

MBL Microbial Diversity Course 2013

Project Report

The Colorful lives of Actinomycetes: Exploring soil and marine actinomycetes, their secondary metabolites and the influence interspecies interactions

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Introduction:

Actinomycetes are gram positive, spore forming, high G+C content filamentous bacteria. They are found in a wide range of habitats including soil and marine environments and various symbiotic relationships. They are the most prolific natural producers of antibiotics which include antibacterial (eg. streptomycin, erythromycin, neomycin), antifungal(eg. candicin), antihelminthic and insecticidal (eg. avermectins) and also, antitumour agents and immunosuppressants(1, 2, 3, 4).

Genome sequencing has revealed that actinomycetes possess many biosynthetic gene clusters, majority of which are yet to be associated with any known secondary metabolites (1). Thus, actinomycetes may be a potentially limitless source of useful therapeutic agents provided they are challenged by the right environment to trigger activation of the gene clusters.

Any phenotypic changes induced by environmental manipulations will be relatively easy to appreciate in actinomycetes as they boast distinctive colony morphologies, produce a great variety of pigments and have aerial branching filaments called hyphae which give them a characteristic fuzzy appearance.

Pigment production in these bacteria are influenced by factors such as composition of the media, pH, extent of oxidation and temperature among others. For example, aerobic conditions and low temperatures boost pigment production. Pigmentation in actinomycetes is linked to respiratory mechanisms, defense mechanisms and UV protection. (3)

Furthermore, morphological differentiation and the diverse secondary metabolism is also influenced by extracellular signalling (1, 7).

This objective of this mini-project was to isolate soil and marine actinomycetes and explore the changes in morphology and secondary metabolites induced by different media and interspecies interactions.

Materials and Methods:

Isolation of soil actinomycetes:

Soil was collected with trowel into a sterile whirl-pak bag from outside Candle House, Woods Hole, MA on 10th July 2013. The soil was slightly moist due to light rain and was incubated at 30°C in a thin layer in a sterile petri dish for 2 days in order to dry it out. This was to avoid potential fungal contamination. One (1) gm of the dried soil was added to 1 ml of sterile PBS, pH 7.2, manually agitated by inverting several times and vortexed for 10 seconds. Serial dilutions were prepared using the supernatant after allowing the soil suspension to settle for 30 minutes. 100 uL of the dilutions were plated on Glycerol-Arginine (AGS) plates. Plates were scanned for prospective actinomycete colonies based on characteristics stated previously with preference for those exhibiting pigmentation and clearance zones of inhibition of surrounding colonies. Five interesting strains were isolated in total.

Isolation of marine actinomycetes:

The following specimens were obtained from the Marine Resources Center (MRC), Woods Hole, MA on 2 July 2013.

1. *Microciona prolifera* (red beard sponge)
2. *Cliona celata* (yellow boring sponge)
3. Sand Sponge



Fig 1. a. Specimens in Sea Water Holding Tank b. Homogenized Sponge material

These were freshly collected from Cape Cod with the exception of the yellow boring sponge which was taken from a seawater tank at the MRC.

A small portion of each specimen was removed using a scalpel, washed with sterile seawater and then homogenized using glass mortar and pestle. The homogenate from each specimen was taken through a serial dilution and plated on AGS plates. The yellow boring sponge was dissected to get the outer layer and inner layers separately for associated microbial community analysis.



Fig 2. Separation of the outer layer of yellow boring sponge

Sea squirts (*Ascidella aspersa*) were obtained from Eel Pond Dock, Woods Hole, MA. They were gently squeezed and the ejected liquid was captured on AGS plates. Serial dilutions were also prepared and plated on AGS plates.



Fig 3. a. Sea Squirts on Eel Pond Dock, Woods Hole, MA b. Squirted liquid captured on AGS plate

Marine sediment obtained from the MRC was also dried and plated following procedure similar to that of the soil.

