# FINAL REPORT

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| **Program:** | **Mobilità Di Breve Durata – STM (Short Term Mobility) Bando 2015** |
| **Project title:** | **Bioprospecting by halophilic microorganisms: toward discovering new antimicrobial compounds** |
| **Instrument:** | **Quality of Life and Management of Marine Resources** |
| **Priority:** | **Identification and Sustainable Use of Metabolic and Genetic Diversity as a Source of New Valuable Products.** |

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| **Start date of project:** | **11/05/2015** |
| **End date of project:** | **22/05/2015** |
| **Duration:** | **10 days** |

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## SUMMARY

In this research project a large set of microorganisms isolated from Antarctic sediments and Salterns of Isla Cristina in Spain was screened for the production of bioactive compounds able to inhibit the formation of biofilm of human opportunistic pathogenic bacteria multi-drug resistant (MDR) such as *Staphylococcus aureus* and *Pseudomonas aeruginosa.*

New media cultures were specifically designed to obtain the vast majority of uncultured microorganisms based on special nutrient requirements. The obtained strains were able to grow on different media cultures that go from 1 to 10% of salinity and at two temperature conditions. The experiments were carried out on a selection of 576 strains based on phenotypic features of the colonies.

Besides of this, the experiment was extended to evaluate the antimicrobial potential to pathogenic bacteria MDR using the same isolates, but was included 3 pathogens more as target. The anti-biofilm assay showed that a total of 14 strains posses *a priori* activity against *Staphylococcus aureus* while 187 strains showed activity against *Pseudomonas aeruginosa.* The antimicrobial activity to pathogenic bacteria MDR showed that 10 strains formed an inhibition halo against pathogens target, this evidenced the inhibitory activity. The results, previously obtained, serve as a good predictor to perform a second screening for anti-biofilm and antimicrobial potential on selected strains. An in-depth study will be carried out in the future, taking into account these data as a biological tool. The purification of the positive strains is ongoing.

# 1. PROJECT DESCRIPTION

Pathogenic bacteria MDR are considered as a major challenge for modern medicine. These microorganisms are responsible for infections, which are difficult to eradicate especially in hospitals and from cystic fibrosis patients. For several years, it is known that both the ability to resist to antibiotics, and the virulence mechanisms are closely related to the production capacity of the biofilm, and then the search for new anti-biofilm molecules has acquired a primary importance. This project aims at the exploitation of new molecules anti-biofilm from halophilic bacteria, isolated in different areas of the world, which proliferate in high salinity conditions. These bacteria, adapted to live in extreme conditions, have developed different coping mechanisms, including the production of secondary metabolites with antimicrobial activity and are considered one of the most promising sources of biomolecules to meet the challenge of multi drug resistant pathogen.

# 2. MATERIAL AND METHODS

## 2.1. Biological material

**Table 1.** Strains from culture collection used

| ANTI-BIOFILM ACTIVITY | ANTIMICROBIAL ACTIVITY |
|---|---|
| *Staphylococcus aureus* | *Staphylococcus aureus* |
| *Pseudomonas aeruginosa* | *Pseudomonas aeruginosa* |
| | *Klebsiella pneumoniae* |
| | *Acinetobacter baumannii* |
| | *Burkholderia cenocepacia* |
| | *Francisella tularensis* |

## 2.2. Environment samples

Antarctic subsea sediments and soils, used in this work, were collected from the deep sea from a sampling expedition of the Italian National Program for Antarctic Research. Samples were kept at -80ºC in sterile falcon tubes. Water samples and

sediments from Salterns of Isla Cristina in Spain were collected in a sampling done on July 2014 under a framework project funded by Spanish Government (National Plan Research and Develop).

### 2.3. **Media Cultures**

The anti-biofilm and antimicrobial assays were achieved using colonies isolated from specific media culture previously designed especially performing for the isolation of different groups of psychrophilic and halophilic microorganisms present in natural environments. This kind of microorganisms not yet culturable can require special conditions or minimum nutrient requirements.

**Luria Bertani (LB) broth and agar.** This medium was used for the growth of pathogens.

**Table 2.** Composition of LB

| Composition | g/l |
|---|---|
| Tryptone | 10 g |
| Yeast extract | 5.0 g |
| NaCl | 5.0 g |
| Distilled water up to | 1000 ml |

Adjusted to pH 7.0 with 1 M NaOH.

