Isolation and characterization of species affiliated with family *Actinomycetaceae*

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Marine Biological Labs (MBL) Microbial Diversity Course, summer 2014 (MD-2014)

Abstract

The study focused on enrichment for actinomycetes from the environment from around Falmouth, MA. The colonies on SAT agar were pink and of about 5mm in diameter. In SAT broth, the white phenotype was sticky to the side of the growth tubes. Gene prediction and phylogenetic characterization of the sticky white aggregates and pink non sticky aggregates was done using 16S rRNA gene sequences from the whole genome. The 16S rRNA gene sequence from the linear chromosome had close similarity with *Streptomyces sp*. The 16S rRNA gene sequence from circular chromosome had close sequence similarity to *Curtobacterium sp*. In the presence of trace elements and additional 1μM $FeCl_3$, the sticky phenotype was changed from white to pink, with mycelia still being formed. Growth at 10uM $FeCl_3$ concentration was compromised with no mycelia being formed. There was neither growth nor mycelia formation at 50 μM 2′-dipyridyl (DIP) concentration. Pigment extraction was done using ethanol as solvent. The pink/red crude extract was analyzed using UV/VIS spectrophotometer. The peak corresponded with 536nm, with a shoulder. Further molecular and biochemical analysis is ongoing.

Key terms: Actinomycetes, elemental analysis, Streptomyces, phylogenetic characterization

Introduction

Actinomycetaceae, a Gram positive family of bacteria is best recognized its ability to produce secondary metabolites and enzymes with novel properties (Bredholt, Fjaervik, Johnsen, & Zotchev, 2008; Zhu et al., 2012). The family includes bacteria in Genus Streptomyces, first proposed by Waksman and Henrici in 1943 and currently encompasses more than 600 species (He et al., 2014). Due to considerable variation in metabolic, GC content and genome sizes that contribute to low congruence level, identifying a new species based on the 16S rRNA gene is problematic (Verma et al., 2013). Because of that, novel taxonomic markers such as SsgA and SsgB proteins which have extraordinary conservation are being considered for the classification of Actinomycetes (Girard et al., 2013). Several microbes in *Actinomycetaceae* family can only be cultivated to date as mixed cultures that may also contain non-actinobacterial strains(Hahn, 2009).

For microbes to grow, they need trace elements as they are essential to life; and that includes iron (Dixon & Stockwell, 2014; Sandy & Butler, 2009). Iron acts as global regulator

for many cellular, metabolic, and biosynthetic processes such as: DNA biosynthesis, formation of heme, electron transport system, synthesis of ATP, oxygen transport, cofactor for enzymes and the reduction of iron I and in the nitrogen cycle(Crosa, 1989; Neilands, 1981; Sandy & Butler, 2009).

During the course, an Actinomycete was isolated from a fire hydrant during the enrichment process for Actinomycetes. The isolate had phenotypes that called for full genome sequencing and characterization.

Aims of the Study

1. Microscopic and growth conditions analysis of isolated colonies
2. Gene prediction and phylogenetic characterization of the two chromosomes using 16S rRNA gene.
3. Determine if iron and iron chelation has any effect on phenotypes
4. Extraction and spectroscopic analysis of colony pigment

Materials and methods

Collection of soil samples

The soil samples were collected from around a rusty fire hydrant on July 7th 2014 from 22 Gardiner RD Falmouth MA, at GPS coordinates 41°31'44.65"N and 70°40'21.52"W. The samples were taken to MBL's Loeb Lab in 50mL falcon tubes for processing and further analysis as part of the Microbial Diversity (MD) summer course endeavors.