A total of 7 marine isolates were obtained.

16S Colony PCR of the isolates:

Extraction: Actinomycetes are gram positive bacteria with thick cell walls which make it difficult to lyse the cells and obtain clean DNA with low concentration of PCR inhibitors. This problem is compounded by the fact that these bacteria also produce numerous secondary metabolites which could also be inhibitors. A number of different extraction procedures were tested. Positive control was an extract of *E.coli* strain 6713. Negative control was all the reagents without the template.

Table 1. Extraction Methods and the corresponding Gel Electrophoresis results

Extraction Method	Gel electrophoresis check of Amplicons
1. Boiling for 10 minutes in NP-40	Failed

2. Incubation in 50mg/ml lysozyme at 37°C for 45 minutes prior to boiling for 10 minutes	Failed (reacted with Promega Go-Taq Green 2X resulting in coagulation in the tube)
3. Incubation in 10mg/ml lysozyme at 37°C for 45 minutes prior to boiling for 10 minutes	Failed (reacted with Promega Go-Taq Green 2X resulting in coagulation in the tube)
4. Vortexing using Power Biofilm kit bead tube and NP-40 for 20 minutes followed by boiling for 10 minutes	Sheared DNA
5. Serial dilutions of the extracts using NP-40, maceration using pipette tips, and addition of DMSO	Failed
6. Extraction using Promega's Maxwell®16 Robot and LEV Blood DNA Kit with dilutions and number of cycles increased to 35	Passed

PCR Parameters:

A mastermix was prepared per reaction as follows

12.5 uL Promega Go-Taq Green 2X Mix

2.0 uL 16S_8F (15 pmol)

2.0 uL 16S_1492R (15 pmol)

6.5 uL Nuclease-free water

To 0.2 ml microcentrifuge tubes, 23 uL of the above master solution and 2 uL of the DNA template from the individual isolated colonies.

Table 2. Thermocycler Parameters:

Step	Temperature	Time
Initial Denaturation	95 °C	2 min.
Denaturation	95 °C	30 sec.
Annealing	46 °C	30 sec.
Extension	72 °C	1.5 min.
Final Extension	72 °C	10 min.
Hold	4 °C	∞

Number of cycles was increased from 30 to 35 to improve PCR yield.

Post-Amplification Purification:

The Amplicons were directly purified using Promega's Wizard® PCR Preps DNA Purification System as per manufacturer's protocol.

The purified amplicons were sent for sequencing and the sequences analysed using Ribosomal Database Project (RDP, Release 10, Michigan State University) and NCBI Basic Local Alignment Search Tool (BLAST).

Community Analysis using 454 pyrosequencing

DNA Isolation:

Extraction was carried out using Mo Bio's Power Biofilm Kit as per manufacturer's guideline.

PCR was carried out using bar coded forward primers using the following protocol.

Table 3. PCR Reaction Concentrations

Ingredient, stock	concentration per sample, uL
Phusion 2x HF MasterMix	15
DMSO, 100%	2.4
Reverse Primer, 25 uM	0.6
Forward Primer, 6.25 uM	2.4
Template DNA	2-8
Water	Fill to volume
Total Volume	30

The amplification was checked using agar gel electrophoresis and sent for sequencing.

The data was analysed using Quantitative Insights Into Microbial Ecology (QIIME, 5).

Culture Media:

Different media with complex carbohydrates and proteins were tested to look for any changes in the colony morphology and secondary metabolites. Yeast extract malt extract/International Streptomyces Project 2 (ISP2), Starch-Casein, R2A(Difco) and AGS (2) were used in this project for growth, pigment analysis as well as for interaction assays.

Spore Stocks:

These were prepared using 0.1% Tween 80 and 40% glycerol and stored at -20 °C.

Binary Actinomycete Interaction Assays:

The assays were set up using 8 of the soil and marine isolates using the spore stocks as per procedure detailed by Seyedsayamdost et al (1).

