assay

The first day, isolated strains were plated on MA and incubated overnight at 30°C to have a dense layer of culture. The same day, some plates were prepared with a small layer (7 ml) of LB agar (25.0 g/L of Luria Broth base (Invitrogen, n°12795-084) and 1.5% (w/v) of BactoAgar (BD, n°214010). The medium was before sterilised at 121°C, 1 atm for 20 min.

The second day, some cells of *Escherichia coli* and *Kocuria rhizophila* (gift of P. Cinà) were added in 5 mL of demineralised water (two different tubes, one for *E.coli* and one for *K.rhizophila*) and the absorbance OD_{600} (Spectrophotometer Jasco, ubest-50, n°2332) of the suspensions were measured. Then 100 µL of the suspension (OD_{600} is between 1.0 and 1.2) was added in 5.0 mL of sterilised soft agar at 45°C (25.0 g/L of Luria Broth base (Invitrogen, n°12795-084) and 0.7% (w/v) of BactoAgar (BD, n°214010) and poured on the plate containing the small layer of LB agar. The plates containing the soft agar were let until solidification.

A plug with a diameter of about 2 cm was cut from MA plates of each dense bacterial layer and put on the soft agar containing *E.coli* or *K.rhizophila*. The plates were incubated overnight at 37°C.

2.2 Independent culture method

2.2.1 DNA extraction

The following method originates from the genomic DNA purification kit by Thermo Scientific (n°K0512).

The coelomic fluid from *P.lividus* was extracted as described in the chapter 2.1.1. After the harvesting and collection in 10 mL sterile tubes (Sarstedt, n°62.9924.284), each sample was centrifuged (Thermo Scientific SL16R, n°75004030) at 2800 G for 10 minutes. The pellet was suspended in 200 µL of TE buffer (10 mM Trizma (Sigma, n°T1503), 1 mM EDTA (Sigma, n° E6758), pH adjusted at 8). The sample was mixed with 400 µL of lysis solution (from the kit) and incubated at 65°C for 5 minutes. 600 µL of chloroform (Sigma, n°32211) was immediately added and the solution was gently mixed by inversion (5 times) and centrifuged at 11,000 G for 2 minutes. Precipitation solution was made by mixing 720 µL of sterile demineralised water with 80 µL of 10x concentrated precipitation solution (from the kit). The upper aqueous phase containing DNA was transferred into a new tube and 800 µL of freshly prepared precipitation solution was added. The solution was gently mixed by serval inversion at RT and centrifuged at 11,000 G for 2 minutes. The supernatant was completely removed and the DNA pellet was dissolved in 100 µL of NaCl solution (from the kit). To eliminate the RNA present in the sample, a RNAse A solution (TermoFisher Scientific, n°EN0531) was added to a final concentration of 0.2 mg/mL, mixed by vortexing and incubated at 37°C for 10 minutes. 300 µL of cold ethanol (Fluka, n°02860) was added for the precipitation of DNA and the sample was stored for 10 minutes at -20°C. Ethanol was removed and the pellet was washed once with a solution of 70% cold ethanol.

The DNA pellet was dissolved in 100 µL of sterilised water by gentle vortexing. 5 µL of the products of extraction were charged on a 1% agarose gel (preparation of the gel is describe in section 2.1.4) and separated by electrophoresis.

2.2.2 DNA Quantification

To determine the DNA concentration and quality of the samples, the spectrophotometer NanoDrop (Nanodrop ND-1000, n°Ed1/0207/152) was used. This instrument is a UV-Visible spectrophotometer and need only 1 µL of sample for the analysis. It quantifies very accurately by measuring the optical density of the sample.

The Nanodrop uses a technology based on surface tension of small liquid volume that exert when it is placed between two neighbouring surfaces. Thus the drop of sample placed on the reading plate, creates a liquid column directly in contact with two optical fibres, this analysis is very simple and fast. The wavelength used to measure the concentration of DNA is 260 nm.

The Nanodrop also provides the degree of purity of the sample, by providing the relationships between:

- 260/280: indicates the contamination by proteins if the ratio is bigger than 1.8.
- 260/230: indicates the contamination by carbohydrates and phenol. The optimal value is 2.2 and lower values indicate contamination.

2.2.3 Amplification of the 16S rRNA gene

The genomic DNA extracts from the coelomic fluid was amplified by PCR reaction of 16S rRNA gene. The primers used were the same than in section 2.1.3 (Table 1). The amplification reaction was carried out in a total reaction volume of 30 µl, using the following quantities of reagents (Table 7):

Table 7: Master Mix used for the amplification of the 16S rRNA gene form microbial genomic DNA.

	Final Conc.	Vol. for 1 rx [µL]	Vol. for 6 rx [µL]
10x PCR reaction buffer	1x	3.0	18
10 mM dNTP mix	200 µM each	0.6	3.6
50 mM $MgCl_2$	1.5 µM	0.9	5.4
Primer 16S_27F	0.2 µM	0.6	3.6
Primer 16S_1492R	0.2 µM	0.6	3.6
Template DNA	-	1	-
***Taq* DNA polymerase (5U/µL)**	1.5 units	0.3	1.8
H_2O sterile	-	23	138
Total	-	30	174

Taq DNA polymerase, 10x PCR reaction buffer and 50 mM $MgCl_2$ (Invitrogen, n°10342-020); dNTP set (Invitrogen, 10297-018). NFW is used as negative control and genomic DNA from *S.coelicolor* as positive control (University sample gave by A.Vassallo).

The programme used for the amplification (Master cycler personal Eppendrof, n°5332 01037) is the following (Table 8):

Table 8: Program used for the amplification of the 16S rRNA gene from microbial genomic DNA.

	Temps	Temperature [°C]	
Initial Denaturation	3 min	94	
Denaturation	45s	94	
Annealing	1 min	50	30 cycles
Extension	1.5 min	72	
Final extension	10 min	72	

The duration of this program is about 2.2 hours. 2 µL of the PCR products were charged on a 1% agarose gel (preparation of the gel is describe in section 2.1.4) and separated by electrophoresis.

2.2.4 Sequencing by minION™ technology

The sequencing of the metagenomics sample has been realised in the laboratories of the Polyclinic of Palermo, under the supervision of Dr Tripodo C. and the technical support of Dr Arancio W. The extracted products have been sent without preparation. The technology used for the sequencing was named minION™ and it was in development. More information on the method used for the preparation of the sample and the analysis can be found on the website of Oxford nanopore technologies [68].

The software used to treat the result is named: What's in my pot (WIMP). It is an EPI2ME workflow for sequence classification. It generates a report that represents the biological diversity present in the sample in real time. The application works on a read-by-read basis by basecalling. WIMP performs real-time taxonomic classification, according to the NCBI taxonomy, by processing MinION™. Wherever possible, the reads are placed at the species or sub-species level. If there is not enough information to identify the species, the read is placed at a higher rank of the taxonomic tree. If no placement is reliable enough, the sequence is labelled as "Could not classify sequence". The WIMP report continues to be updated as more reads are processed through the workflow until it is stopped by the user [69].

WIMP is designed to work for any organism for which a reference is available via the NCBI RefSeq database for viruses, bacteria, and fungi [70]. The NCBI taxonomy is used as it is a curated resource based on public sequence databases where users can find details on the organism identified based on a unique NCBI taxonomy ID. Currently, WIMP combines three databases to support identification of bacteria, viruses, and fungi.

The classification method of WIMP relies on a data structure that maps all k-mers of length 24 present in the reference database to nodes in the NCBI taxonomy tree. Due to this pre-processing, new reads can be quickly classified by looking up k-mers (rather than aligning against the original reference). WIMP uses the kraken algorithm [71] to map all k-mers, calculate the LCA (least common ancestor), and filter with a minimum score threshold of 0.001 to determine what is the most likely placement for a query in the taxonomy tree. The output is a taxon ID for which a classification score is computed.

3 Results

3.1 Dependent culture method

3.1.1 Isolation of bacterial strains from coelomic fluid of *P.lividus*

The sea urchin was externally sterilised with a Bunsen and the coelomic fluid was harvesting with sterile syringe. The dilution 10 and 100 fold of coelomic fluid was realised with sterilised seawater. 20 µL of each sample were dispensed on MA and the plates were incubated overnight at 30°C. A total of $4.4*10^5$ bacteria isolated/mL was obtained from the coelomic fluid of *P.lividus*. The results obtained are shown on the Figure 9.