**Artificial Seawater (SW)**

In order to increase and recover the vast majority of microorganisms, specific media cultures were designed using artificial seawater (SW) and seawater from the Stazione Zoologica (SZN) of Naples.

Different nutritional approaches were considered to promote the growing of new microorganisms with special metabolic requirements.

Concerning the isolation of microorganism we have tested three different culture media: SW minimum medium to 8 different salt concentration (Table3) and SWSZN medium enriched and SWSZN supplemented with tryptone and glycerol (Table 4).

**Table 3.** Composition of artificial seawater 30% (SW30) according to Subov 1931.

| Subov's salts solution 30% | |
|---|---|
| **Composition** | **g/l** |
| NaCl (Panreac) | 234.0 g |
| $MgCl_2 \cdot 6H_2O$ | 39.0 g |
| $MgSO_4 \cdot 7H_2O$ | 61.0 g |
| *$CaCl_2$ | 1.0 g |
| KCl | 6.0 g |
| $NaHCO_3$ | 0.2 g |
| NaBr | 0.7 g |
| Distilled water up to | 1000 ml |

* $CaCl_2$ were dissolved separately in small amount of distilled water (20 ml). This is for avoid the formation of insoluble complexes of $CaCO_3$. Add to the solution when all components are well mixed.

**Medium artificial seawater minimum (SW minimum)**

These media were prepared dissolving all components in different volumes of artificial seawater SW30 (Subov's salts solution 30%) according the concentrations of salinity required for the culture for halophilic microorganisms.

**Table 4.** Media used for the isolation of moderately halophilic and/or psychrophilic microorganisms in different salt concentrations

| COMPOSITION | g/l | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Casaminoacids | 0,5 | | | | | | | |
| Yeast extract | 1.0 | | | | | | | |
| Sodium pyruvate | 0.1 | | | | | | | |
| Peptone | 1.0 | | | | | | | |
| Agar powder (solid media) | 20.0 | | | | | | | |
| **Salinity** | **0%** | **1%** | **3%** | **5%** | **10%** | **15%** | **20%** | **25%** |
| **Add SW30 (ml)** | - | 33.3 | 100 | 166.6 | 333.3 | 500 | 666.6 | 833.3 |
| Distilled water up to | 1000 ml | | | | | | | |

All media were prepared using deionised distilled water ($dH_2O$) and sterilised by autoclaving at 121 °C for 15 min.

**Media prepared with natural seawater (SWSZN)**

**SWSZN enriched:** Used a variety of nutrients but less than 1% for stimulate de production of secondary metabolites.

**SWSZN Tryptone/Glycerol**: Is a medium with amino acids as nitrogen source and low carbon amounts.

**Table 5.** Media used for the isolation of moderate halophilic bacteria (in g/liter)

| Composition | SWSZN enriched (g/l) | SWSZN Tryptone/Glycerol (g/l) |
|---|---|---|
| Casaminoacids | 1.0 g | - |
| Yeast extract | 1.0 g | - |
| Glucose | 1.0 g | - |
| Peptone | 1.0 g | - |
| Tryptone | - | 1.0 g |
| Glycerol | - | 0.5 g |
| Agar powder (solid media) | - | 20.0 g |
| Seawater (SZN) up to | 1000 ml | |

Dissolved this components in 1L of sea natural water from Stazione Zoologica Napoli (SZN) and sterilize by autoclaving.

In this case we did not adjust the pH, it was considered the natural pH from the original seawater that corresponds to pH 7.7.

### 2.4. Cultivation

**Pathogens**. Different strains tested were grown overnight at 37$^{o}$C in Luria Bertani (LB) broth and agar.

**Sediment treatment.** About 1g sediment was resuspended in 9 ml of SZN water. Serially dilutions up to $10^{-5}$ were prepared.

**Isolation of psychrophilic/halophilic bacteria.** Aliquots of 100 ul of serial sample dilutions up to $10^{-5}$ were plated onto agar plates with different medium designed.

**Growing conditions.** Plates were incubated at two different temperatures: 4ºC and 15ºC for 20 days. Plates were periodically checked for the appearance of new colonies.

* After this incubation period was possible to obtain the biologic material to perform the antibiofilm and antimicrobial assay.