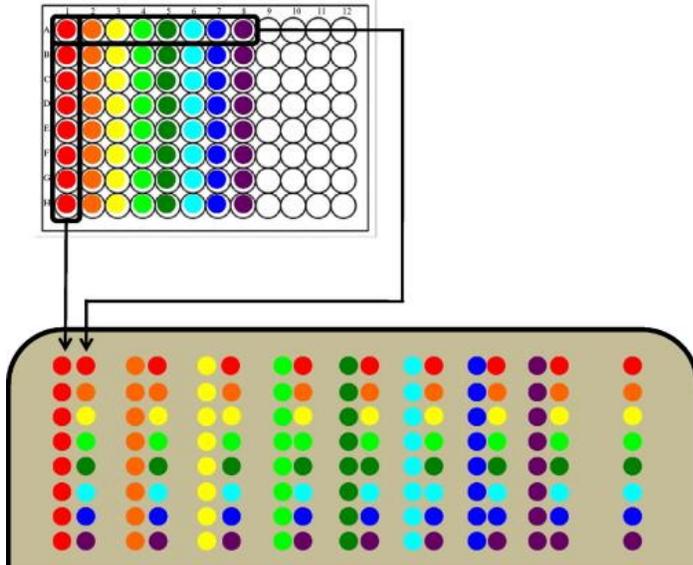


Fig 4. General scheme for Interaction Assay (Seyedsayamdost et al)

ISP2, R2A, Starch-Casein and AGS were tested at first with 4 isolates array and AGS was identified as giving the most pigmentation and sporulation and selected for the subsequent 8 isolates array interactions. Here each isolate is challenged with every other isolate 4 times separately. It is set up using 96 well plates and multi-channel pipettor to place 1 ul of spore stock of the different isolates 0.5 cm apart as depicted by the schematic in Fig. 1 on rectangular AGS plates.

Pigment Extraction:

Solid State Fermentation:

Solid State fermentation is a technique in which complex solid substrates such as bran and bagasse are broken down slowly by microorganisms resulting in industrial-level production of bioactive compounds (6). Crude pigment production was facilitated by SSF using wheat bran as per Selvameenal et al (3). For this, spores from 5 day old plates of 4 of the isolates were inoculated in ISP2 liquid media and incubated for 2 days in rotary shaker at 28 °C. Five (5) ml of this liquid culture was then added to 10 gms of sterilized wheat bran and incubated at 28 °C for 7 days.



Fig 5. a) Pre-fermentation wheat bran and liquid culture in ISP2 b) Pigmentation post-fermentation c) Maceration of biomass with mortar and pestle using ethyl acetate

Crude Pigment Extraction:

The extraction was carried out by adding 50 ml of ethyl acetate to the fermented wheat bran and macerating the mix using a mortar and pestle and straining out the pigment using sterilized cheesecloth. ~ Thirty (30) ml of pigment was extracted using this method per sample.

Direct Pigment Extraction from Plates:

Pigments were also extracted directly by cutting the agar from minimum 10 days old plates into small pieces and adding 25 ml of ethyl acetate so as to cover the agar completely. The extracted pigments (~15 ml) were removed by pipetting and stored as described above. For isolates with diffusible pigments, water was also used for pigment extraction similar to the process with ethyl acetate (3, 8).

All the extracted pigments were stored in sterilized vials and flasks and protected from light by covering with aluminium foil.



Fig 6. Extracted pigments from Isolates #4, #2, #1, #3, #9 and #6 (See Table 4)

UV-Visible Spectroscopy:

This was carried out on all the extracts using Cary UV-Visible spectrophotometer and Cary WinUV software with baseline correction. A quartz cuvette was used. Apart from ethyl acetate and water based extracts, quick extraction was also performed using the liquid cultures and

30% w/v sucrose for the pigment analysis of 4 of the samples. Spectrum was scanned between 250 nm and 1000 nm.