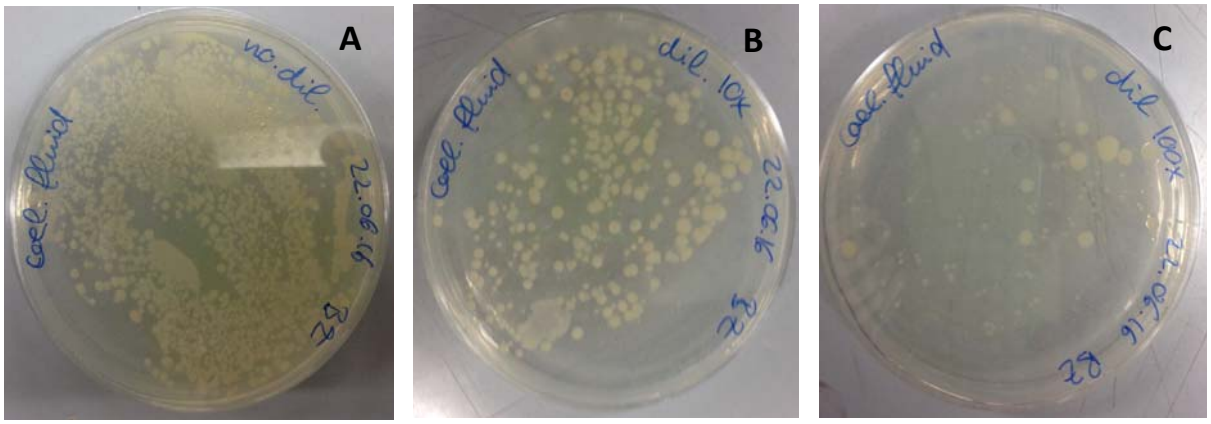


Figure 9: Isolation of the strain from the coelomic fluid on MA. (A) No dilution, (B) 1/10 dilution and (C) 1/100 dilution.

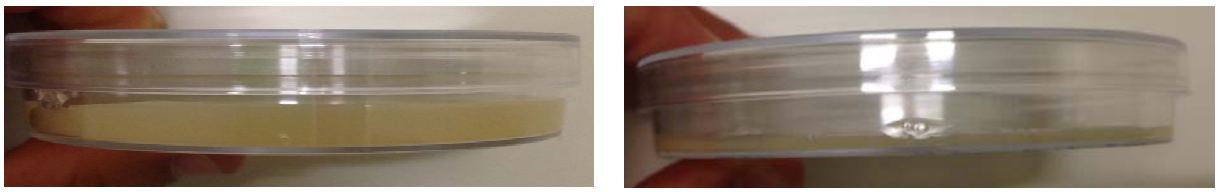


Figure 8: Diminution of the MA thickness after 3 weeks at 30°C.

The Figure 8 shows the diminution of the MA thickness after 3 weeks at 30°C caused by the production of hydrolytic enzymes from the isolated strains 1, 4, 5, 6, 7, 9 and 10.

A plate without bacteria has been used to confirm that the diminution of the thickness was not a cause of evaporation. The result obtained shows any difference of thickness.

The phenotypic analysis of pure culture suggested that 12 different bacterial strains have been isolated from the coelomic fluid. The Figure 10 regroups the picture of each clone derived from a pure colony.

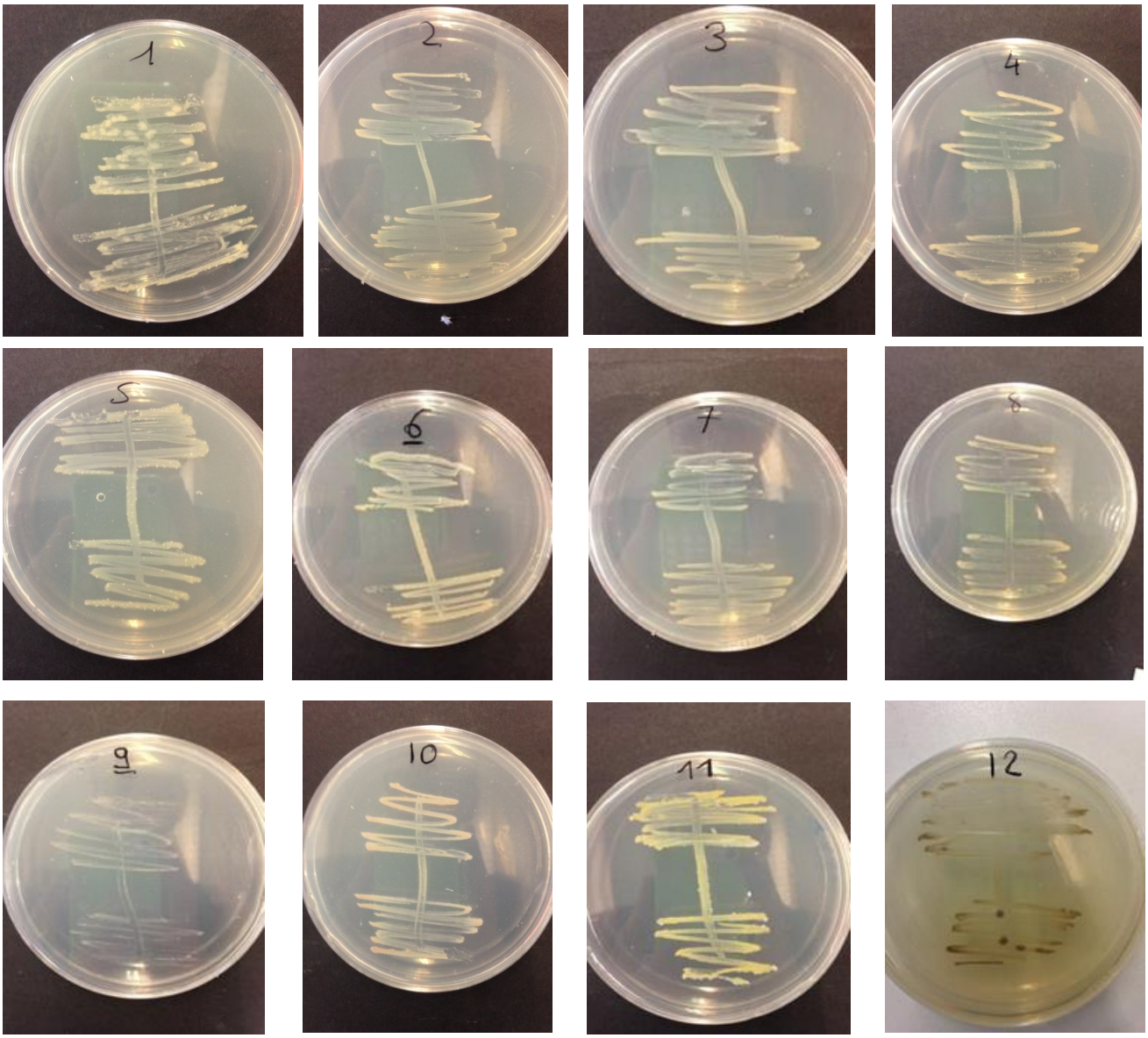


Figure 10: Pure colony on MA isolated from coelomic fluid after 5 days at 30°C.

The picture of the Figure 10 was taken after 5 days of incubation at 30°C because the strain 9 needed this period to growth.

The bacterial colonies from the strain 1 to 7 have a light yellow - cream colour and are opaque. The form of the colonies is circular with raised elevation, regular margin and smooth surface. The strain 1 presents a blue luminescence after one night of incubation and non-luminescence after 2 days of incubation. The colonies from the strain 8 and 10 have a light orange colour and are opaque. The form is circular with raised elevation, regular margin and smooth surface. The colonies from the strain 9 have a light pink colour and are translucent. The form is circular with raised elevation, regular margin and rough surface. The colonies from the strain 11 have an intense yellow colour and are opaque. The form of the colonies is irregular with raised elevation, undulate margin and smooth surface. The colonies from the strain 12 have a brown colour and are translucent. The form of the colonies is circular with raised elevation, regular margin and smooth surface.

3.1.2 Colony PCR

A PCR colony was realised for each strains isolated. The region amplified by the primers used is the 16S ribosomal RNA (see Figure 7). The experimental PCR conditions and preparations are detailed in section 2.1.3. The final volume of the reaction is 50 µL. 2 µL of PCR products were charged on a 1% agarose gel and separated by electrophoresis. The Figure 11 and 12 show the results obtained.