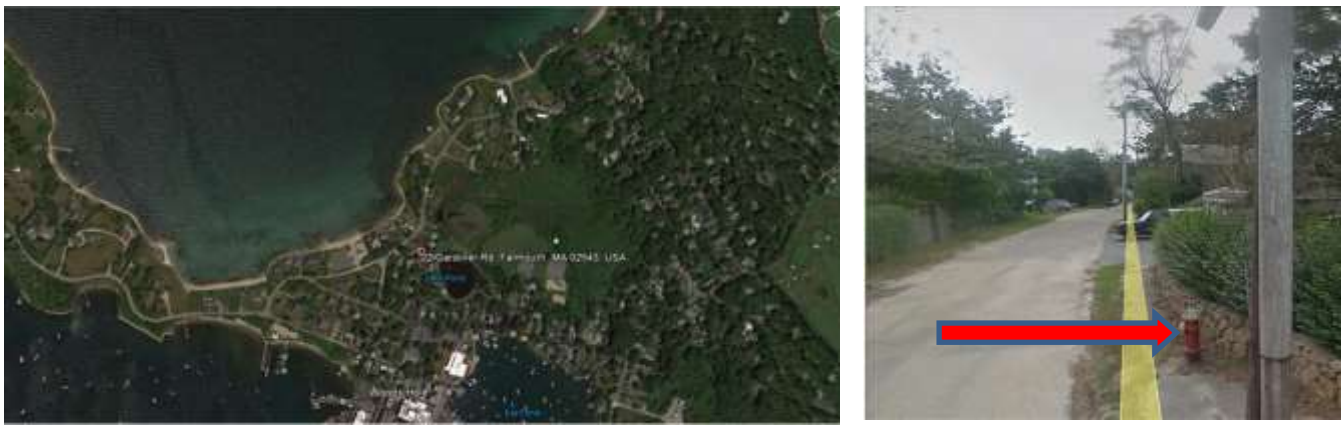


Fig 1: Sampling site

Isolation of Actinomycete bacteria

Following the protocol adapted (Elnakeeb & Lecheval.Ha, 1963), 1g of debris-free soil was spread over petri plates and left on the bench overnight. The soil was then sifted into fine powder and mixed with 1 g of Calcium carbonate ($CaCO_3$). Petri plates were then covered with a wet Whatman paper to maintain the plate in humid conditions and left at room temperature for another day. The batch was then transferred into falcon tube and mixed with 50mL sterile FW-base (1X) and vortexed.

The mixture was allowed to settle for 30 min before making dilutions. Then 50uL to 100uL of serially diluted supernatants (1, 1:10, 1:100, 1:1000 and 1:10,000) was spread plated onto Starch Arginine Tryptophan (SAT) medium and incubated at 30°C under aerobic conditions and monitored for 3 -5 days. Re-streaking was done severally until pure colonies were obtained.

Growth in aerobic conditions

Test for anaerobic and microaerobic growth

Autoclaved SAT broth media was cooled in nitrogen before being dispensed into growth bottles that had been kept anaerobic chamber or in hypoxic chamber for 3 hrs. Some

bottles were kept in hypoxic chamber whereas the sealed anaerobic bottles were then incubated while shaking at 250 rpm at 25°C and monitored for 2-5 days.

DNA Extraction and Complete genome sequencing

Promega Wizard Genomic DNA Purification Kit (Madison, WI) with 1 Hr Lysozyme digestion protocol was used for DNA extraction. For library preparation, sheering was done at 6,400 rpm at 200ng/µl and library size cut off was at 4kb. Long read Pacific Biosciences (PacBio) sequencing using P5C3 sequencing chemistry was done following a workflow that included: Sequencing library preparation and QC; Monitoring sequencing performance; HGAP *De Novo* Genome Assembly with CELERA8; polishing of genome sequence and determination of the epigenome.

Gene prediction and phylogenetic characterization

To predict 16S rRNA genes from the whole genome, RNAmmer 1.2 Server (http://www.cbs.dtu.dk/services/RNAmmer/) was used(Lagesen et al., 2007). NCBI Blast of the 16S was done for the 16S rRNA genes sequences from both the linear chromosome (MR_MD2014) and circular chromosome (CCM_MD2014) of the isolate and both are closely related microbes in the actinobacterial lineages.