Fluorospectrometry:

Broad spectrum fluorescent analysis was carried out on one of the samples using NanoDrop 3300.

Antimicrobial Activity of Extracted Pigment:

Filter paper discs dipped in the pigment extract were placed onto a freshly spread-plated E.coli strain 6713 on LB agar plate. OD was 1.044.

E. coli 6713 Overlay on Co-culture

Overnight culture of E. coli strain 6713 (OD= 1.819) was added to 1% LB agar (~45 °C) to prepare 1% v/v mix which was poured over the interaction assay plates.

Results and Discussion:

Isolation of soil and marine actinomycetes and 16S colony PCR

Table 4. Classification of isolates based on 16S, pigmentation, inhibitory action and habitat

Isolate #	Sequences producing significant alignment (BLAST), max identity (97-99%)	Pigmentation on AGS media	Clearance zone on initial plate for isolation	Habitat
1.	strep	Dark golden	No	Soil

	polychromogenes, lavendulae, flavotrichini, bikiniensis	brown		
2.	Streptomyces prunicolor, 98%	Dark brown*	-	Soil
3.	Streptomyces narbonensis, 98%	Yellow brown	No	Soil
4.	Streptomyces polychromogenes, lavendulae, flavotrichini, bikiniensis	Pale yellow brown	Yes	Soil
5.	Streptomyces fumigatiscleroticus, (98%), poonensis, spiralis	Indigo	No	Soil
6.	Streptomyces seoulensis, 98%	Lavender*	-	Soil
7.	Streptomyces aureus, silaceus	Yellow*	-	Soil
8.	Streptomyces sanyensis, 99%	Greyish-green	No	Yellow Sponge
9.	Streptomyces anulatus	Yellow-green	Yes	Sea Squirt
10.	Not sequenced	Dark Purple	No	Marine Sediment
11.	Not Sequenced	Dark Pink	No	Marine Sediment
12.	Not Sequenced	Dark Green	No	Red Sponge
13.	Not Sequenced	Coral	No	Sand Sponge

14.	Not sequenced	Orange Yellow	No	Sand Sponge
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* Isolated by fellow MBL MD 2013 student Yosmina Tapilatu.

Of the two marine, actinomycetes, #8, from yellow boring sponge, closely matched the description of *Streptomyces sanyensis* as dark yellow-brown colonies with white to grey aerial spores. It was originally isolated from Mangrove sediment. The other one, #9, from Sea Squirt, also, looks morphologically similar to *S. anulatus* with yellow-green pigmentation. It was originally isolated from recirculating aquaculture which corresponds well with the yellow sponge being from the tank at the MRC.

A DNA extraction kit which includes purification steps for removal of potential inhibitors such as the Mo Bio PowerSoil or PowerBiofilm or Promega's LEV Blood DNA kit is recommended for DNA extraction from actinomycetes. The results point more towards presence of inhibitors which causes the PCR to fail rather than difficulty in the cell wall lysis.

Community Analysis: Sponge Symbionts

Sponges are filter-feeding metazoans. Sponge-associated communities encompass a diverse range of microbes such as bacteria, cyanobacteria, dinoflagellates, diatoms and archaea. They serve important functions such as stabilization of the sponge skeleton, uv defense, and consolidation of nutrients. They can be exosymbionts or endosymbionts depending on whether they are associated with the outer or inner layer of the sponge respectively. They can also be intracellular symbionts occupying the inside of the sponge cells (4).