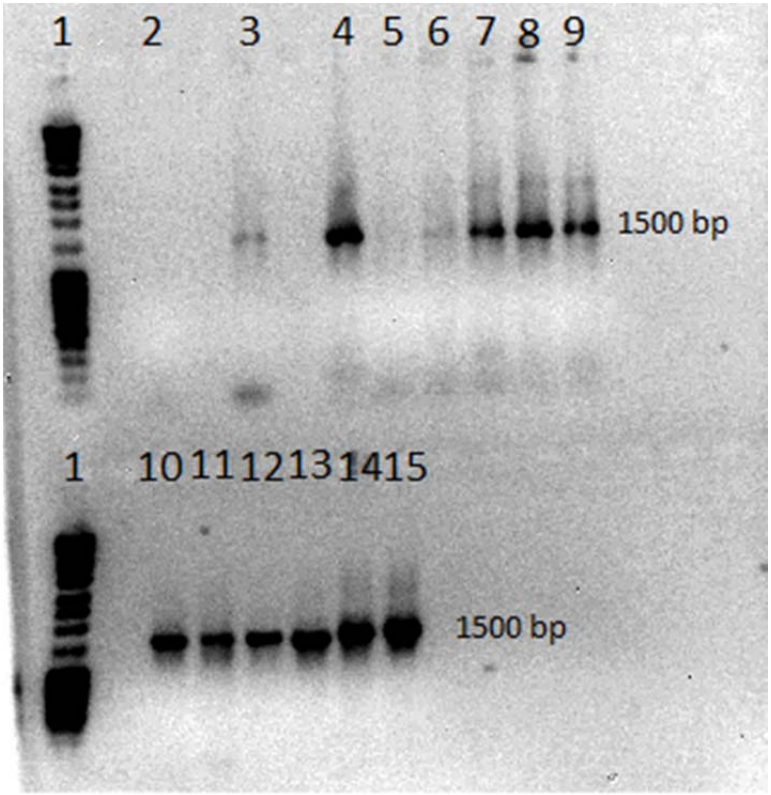


Figure 12: Electrophoretic analysis of colony PCR products (1%, 100V for 1h). 1) DNA ladder mix 2) Negative control 3) Positive control 4-15) Strains 1-12

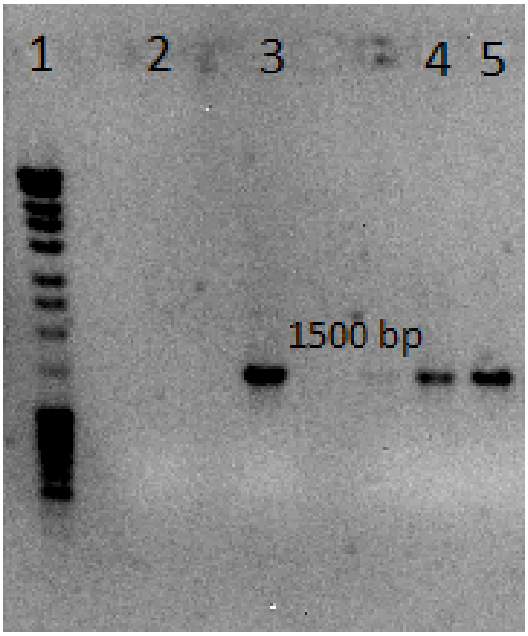


Figure 11: Electrophoretic analysis of colony PCR products (1%, 100V for 1h). 1) DNA ladder mix 2) Negative control 3) Positive control 4-5) Strains 2-3

The results obtained for the first amplification of the strain 2 and 3 (Figure 12) were not as expected. The amplification of these 2 strains was repeated. No band is visible in lane 2 (for the both gel used) and confirms that the MM and PCR water are not contaminated. The band at 1500 bp in lane 3 (for the both gel used) confirms that the PCR reaction worked fine and the functionality of the primers used (see section 2.1.3 for more details). The amplified DNA has a length of 1500 nucleotides which is confirmed by the gel electrophoresis.

3.1.3 Purification of amplified DNA

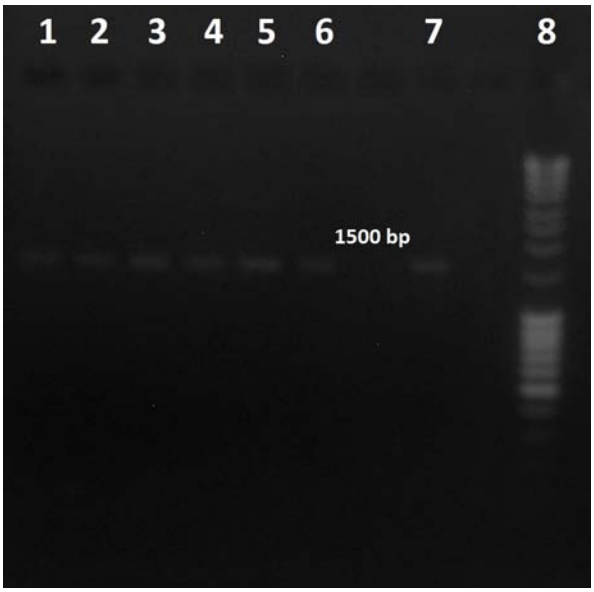


Figure 13: Electrophoretic analysis of colony PCR products after purification.
1-7) Strains 1-7 8) DNA ladder mix

After the PCR colony and before the sending of the samples for the sequencing, a purification step should be realised. This step was realised to eliminate all residues of the reaction and to obtain clear result during the sequencing. The final volumes were 50µL (two elution). 1 µL of PCR products were charged on a 1% agarose gel and separated by electrophoresis. The Figure 13 shows the result obtained after purification of PCR products of the strains 1-7.

The band of 1500 bp obtained in the lanes 1-7 is expected and confirms that the purification step worked well. The results obtained for the strains 8 to 12 were the same, unfortunatly the picture of the result was deleted. The concentration of purified products is almost 20 ng/µL, sufficient for the sequencing.

3.1.4 Sequencing and bioinformatics analysis

The amplified products obtained were sent, after the purification step, to Macrogen Company for the determination of the nucleotides sequence. Two chromatograms of each strain were obtained, one for the amplification with the forward primer (16S_27F) and one for the amplification with the reverse primer (16S_1492R). After the selection of the best amplified region (quality and separation of the pics) from each chromatogram, the forward and reverse sequences were aligned with each other in order to reconstruct almost the entire region of the 16S gene and thus to obtain clean sequences.

The entire sequence for each strain was analysed with the online software BLAST to find a correlation between the isolated strain and a microorganism already known (present on the BLAST database).

The phylogenetic tree of all isolated strains was realised with the software MEGA 7. The entire sequence of each strain and the 10 first complementary sequences found with blast were used to build the tree. If a complementary sequence appears more than one time, the double was deleted. The phylogenetic tree obtained is shown in the Figure 14.

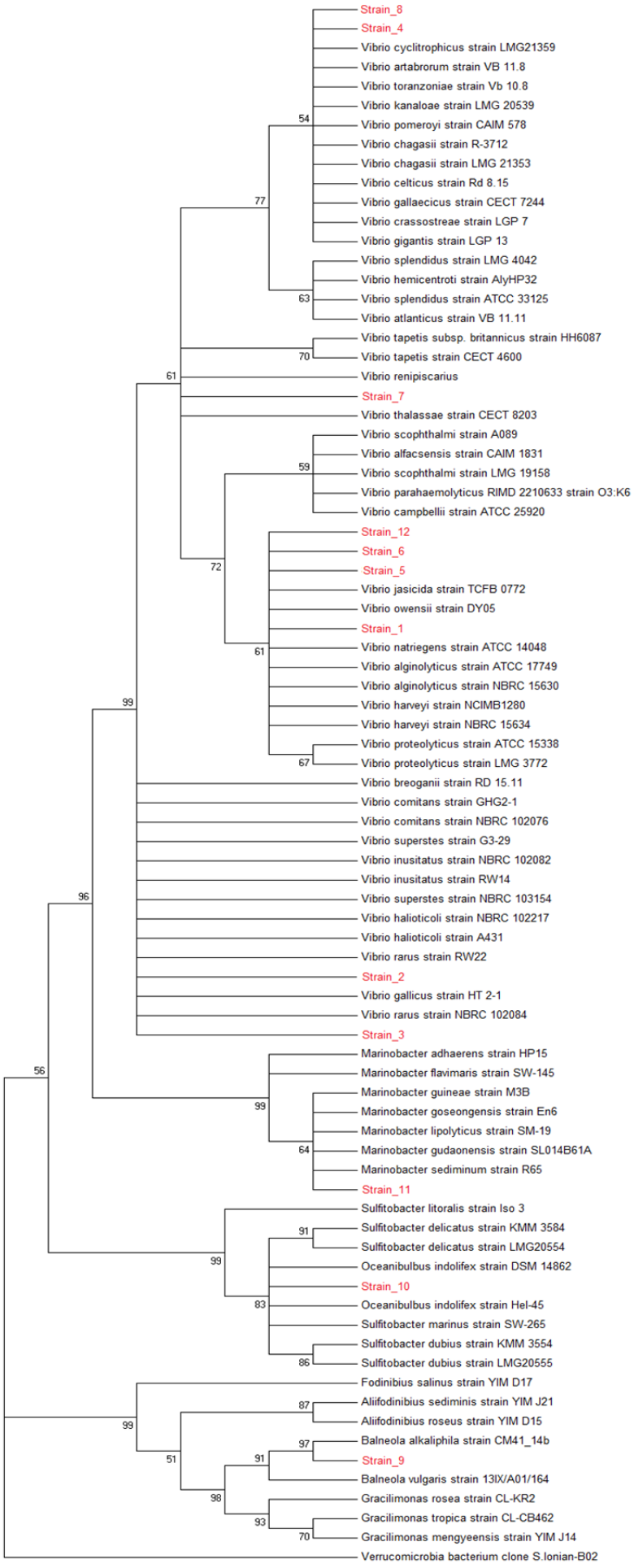


Figure 14: Phylogenetic tree of the isolated strains from the coelomic fluid of *P.lividus*.