For phylogenetic characterization, the 16S rRNA sequences were downloaded and multiple aligned using MUSCLE v.3.7(Edgar, 2004) on Geneious software V.6.18 with a CLUSTALW cost matrix. To ensure quality alignment, Gblocks program v0.91b was used to eliminate poorly aligned positions and divergent regions (Castresana, 2000). PhyML program v3.0 aLRT (Guindon & Gascuel, 2003) one click mode (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi)was used to make phylogenetic tree using the maximum likelihood method. For graphical representation of the tree, Treedyn v198.3 (Chevenet, Brun, Banuls, Jacq, & Christen, 2006) was used. Branches were collapsed if they had branch support value smaller than 50% and a number of bootstraps smaller than 100.

Effect of iron and iron chelation on phenotypes

To possible involvement of iron in phenotypes observed, isolates were inoculated into two types of treatments (one with trace elements and the other without) of 2,2′-dipyridyl (Dip) and iron concentrations ranging from 200, 150, 100 and 50 µM Dip concentration and 1, 10, 50 and 100 µM $FeCl_3$ concentration. As control, regular SAT broth was used.

Analysis of red pigment

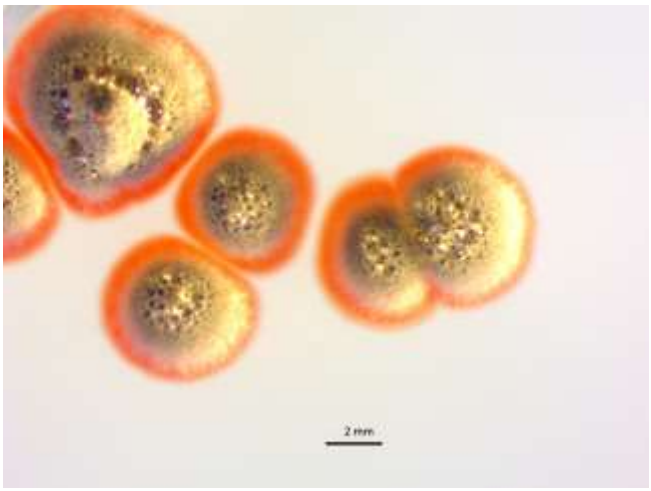
The red pigment of the colonies was extracted using 7 organic solvents: water, 70% and 95% ethanol, formalin, acetone, phenol, methanol and DMSO. Spectral analysis of the crude

extract was performed with a Cary 60 UV-VIS Spectrophotometer (Agilent Technologies, Basel, Switzerland). The wavelength was set between 300nm and 800nm.

Results and discussion

Isolates characterization

The isolates best grew in aerobic conditions on SAT agar, producing pink colonies (Fig. 2a). Scanning electron microscopy showed typical mycelia growth for actinomycete whose thickness was about 0.5µm (Fig. 2b).



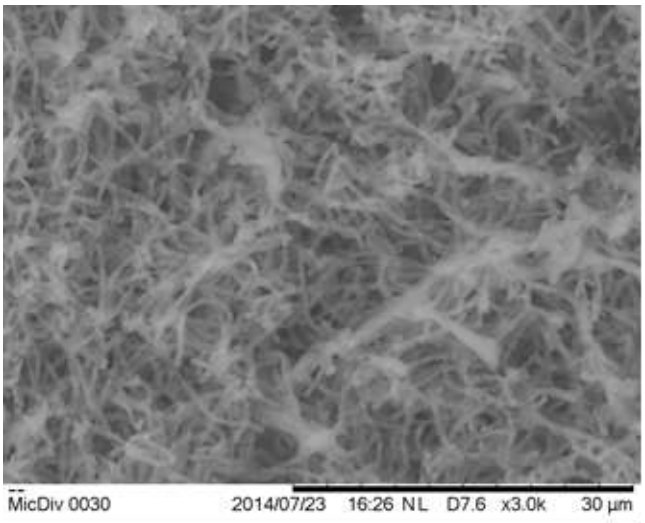
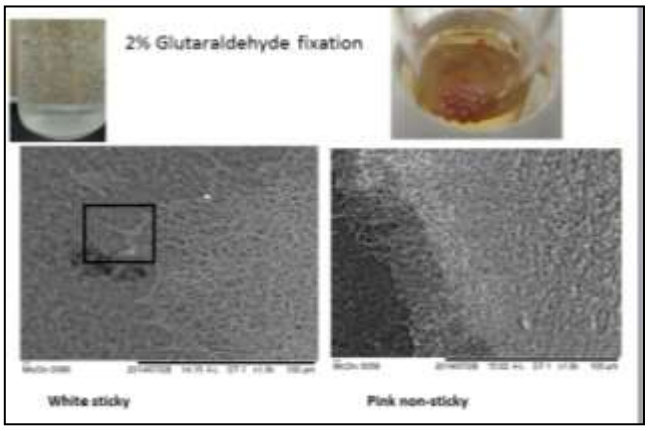