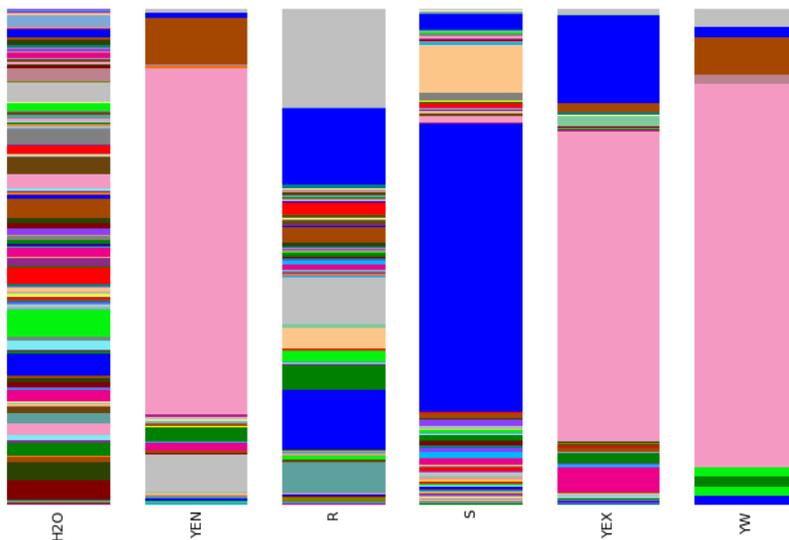


Fig 7. Sponge associated microbial community: a. Water b. Internal Layer Yellow Sponge c. Red Sponge d. Sand Sponge e. External Layer Yellow Sponge

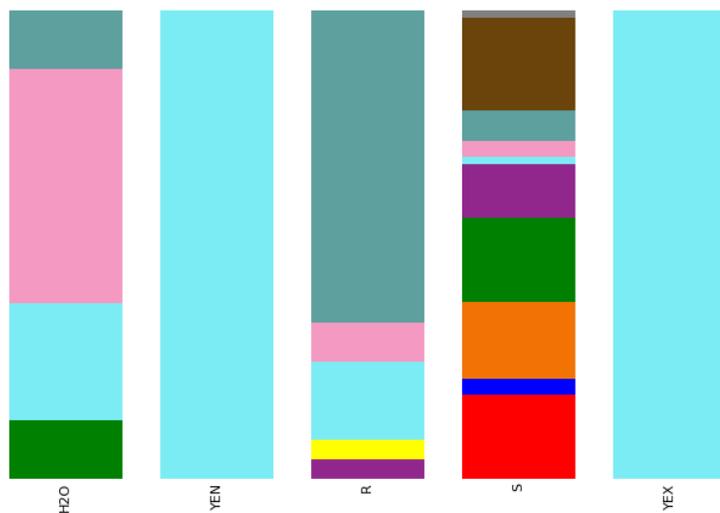


Fig 8. Sponge associated actinomycetes a. Water b. Internal Layer Yellow Sponge c. Red Sponge d. Sand Sponge e. External Layer Yellow Sponge

To differentiate between sponge-associated bacteria and bacteria present in the water filtered, 454 sequencing was also performed on the water in which the sponges were handed over from the MRC. Preliminary analysis of the data shows that the water sample has a much more diverse community and that the sponges appear to select for specific OTUS which differ among the different sponges. Zooming onto the actinomycetes in the communities, one can see that the red sponge exhibits the greatest diversity while the yellow sponge has the least. One is unable to appreciate any difference external versus internal layer communities of the sponge at this level.

Effect of different culture media:

There were some changes observed in a few of the isolates as a result of different culture media. A more rigorous and uniform evaluation of changes in all the isolates is warranted. Isolate #2 did not produce dark brown pigmentation on ISP2 media as opposed to copious amounts of it on AGS.

Isolate#6 produce pink pigmentation on starch-casein instead of purple as seen on AGS.

Isolate #9 produced yellowish green colonies on starch casein while it was greyish green on AGS. The colony morphologies were also different with the former having grey spores, heaped center, beads of secondary metabolites while the latter had a yellow concave center with ring like pattern of the colony.



Fig 9. Isolate #9 on a. starch casein b. AGS

Pigment extraction and analysis: UV-Visible Spectroscopy:

Solid State fermentation produced the best results for pigment extraction yielding good volume of relatively pure extract.