The phylogenetic tree obtained for the 12 strains isolated shows that 3 different classes of bacteria were present in the coelomic fluid of *P.lividus*. The strain 1 to 8, 11 and 12 belong to the Gamma-Proteobacteria class. The strain 9 belongs to the Spingobacteria class and the strain 10 belongs to the Alpha-Proteobacteria class. The genera of the isolated strains are the following: *Vibrio, Marinobacter, Balneola* and *Oceanibulbus.* The only sequencing of the 16S rRNA region is not enough to determine precisely the species of the isolated strains.

The Figure 15 is a zoom focused on the genus *Marinobacter* (class: Proteobacteria). The isolated strain present in this group is the 11. The species of this strain is close to the species *M.sediminium* with 97% sequence similarity. *M.sediminium* is a marine Gram-negative, aerobic and heterotrophic bacterium. It requires sodium ions for the growth and it is an oxidase- and catalase-positive species. Cells are motile and rod-shaped. It was isolated in marine coastal sediments from Russia, Peter the Great Bay and Sea of Japan [72].

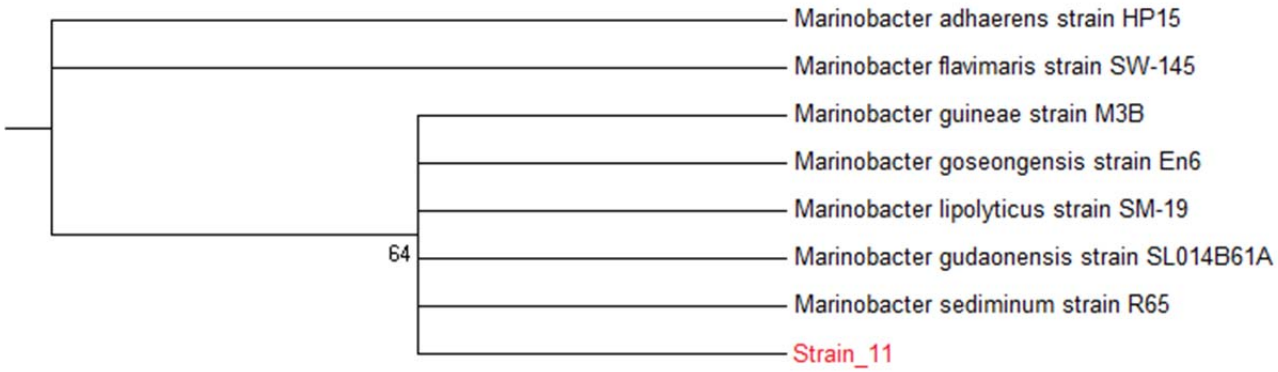


Figure 15: Phylogenetic tree zoomed to show the affiliation of the isolated strain 11 to the genus *Marinobacter.*

The Figure 16 is a zoom focused on the genus *Balneola* (class: Sphingobacteria). The isolated strain present in this group is the 9. The species of this strain is close to the species *B.alkaliphila* with 99% sequence similarity. *B.alkaliphila* is a marine Gram-negative, aerobic bacterium. It forms pale-orange colonies on MA and cells are non-motile rods. It was isolated from water column in the bay of Banyuls-sur-Mer [73].

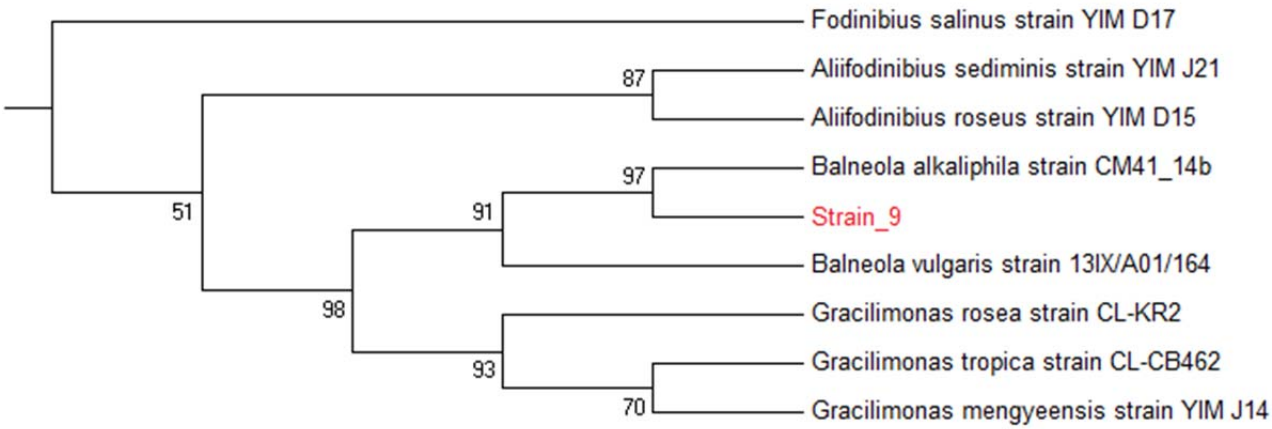


Figure 16: Phylogenetic tree zoomed to show the affiliation of the isolated strain 9 to the genus *Balneola.*

The Figure 17 is a zoom of the genus *Oceanibulbus* (class: Alphaproteobacteria). The isolated strain present in this group is the 10. The species of this strain is close to the species *O.indolifex* with 99% sequence similarity. *O.indolifex* is a marine Gram-negative, strictly aerobic and non-fermentative bacterium. It requires sodium and sea salts for growth. Cells are non-motile irregular rods. It was isolated in the North Sea water from a depth of 10 m [74].

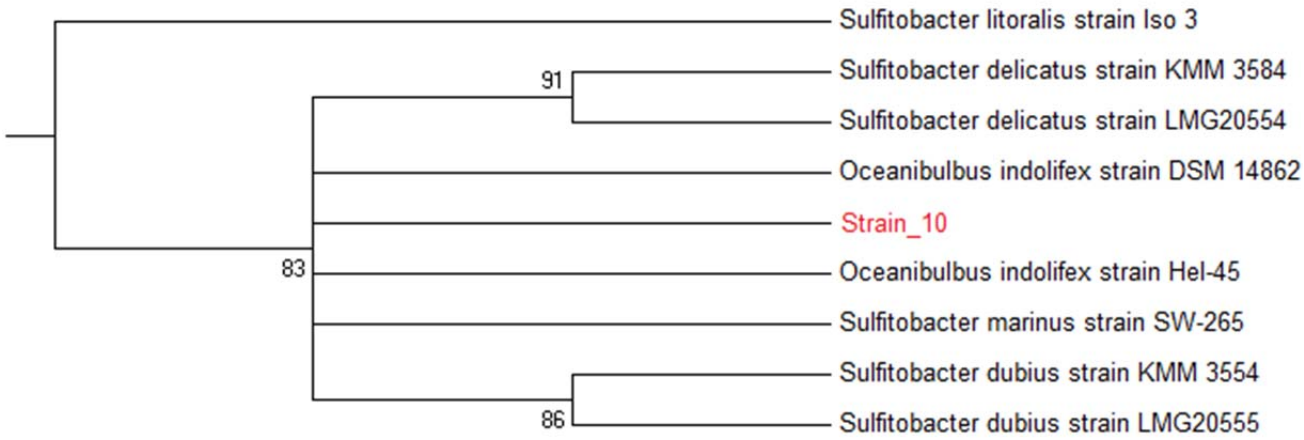


Figure 17: Phylogenetic tree zoomed to show the affiliation of the isolated strain 10 to the genus *Oceanibulbus.*

The Figure 18 is a zoom focused on the genus *Vibrio* (class: Gammaproteobacteria). The isolated strains present in this group are the 1 to 8 and the 12. *Vibrio* genus is one of the most abundant cultivable microorganisms from sea environment [75]. The specie of the strains 4 and 8 is close to *V.cyclitrophicus* with 99% sequence similarity. *V.cyclitrophicus* is a marine Gram-negative, anaerobic facultative and motile bacterium and need a defined media using ammonium salts as the sole nitrogen source. This specie was isolated from eagle harbour in Puget Sound, Washington [76]. The specie of the strain 7 is closed to *V.renipiscarius* with 99% sequence similarity. *V.renipiscarius* is a marine Gram-negative, anaerobic facultative and motile bacterium. Cells are coccoid to rod-shaped. This specie is able to ferment the glucose without gas production and reduce nitrate to nitrite. It was isolated from cultured gilthead sea bream (*S. aurata)* [77]. The harveyi clade contains the stain 1, 5, 6 and 12. The species of the strain 5, 6 and 12 are close to *V.jasicida* with 99% sequence similarity. *V.jasicida* is a marine Gram-negative bacterium, forms normally no pigmented colonies in MA, and it is responsible of different diseases on sea animals (vibriosis); it was isolated from packhorse lobster phyllosoma with luminous vibriosis [78-79]. The strain 12 presented a brown pigmentation on MA, as *V.cholera* and *V.anguillarium* [80-81]. However these two species were not proposed for a good correlation after the blast analysis. The specie of the strain 1 is close to *V.owensii* with 99% sequence similarity. *V.owensii* is a marine Gram-negative, anaerobic facultative and motile bacterium. Cells are slightly curved rod (= *Vibrio* phenotype) [82]. The species of the strain 2 and 3 are close to *V.rarus* with 99% sequence similarity. *V.rarus* is a marine Gram-negative, anaerobic facultative, chemo-organotroph and non-motile bacterium. It was isolated from the gut of the Californian red abalone (*H. rufescens)* [83].