Fig. 2a Pure pink colonies on SAT agar

2b: Scanning electron micrograph

Upon growth in SAT broth, two phenotypes were observed; white sticky aggregates and pink non sticky aggregates. There was a clear difference when the two phenotypes were fixed using 2% glutaraldehyde and analyzed by Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) for elemental analysis indicated possible EPS involvement in stickiness (Fig 3a) and probable influence of Carbon:nitrogen:phosphorus as factor (Fig. 3b to attachment to surfaces as earlier speculated (Thompson, Gray, Lindsay, & von Holy, 2006).



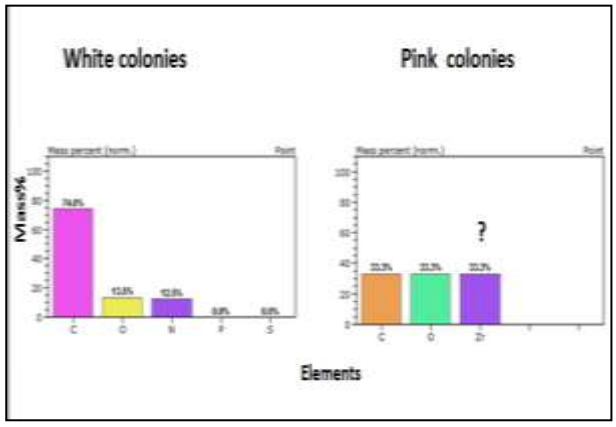


Fig. 3 (a) SEM for white and pink phenotypes

3(b): EDS elemental analysis

Growth in anaerobic and micro anaerobic conditions

There was no growth under anaerobic conditions. But there was growth in microaerobic conditions as seen in figure 4a and 4b below.

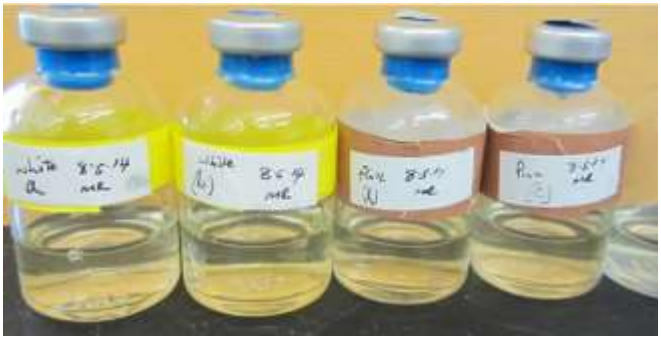


Fig. 4 (a) No growth in anaerobic conditions

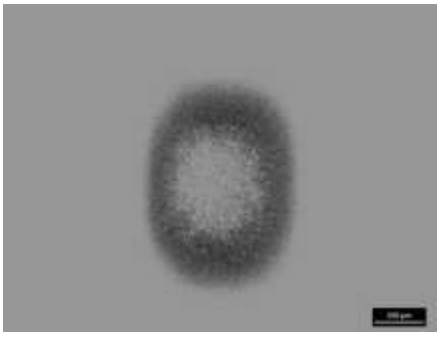


Fig. 4 (b). Growth in microaerobic

Whole Genome Sequencing and gene prediction

Assembly into finished genome yielded two chromosomes. The linear chromosome had a genome size of 8, 274,043 bp with GC content of 72.13%. The circular chromosome had a genome size of 3, 443,800 bp with GC content of 71.95%. The epigenome had five active methyltransferases. After annotation, subsystem statistics information was accessed using RAST server through the SEED model (Aziz et al., 2008) as shown in figure 5 below. Detailed analysis is ongoing.