**Colonies selection.** Single colonies were carefully picked and placed into microtiter wells, which contain the same medium where the strains were obtained and incubated under the appropriate conditions.

### 2.5. **Maintenance of strains**

Isolated colonies were maintained in microtiter plates by adding sterile glycerol as cryoprotectant to the wells to a final concentration of 20 % (v/v).

Adhesive films were secured on top of the plates for freezing at -80ºC for long-term storage until requested.

Agar plates were stored at 4ºC in sealed bags to prevent them drying out and the salts from crystallising.

### 2.6. **Anti-biofilm assay**

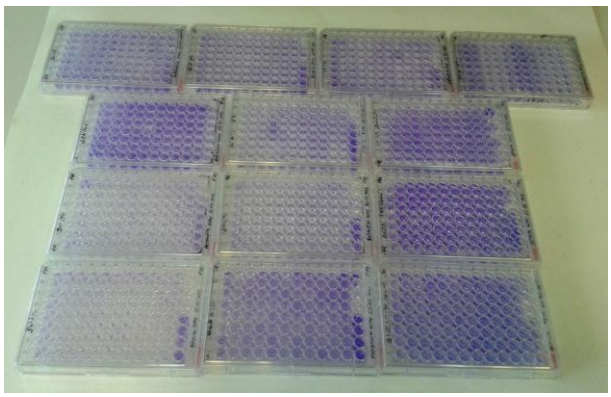

**Figure 2.** Multiwell plates for anti-biofilm activity

The strains selected grown on the microtiter plates were pelleted by centrifugation at 3700 rpm for 30 min. About 80 µL of supernatant was recovered and transfer to another microtiter following the same order of original plate.

To the supernatants (80 µL) was inoculated to each well about 100 µL of cells of pathogen to $OD_{600}$ of 0.4. Controls were included on the microtiter. For negative controls was used LB broth without inoculation and for positive controls were used the pathogen grown on LB without supernatants of strains cells tested.

After incubation for 48 hours the wells of the microtiter plate were emptied and washed three times with PBS. On this washing was used for remove the residual cells on the well and keep the adhered biofilm to the wall and bottom in-the well.

To the microtiter air dried was added a solution of Cristal Violet 0.01% in water. After 20 minutes of staining, the crystal violet solution was removed and washed again for three times.

Finally the bound dye was dissolved using 200 µL of 95 % (v/v) ethanol and then was measured on spectrophotometer to an $OD_{595}$.

**Interpretation**

The values obtained were reported as percentage of biofilm formation in each well compared to positive controls.

A threshold value corresponding to 30% of maximum biofilm formation was fixed in order to avoid consider false positives.

**Positive:** It is considered positive for anti-biofilm activity if the percentage is similar or under the average value of the negative controls. On basis on these results were considered positive only those that shown a value less than 30%.

**Negative:** It is considered negative for anti-biofilm activity if the percentage is over the average value of the positive controls. On basis on these results were considered negative only those that shown a value more than 30%.

### 2.7. **Antimicrobial assay**

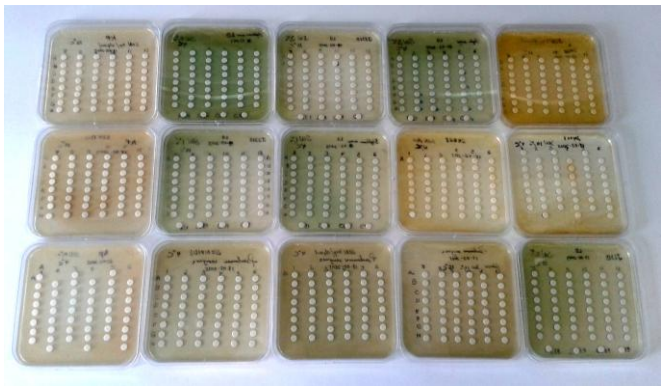

**Figure 1.** Plates with filter disc with all strains tested for antimicrobial assay

This screening was achieved using the agar diffusion method (Kirby-Bauer) to determine the susceptibility of the pathogens against all set of strains. The following protocol was followed.