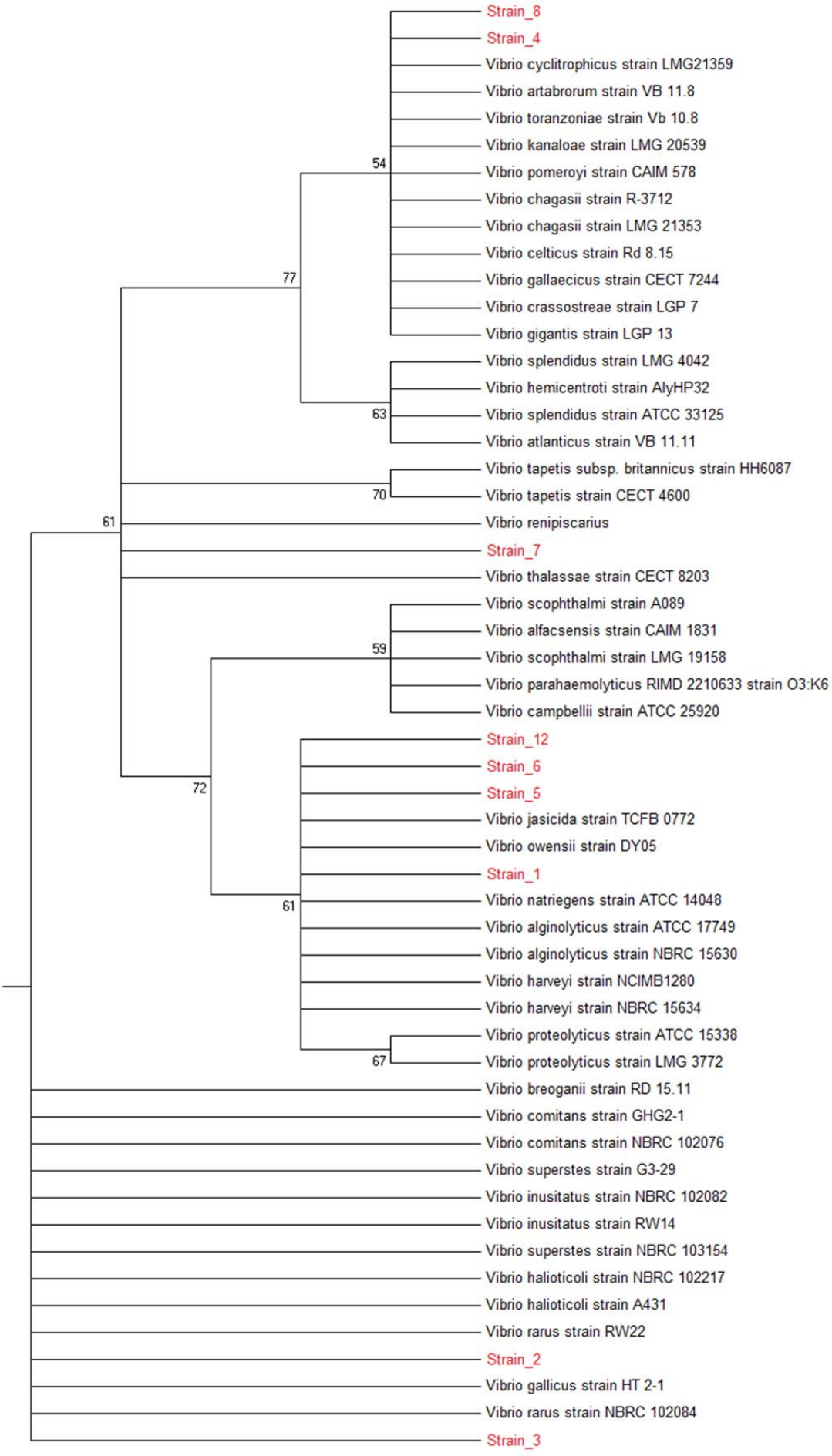


Figure 18: Phylogenetic tree zoomed to show the affiliation of the isolated strains 1-8 and 12 to the genus *Vibrio*.

3.1.5 Zymographic analysis

To evaluate the activity of extracellular proteases produced by the isolated strains, 5 µL of SGS of each isolated strain (without strain 9 because it was not cultivable in liquid culture, no growth was visible after 5 days of incubation at 30°C and 200 rpm) were added to 5 mL of MB and incubated overnight at 30°C. The cultures were then centrifuged at 1800 G for 20 min at 4°C and the supernatant was recovered. The gel used contains as substrate for the proteases a concentration of 0.54 g/L of gelatine. 15 µL of a mix supernatant:loading buffer (1:1) and 8µL of ladder were charged per well for each gel. The duration of the migration was 1h30min.

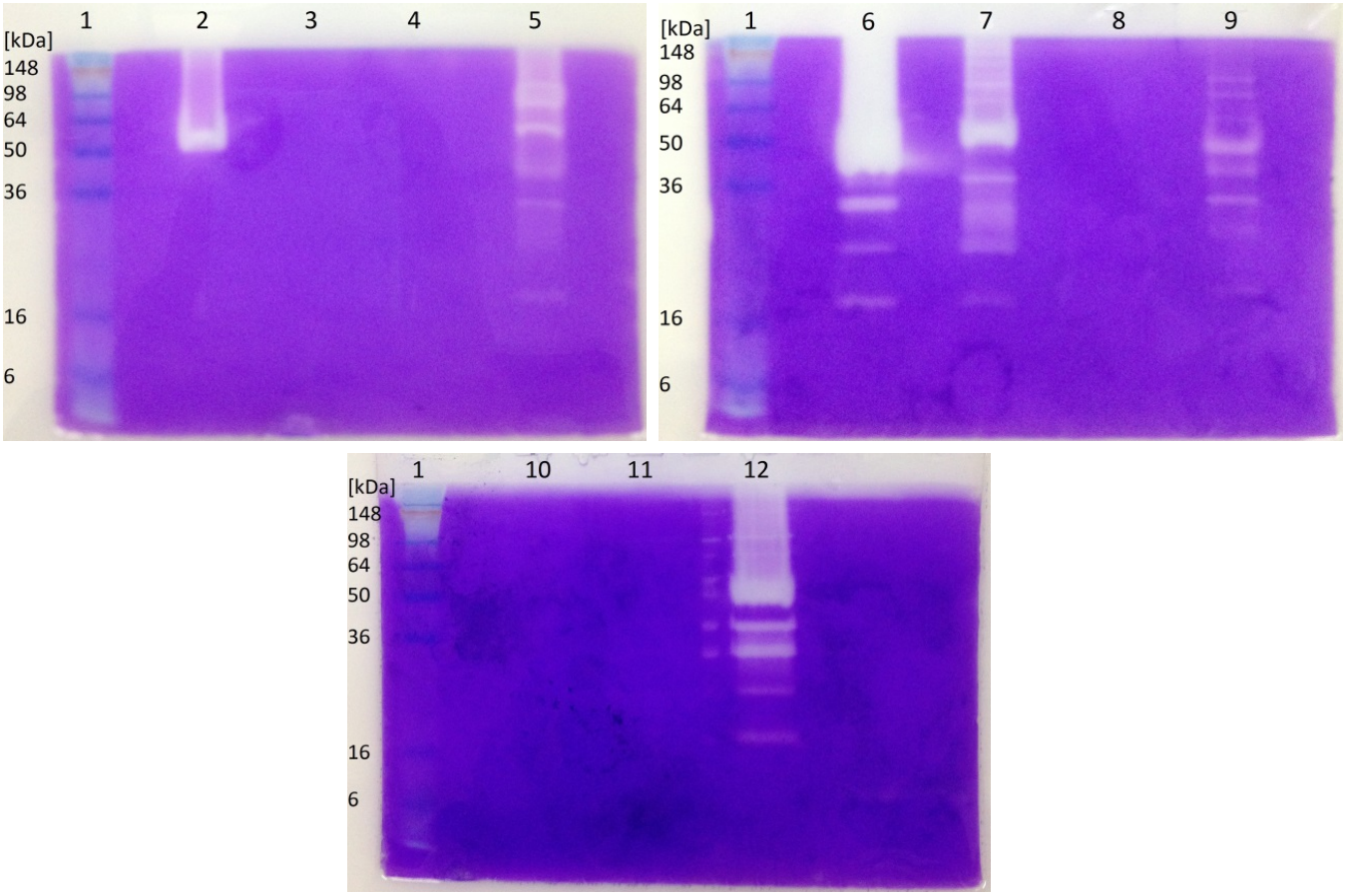


Figure 19: Zymographic analysis of the isolated strains. Migration parameters: 100 W, 160 V and 15/25 mA
1) SeeBlue ladder 2-9) Strains 1-8 10-12) Strains 10-12

The Figure 19 shows the results obtained for the protease assay. Some bands are visible in lanes 2, 5, 6, 7, 9 and 12 which mean that the spent medium of these strains contains proteases and these enzymes are actives at room temperature. The spent medium of the sample 3, 4, 8, 10 and 11 does not contain proteases; no band is visible in these lanes. This analysis is qualitative and the ladder is used as indication. It is not possible to determine precisely which kinds of enzymes are present with this assay.