The spectra generated with the pigments from the different extractions were all non-specific with no prominent maximum absorbance peaks generated in the visible range. There was strong absorptions <260 nm for all the pigments which may be indicative of polyene compounds which are biologically active.

Pigment extracted from #9 lost its yellow green coloration which indicated a compound reacting with oxygen. This is a characteristic trait of flavins which have fluorescence activity. This was tested with fluorescent spectroscopy which confirmed fluorescence and 420 nm uv filter under 100x objective showed autofluorescent spots(7) in the mycelial mat of the colony. Thus, the pigment may be a flavin such as riboflavin.

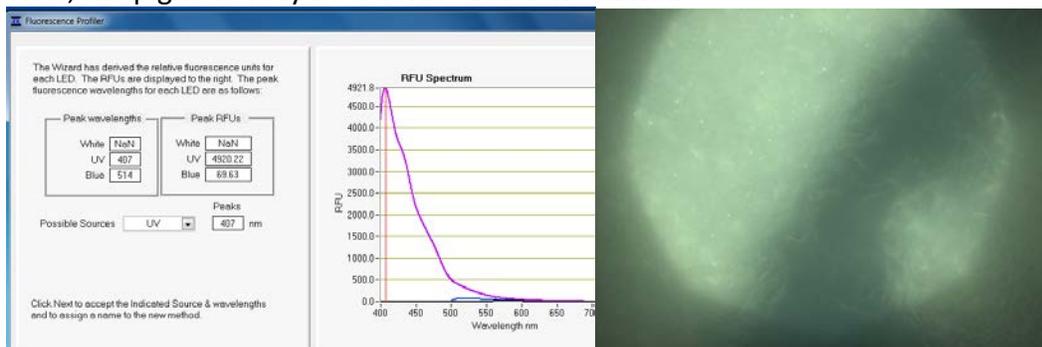


Fig 10. a. Fluorescence spectra b. Autofluorescence

The pigment soaked discs from all the isolates tested except #9 (extracts from both the Starch casein and AGS plates) formed a zone of clearance on the E. coli spread plate. The negative control with ethyl acetate showed no such zone of inhibition, thus indicating that the pigments have antibacterial activity against the tested strain.