On the surface of an agar plate LB was inoculate homogeneously the pathogens corresponding to 0.8 to 1.0 $OD_{600.}$ Over the agar plate were placed paper discs filter impregnated with 50 µL supernatant of exhaustive cultures of strains picked. The components present on the filters will diffuse radially from the filter paper.

Plates were incubated for 18-24 hours at 37 ° C.

**Interpretation**

The presence of inhibition halos indicate the susceptibility of the pathogen to some substances present on the supernatant that show an antagonist effect, this is because are present secondary metabolites able to inhibit the growth of the pathogen tested.

On this first screening, the concentrations of the inhibitory substance in 50 µL of supernatant in the filter disc is unknown, therefore was not possible establish the results expressed as: Sensitive (S), Intermediate or Moderately sensitive (I) and resistant (R).

## 3. RESULTS AND DISCUSSION

### 3.1. **Colonies selection**

A wide variety of microorganisms were possible to obtain by the direct dilution plate method in all new media cultures developed. It was not isolated any microorganism from 15% to 25% of total salts probably because the salinity of sediment was too low for cultivate extreme microorganisms halophilic archaea that were not present in Antarctic sediments. So the most likely is that all microorganisms are moderately halophilic bacteria.

Culture analysis of material indicated diverse populations of psychrophilic and halophilic bacteria.

A total of 576 strains were picked by random selection using as criteria the cell morphology and colony pigmentation avoiding take those repetitive. It was selected 96 colonies from each 6 different media culture where was possible grown previously at 4ºC and 20ºC: SWSZN enriched, SWSZN Tryptone/Glycerol and SW1%, SW3%, SW5% and SW10%.

### 3.2. **Antibiofilm activity**

**Table 6.** Positives strains with anti-biofilm activity against pathogens tested

| MEDIUM | MICROORGANISM | |
|---|---|---|
| | *Stapyilococcus aureus* | *Pseudomonas aeruginosa* |
| SW 1% | 0 | 8 |
| SW 3% | 7 | 53 |
| SW 5% | 0 | 27 |
| SW 10% | 6 | 75 |
| SWSZN Tryptone/Glycerol | 0 | 0 |
| SWSZN enriched | 1 | 24 |
| TOTAL POSITIVE STRAINS | 14 | 187 |

The anti-biofilm activity of the strains tested against *Staphylococcus aureus* showed 14 strains positives while a 187 strains showed be positives for anti-biofilm activity against *Pseudomonas aeruginosa.* It is observed that the number of strains with activity for *S. aureus* was very low comparing with the results obtained for *P. aeruginosa* that showed a wide number of positives with anti-biofilm activity. Since these results correspond to an initial screening, a second screening will be approached in order to define the strains in which can it work on.

### 3.3. **Antimicrobial assay**

The suitable approach of the method used has permitted to detect 14 strains that showed inhibitory halos from 576 discs tested with supernatants of the strains picked. This first result can be considered as antimicrobial potential. The major inhibition activity was observed in *Pseudomonas aeruginosa.*

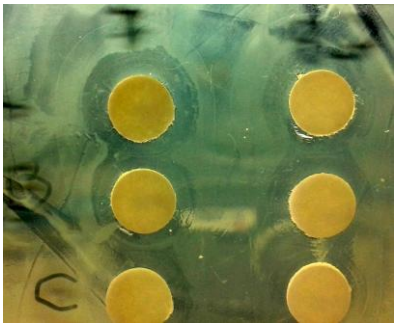

**Figure 3.** Halos of inhibition on the plate inoculated with *Pseudomonas aeruginosa.*

In terms of overall these prior obtained results manifest a possible antimicrobial activities. The success of these first results obtained will be driven/derived to a second confirmatory screening and the subsequently procedures to conclude with the purification of the compound. The purification of the positive strains is ongoing.

## CONCLUSIONS

1. A large number of the strains (576) were isolated from Antarctic sediments and salterns of Isla Cristina in Spain.

2. The vast majority of isolates showed the ability to growth to low temperatures and salt concentration demonstrating their versatile metabolism.

3. The anti-biofilm activity has been investigated in this study; it has determined that 14 strains showed anti-biofilm activity against *Staphylococcus aureus* and 187 strains against to *Pseudomonas aeruginosa.*

4. Ten isolates showed halos of inhibition around the discs tested against *Pseudomonas aeruginosa*. Antimicrobial properties will be studied of these microorganisms.