3.1.6 Native SDS-Page

To evaluate the production of extracellular enzymes by the isolated strains, 5 µL of SGS of each isolated strain (without strain 9, same reason as section 3.1.5) were added in 5 mL of MB and incubated overnight at 30°C. The cultures were then centrifuged at 1800 G during 20 min at 4°C and 2 mL of the supernatant was lyophilised and resuspended in 150 µL of demineralised water (concentration 13.3x). 15 µL of a mix supernatant concentrated:loading buffer (1:1) and 8µL of ladder were charged per well for each gel. The duration of the migration was 2 hours.

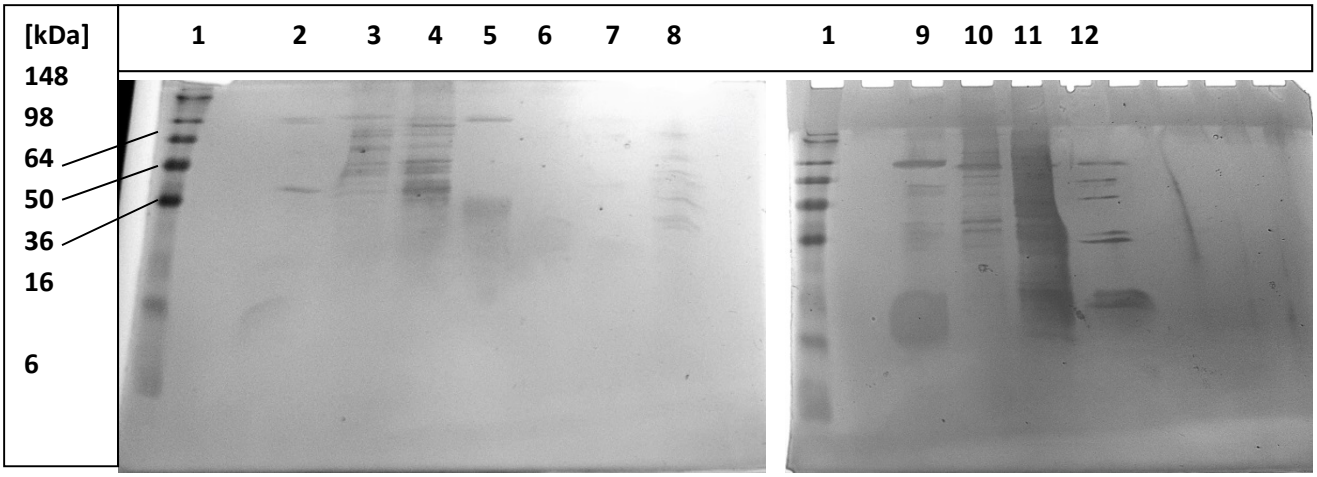


Figure 20: Native SDS-Page of the isolated strains. Migration parameters: 100 W, 160 V and 15/25 mA
1) SeeBlue ladder 2-9) Strains 1-8 10-12) Strains 10-12

The Figure 20 shows the results obtained for the native SDS-Page analysis. Some bands are visible in the lane of all isolated strains (difficult to see on the Figure 20). The spent medium of all isolated strains (without strain 9, same reason as section 3.1.5) contains extracellular proteins after 24 hours of culture in MB at 30°C. This analysis is qualitative and the ladder is used as indication. It is not possible to determine precisely which kinds of proteins are present with this assay. However, the experiment to identify by mass spectrometry the kinds of protein produced is in progress (in Naples).

3.1.7 Assay of cellulase activity

To evaluate the activity of extracellular cellulases produced by the isolated strains, 5 µL of SGS of each isolated strain (without strain 9, same reason as section 3.1.5) were added in 5 mL of MB and incubated overnight at 30°C. The cultures were then centrifuged at 1800 G for 20 min at 4°C and 2 mL of the supernatant was lyophilised and resuspended in 150 µL of demineralised water (concentration 13.3x). The gel used contains as substrate for the cellulases a concentration of 0.2% (w/v) of CMC. 15 µL of a mix supernatant concentrated:loading buffer (1:1) and 8µL of ladder were charged per well for each gel. The positive control used is a cellulase produce by a strain of *A.niger*. The duration of the migration was 1h30min.

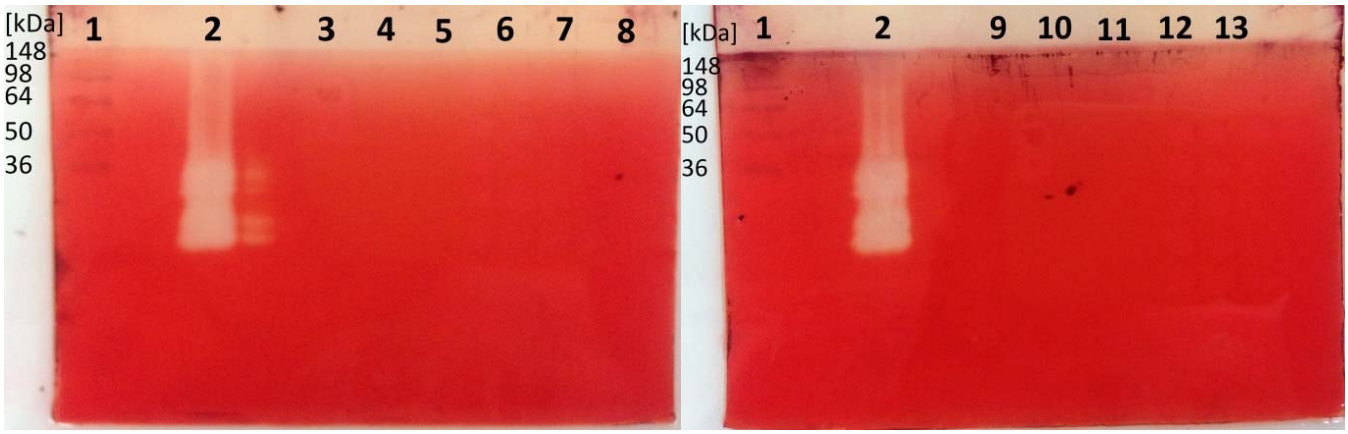


Figure 21: Cellulase assay. Migration parameters: 100 W, 160 V and 15/25 mA.
1) SeeBlue ladder 2) Positive control 3-10) Strains 1-8 11-13) Strains 10-12

The Figure 21 shows the results obtain for the cellulase assay. The bands visible on the lane 2 are expected and confirm that the test work well for a qualitative detection of cellulase secreted. No band is visible on the lane of each sample, the spent medium of isolated strains don't contain active cellulase at RT.

3.1.8 Assay of esterase activity

To evaluate the activity of extracellular esterases produced by the isolated strains, 5 µL of SGS of each isolated strain (without strain 9, same reason as 3.1.5) were added in 5 mL of MB and incubated overnight at 30°C. The cultures were then centrifuged at 1800 G for 20 min at 4°C and 15 µL of the supernatant was plated as a drop on agar plates containing ester myristate. The plates were incubated at 37°C overnight.

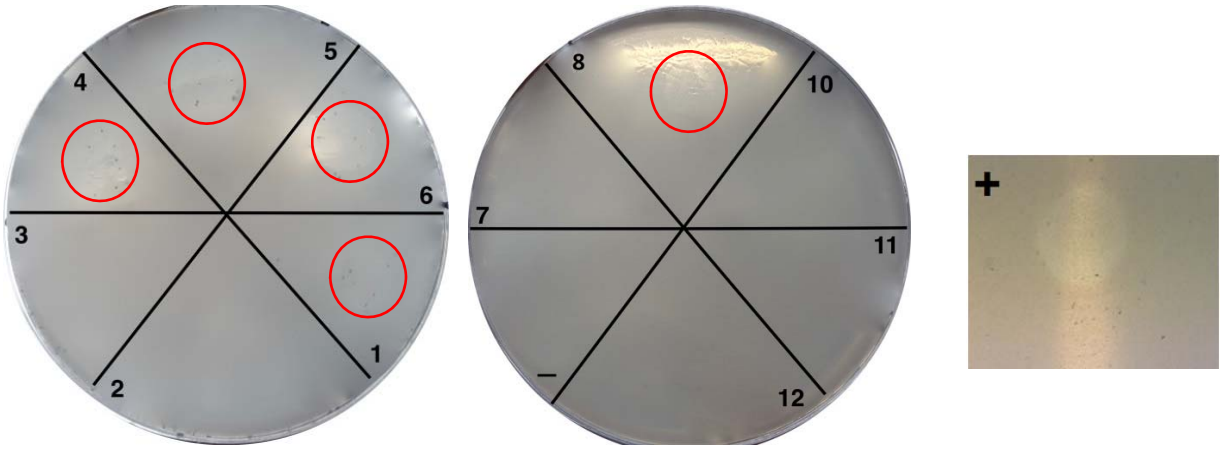


Figure 22: Esterases assay on agar plate containing ester myristate after an overnight incubation at 37°C.
1-12) Strains 1-12 -) Negative control +) Zoom on the strain 1

The Figure 22 shows the results obtained for the esterases assay. A clear halo has been observed for the isolated strains 1, 4, 5, 6 and 8 which mean that the spent medium of these bacterial strains contain esterases and these enzymes are active at 37°C. The red circle is used to show the positive result because it is difficult to see them directly from the pictures. The picture + is a zoom on the strain 1, a clear halo is actually visible. The spent medium of the strains 2, 3, 7, 10, 11 and 12 does not contain any esterases, no clear halo is visible in the delimited space. This analysis is qualitative; it is not possible to determine precisely which kinds of enzymes are present with this assay.