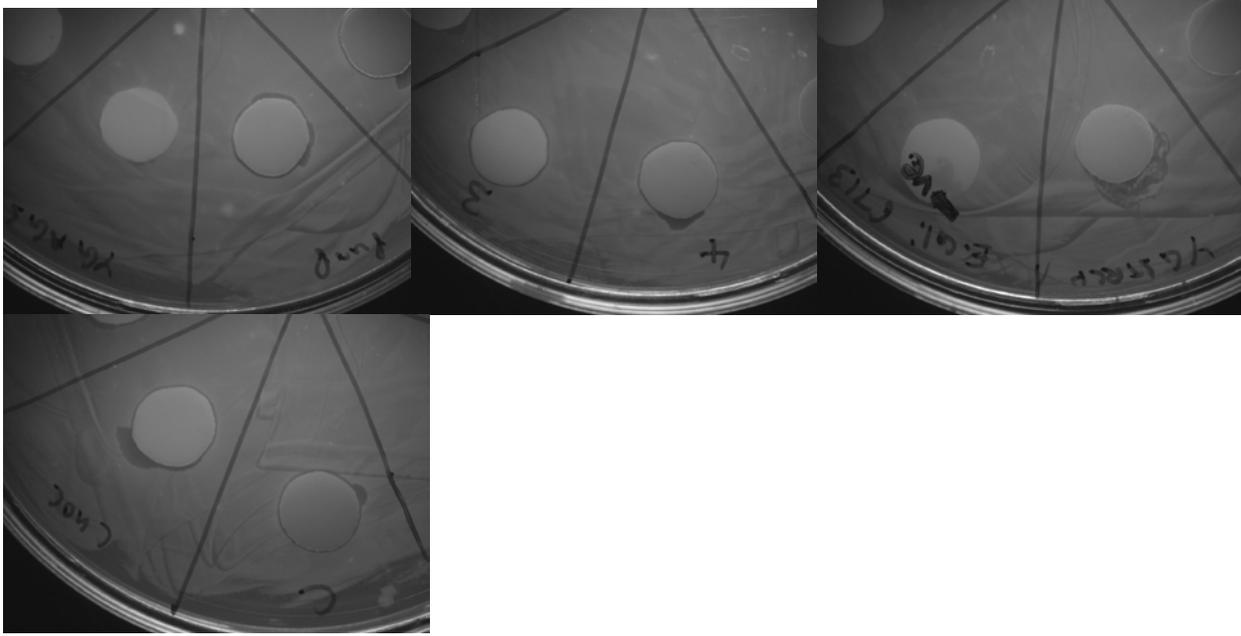


Fig 11. Zone of Inhibition in *E. coli* 6713 lawn a. #9(AGS, no clearance zone) and #6 b. #1 and #3 c. #2 and #4 d. No zone of Inhibition seen around Negative Control of ethyl acetate and # 9 (starch-casein).

Purification of the crude extracts would greatly benefit both the UV-vis spectroscopy as well as the antibacterial tests. Other methods of pigment analysis such as Micro-Raman Spectroscopy need to be explored (9).

Binary Interaction Assays:

The negative control showed good growth of all the tested isolates. Every interaction was in quadruple. Many of the interactions led to inhibition of sporulation and/or colony formation. Changes in pigmentation was difficult to evaluate due to production of diffuse pigmentation by some of the strains which masked any probable changes in the colour or any increase or decrease in pigment activity. For this, isolated pairwise interactions need to be carried out. A method to quantify these interactions in addition to the visual evaluation would greatly benefit this assay. The degree of inhibition/enhancement also needs to be taken into account.

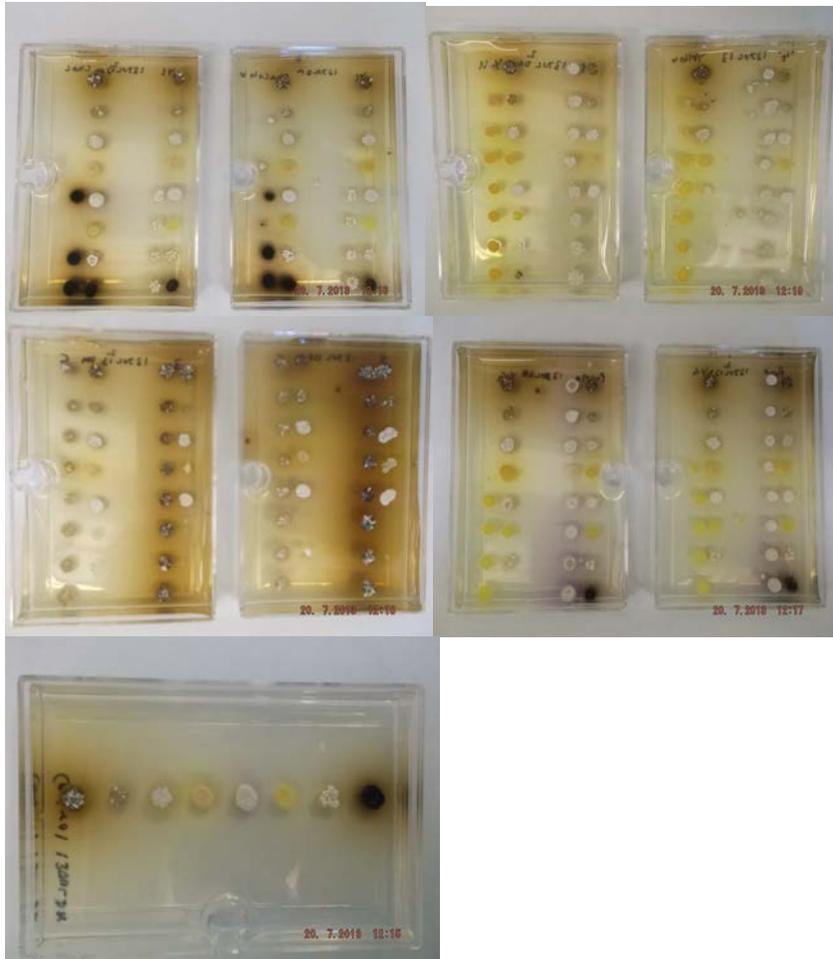


Fig 12. Interactions of all isolates against a. #2 and #8 b. #7 and #3 c. #9 and #6 d. #4 and #1 f. Negative Control Plate showing the growth and pigmentation of all 8 isolates

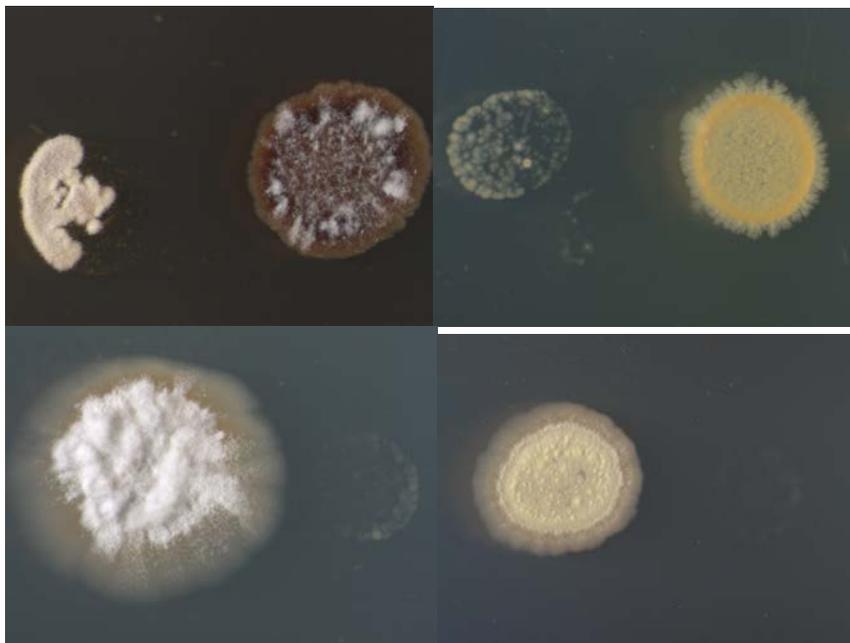




Fig 13. Partial and complete inhibitions caused by interactions

Antibiotic production may similarly be stimulated or repressed by an interaction. An ~ 0.8 cm zone of clearance was observed around Isolate #4 on the negative control plate. No other zones of inhibition were observed in any of the other plates



Fig 14. Zone of Inhibition in E.coli agar overlay around Isolate #4

It would be a worthwhile strategy to test the interactions for biological activity against bacteria and fungi from the soil/marine environment that the test actinomycetes were isolated from.

Table 5. Binary Interactions: "0"=No Change "-"=Inhibition "+"=Enhancement of Test Isolates #1-4 and #6-9 in the 1st column when challenged by the corresponding isolate in the 1st row

Test Isolates ↓	#1	#2	#3	#4	#6	#7	#8	#9
#1	0	0	-	0	0	0	0	0
#2	-	0	-	-	0	-	0	-
#3	0	0	0	0	0	0	0	0
#4	0	0	0	0	0	0	0	0
#6	0	+	0	0	0	0	0	0

#7	-	0	-	0	0	0	0	0
#8	-	0	-	-	0	-	0	0
#9	-	0	-	-	0	-	0	0

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