3.1.9 Assay of lipases activity

To evaluate the activity of lipases produced by the isolated strains, 5 µL of SGS of each strain were plated on MA containing rhodamine-B and olive oil (substrate for lipases) and incubated 10 days at 30°C. The lecture of the results was realised under UV lamp.

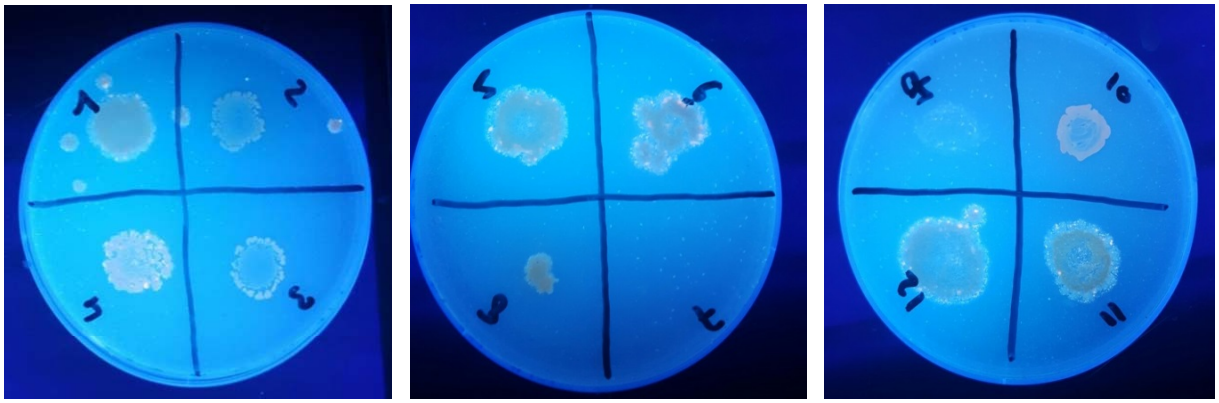


Figure 23: Lipases assay on MA containing rhodamine-B and olive oil after 10 days of incubation at 30°C. 1-12) Strains 1-12

The Figure 23 shows the results obtained for the lipase assay. An orange-pink fluorescent is visible for the delimited spaces (formed by the colonies) 1, 4, 5, 6, 11 and 12 which mean that these bacterial strains produce lipase and these enzymes are actives at 30°C. The bacterial culture of the sample 2, 3, 8, 9 and 10 don't contain lipase; no orange-pink fluorescent is visible in the delimited space. For the strain 7, no growth is visible. This strain is probably lipid intolerant. This analysis is qualitative; it is not possible to determine precisely which kinds of enzymes are present with this assay.

3.1.10 Antimicrobial assay

To determine if the isolated strains produce antibacterial compounds, two assays were realised in soft agar: the first one with a strain of *Escherichia coli* (Gram-negative bacteria) and the second one with a strain of *Kocuria rhizophila* (Gram-positive bacteria). A plug of 2 cm of diameter from the culture of each isolated strain was placed on the soft agar containing the tester strains and the plates were incubated overnight at 37°C.

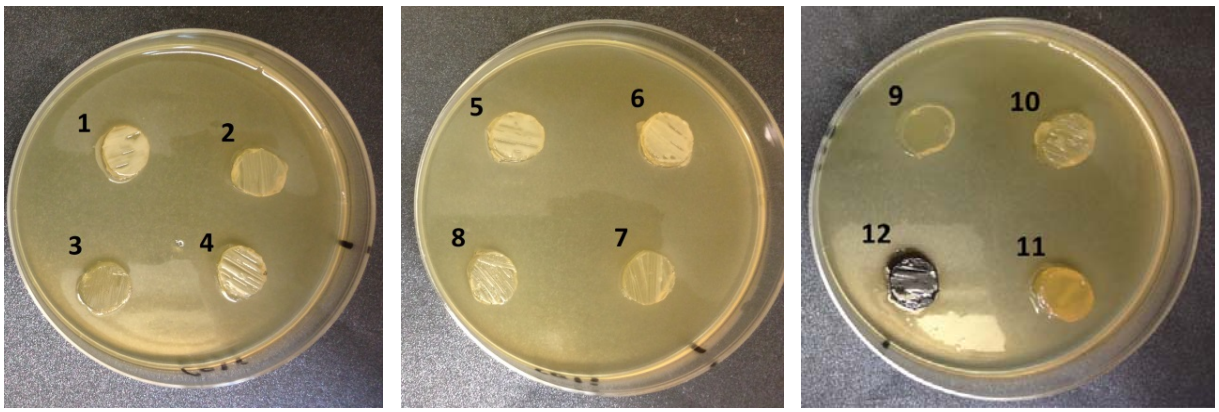


Figure 24: Antimicrobial assay with a Gram-negative strain (*E.coli*) in soft agar. Incubation overnight at 37°C. 1-12) Strains 1-12

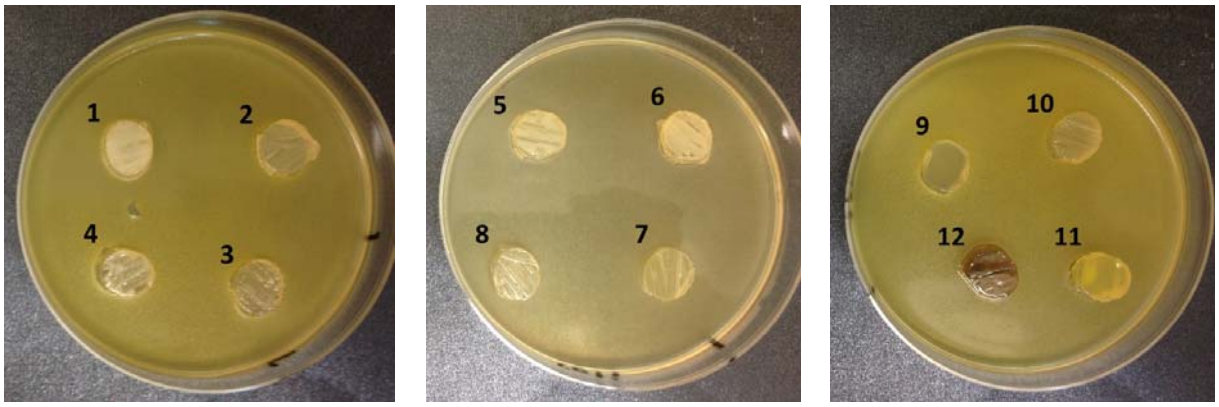


Figure 25: Antimicrobial assay with a Gram-positive strain (*K.rhizophila*) in soft agar. Incubation overnight at 37°C. 1-12) Strains 1-12

The Figure 24 and 25 show the results obtained for these two assays. The bacterial strains *E.coli* and *K.rhizophila* have been chose because these two strains are commonly used for antimicrobial assays. The isolated strains don't produce any antibacterial compound against Gram-negative and Gram-positive bacteria; no clear halos are visible around the culture circle of each strain.

3.1.11 Summary of the results

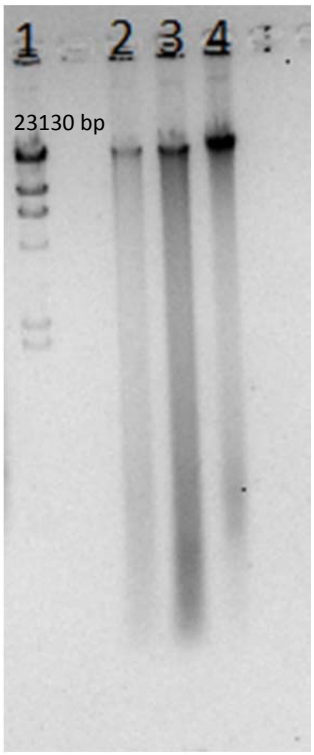
The table 9 provides an overview of the results obtained with the culture dependent method. For more details see the correspondent section.

Table 9: Summary of the results obtained with the culture dependent method.

Identification (sect. 3.1.1 and 3.1.4)		Extracellular enzymes (sect. 3.1.5-9)				Antimicrobial (sect. 3.1.9)	
Strains	**Closed to**	**Proteases**	**Cellulases**	**Esterases**	**Lipases**	***E.coli***	***K.rhizophila***
1	*V.owensii*	+		+	+		
2	*V.rarus*	-		-	-		
3	*V.rarus*	-		-	-		
4	*V.cyclitrophicus*	+		+	+		
5	*V.jasicida*	+	-	+	+		
6	*V.jasicida*	+		+	+	-	-
7	*V.renipiscarius*	-		-	NA		
8	*V.cyclitrophicus*	+		+	-		
9	*B.alkaliphila*	NA	NA	NA	-		
10	*O.indolifex*	-		-	-		
11	*M.sediminum*	-	-	-	+		
12	*V.jasicida*	+		-	+		

3.2 Independent culture method

3.2.1 DNA extraction



After the harvesting of the coelomic fluid from 3 different *P.lividus*, the DNA present in each sample was extracted following the method in section 2.2.1. 10 µL of sample were charged in a 1% agarose gel and separated by electrophoresis. The Figure 26 shows the results obtained.

The band at 23 kb in lanes 2, 3 and 4 represents the total DNA and confirms that the protocol for extraction worked fine. The extract DNA has been quantified following the method in section 2.2.2. The results obtained are the following:

Table 10: Quantification of the DNA extracted.

N° Extraction	DNA conc. [ng/µL]
1	13.7
2	25.8
3	29.5

The ratio 260/280 and 260/230 were as expected and confirm that the DNA samples were not contaminated by proteins, carbohydrates and phenol.

Figure 26: Electrophoretic analysis of the DNA extracted from the coelomic fluid of *P.lividus* (1%, 100V for 60 min). 1) Marker 2 in bp 2-4) Extraction 1-3

3.2.2 Amplification of the 16S rRNA gene

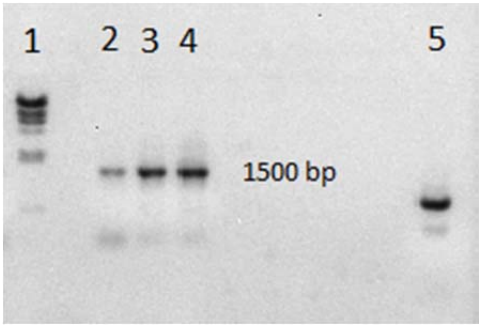


Figure 27: Electrophoresis analysis of the amplified products of extraction (1%, 100V for 1h). 1) DNA ladder 2-4) Extraction 1-3 5) Positive control

To confirm the presence of bacterial DNA, a PCR reaction was realised with the product of extraction. The region amplified by the primers used is the 16S ribosomal RNA. The experimental PCR conditions and preparations are detailed in section 2.2.3. The final volume of the reaction is 30 µL. 1 µL of PCR products were charged on a 1% agarose gel and separated by electrophoresis. The Figure 27 shows the results obtained.

The band at 1500 bp in lane 5 confirms that the PCR reaction worked fine and the functionality of the primers used (16S_27F and 16S_1492R). This band is more below in the lane than the other because the positive control was charged before the sample to test the viability of the loading buffer. The amplified 16S DNA has a length of 1500 nucleotides which is confirmed by the electrophoresis gel.

3.2.3 Sequencing by minION™ technology

The results of DNA sequencing obtained in the laboratory of Dr Tripodo, Polyclinic of Palermo, for one DNA sample are reported in Figure 28 (the sequencing of others samples is in progress). The Figure 28 shows the phylogenetic tree constructed online by the minION™ system and the abundance relative of each strain present. The reading of the tree was stopped at the genus level.

Figure 28: Phylogenetic tree and relative abundance of each strain obtained after the sequencing, by minION™ technologies.

Two different genus of yeast were found, the genus *Aspergillus* and *Nauvomozyma* (both belonging to Ascomycota). The bacterial strains represented, at the genus level, are the following: *Lactococcus* and *Clostridium* (both belonging to Firmicutes); *Phytoplasma* and *Mycoplasma* (both belonging to Tenericutes); *Corynebacterium* (belonging to Actinobacteria); *Vibrio, Escherichia, Advenella* and *Sorangium* (all belonging to Proteobacteria); *Prochlorococcus* (belonging to Cyanobacteria); *Mesotoga* (belonging to Thermotogae); *Thermodesulfatator* (belonging to Thermodesulfobacteria); *Melioribacter* (belonging to Ignavibacteria); *Ilyobacter* (belonging Fusobacteria). And two different genus of Archaea were found, *Methanocella* and *Methanococcus* (both belonging to Euryarchaeota). However, the sequencing of more than one sample is necessary to identify the most represented genera in the coelomic fluid of *P.lividus*.

4 Discussion and conclusion

The quantity of coelomic fluid inside a healthy sea urchin adult is about 8-12 mL and corresponds to more than 70% of the internal space of the test, which appears to be involved in the defence of the internal organs [84]. To live and survive, the sea urchin has to be able to defend itself against the external predators and also against pathogen microorganisms present in the marine environment that can enter inside the test cavity [85-86]. In this case, this invertebrate has developed defence responses based primarily on immune cells and humoral factors present in the coelomic fluid [83]. The presence of five different types of cells in the coelomic fluid, classified as amebocytes, haemocytes, spherical cells, progenitor cells and crystal cells, and responsible for a wide range of functions such as cell recognition, phagocytosis, cytotoxicity, antibacterial activity and inflammatory reactions made coelomic fluid is considered sterile [60 and 87-90].

With this work, it was confirmed [61] that the coelomic fluid of the sea urchin species belonging to the Mediterranean *Paracentrotus lividus*, has its bacterial microbiota and that microbiota produces some extracellular enzymes as protease, lipases and esterases. No active cellulase and antimicrobial substance have been found from these isolates.

The presence of bacteria in coelomic fluid was demonstrated with two different approaches: culture-dependent and culture-independent method. The first approach has allowed the identification of species closely related to the genus: *Vibrio*, *Marinobacter, Balneola* and *Oceanibulbus*. Among these, 9 isolated strains are from *Vibrio* genus and one strain represents each other genus. The bacterial phyla identified by this approach are *Proteobacteria* and *Bacteriodetes*. These two phyla were also isolated from the coelomic fluid of *P.lividus* collected in a different geographic area, during the first study [61]. The culture-independent approach has partially confirmed the results of the culture-dependent approach. Indeed, only the *Vibrio* genus was found equally present in the metagenomics sample sequenced. However, just one of the metagenomics DNA samples was sequenced and the culture dependent method was realised on MA. In addition, as only 1- 1.5% of the bacteria are cultivable, the culture-independent approach provides knowledge on microorganisms that cannot grow in the laboratory, but that may be present in major proportion in the sample analysed.

The independent culture analysis has also identified bacterial phyla not isolated by the culture-dependent method, as *Firmicutes*, *Tenericutes*, *Actinobacteria*, *Cyanobacteria*, *Thermosulfobacteria*, *Ignavibacteria* and *Fusobacteria*. One phylum of Archaea, *Euryarchaeota* has been sequenced. The genera isolated from this phylum (i.e. *Methanococcus*) are known to be present in polluted water as mudflat [91]. Two different genera of yeast (belonging to *Ascomycota*) were also found with this second method. Nevertheless, this strain derives probably from the environmental pollution because these strains are known to be ubiquitous [92].

Preliminary data have shown that the microbiota isolated from the coelomic fluid of *P.lividus* don't produce antimicrobial substance against Gram-negative and Gram-positive strains as *E.coli* or *K.rhizophila*. However, it can be interesting in the future to realise this assay with some marine bacterial strains to determine if specific protein are produce to inhibit these strains or to improve the condition of growth as symbiosis model.

The assays realised in this study to characterise the extracellular products of the isolated strains were qualitative. The results obtained show that different enzymes are secreted and active at room temperature. A more precise characterisation using specific substrates, inhibitors, different temperatures and a mass spectrometric analysis has to be realised to identify which kinds of enzyme are produced and determine if these products that could be exploited in biotechnological process.

In addition, studies on interactions between microorganism and marine organisms are providing additional tools to understand phenomena of symbiosis, development and morphogenesis. It has been shown that some strains belonging to the genus *Pseudoalteromonas*, promote the larval settlement of a species of sea urchin, *Heliocidaris erythrogramma* [93]. This specie has a direct development, from the larva formed to the adult forms, instead of a first passing through an intermediate stage, as for *P.lividus*. Then it would be interesting to determine which role has the bacterial microbiota contained in the coelomic fluid for the development and morphogenesis of *Paracentrotus lividus* larvae and investigate which kind of bacterial strains are present during the first hours of the fecundation of the eggs.

The sea urchin is an organism used in many scientific and economical fields as marine ecology, fishery and molecular embryology. That is why, generate detailed information on this issue has a big interest. In conclusion, the results obtained show that the coelomic fluid of *P.lividus* contains its own microbiota and can be helpful to understand the biological functions of this fluid.

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