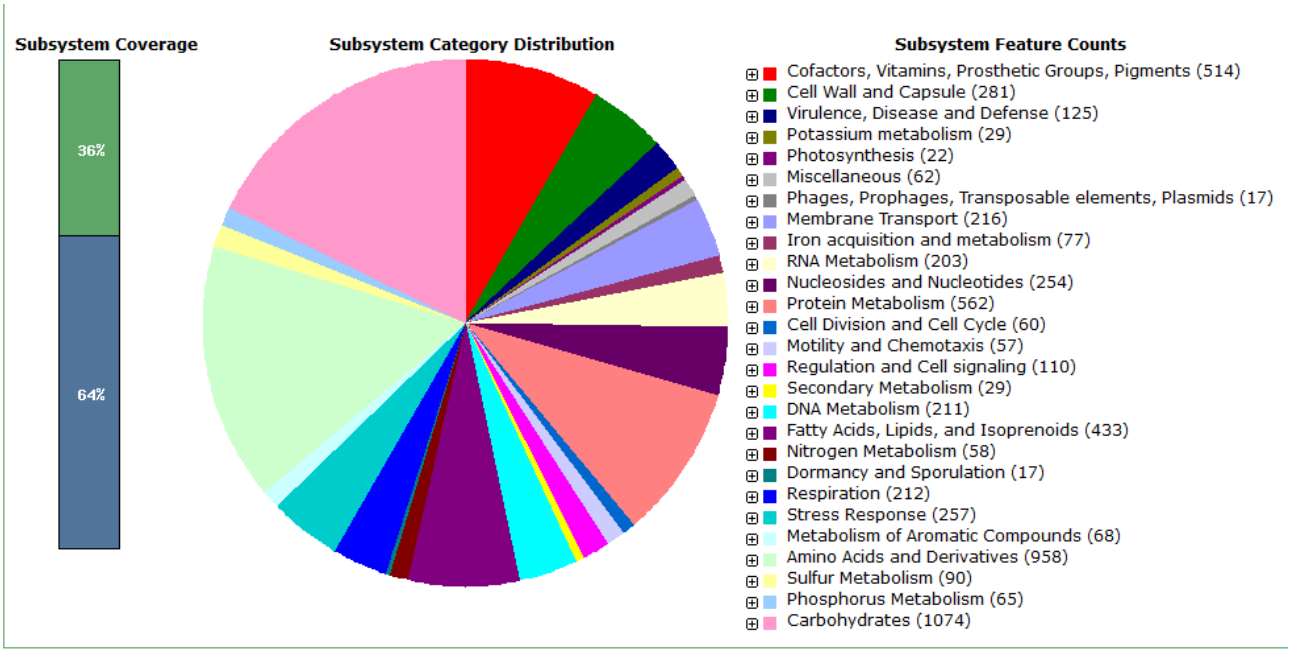


Fig. 5: Subsystem Statistics

Phylogenetic tree based on 16SrRNA gene

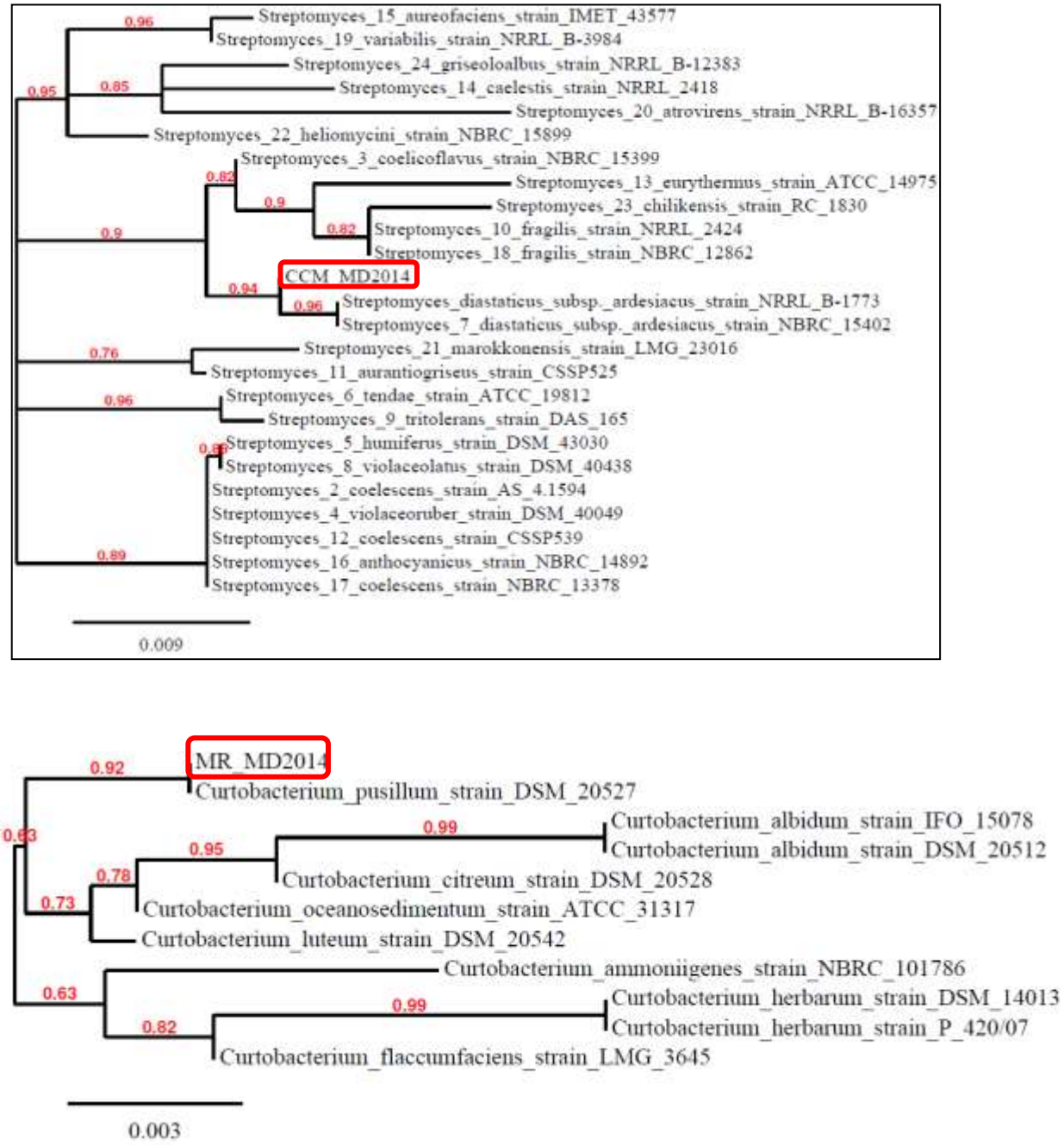


Fig. 6: Phylogenetic characterization using molecular markers

Using 16SrRNA gene (sequences in appendix 1) for phylogenetic characterization, it was demonstrated that the two chromosomes are of the actinobacterial lineages, with one

belonging to *Streptomyces sp*., and the other to *Curtobacterium sp*. (Fig. 6). This genome may consist of two microbes in an obligate symbiotic relationship. Preliminary metabolic profile analysis has given clues towards this, but it is perhaps too early to be very assertive at this stage until it is experimentally proved.

Effect of iron and iron chelation on phenotypes

Growth was observed at 1µM FeCl3 concentration with or without other trace elements. However, mycelia formed under this conditions was different (shiny with occasional space) compared to the mycelia from regular growth media which was thick and uniformly distributed (Fig. 7). Also, with 1µM $FeCl_3$ without other trace elements being added, the pink/red phenotype was sticky (Fig. 7)

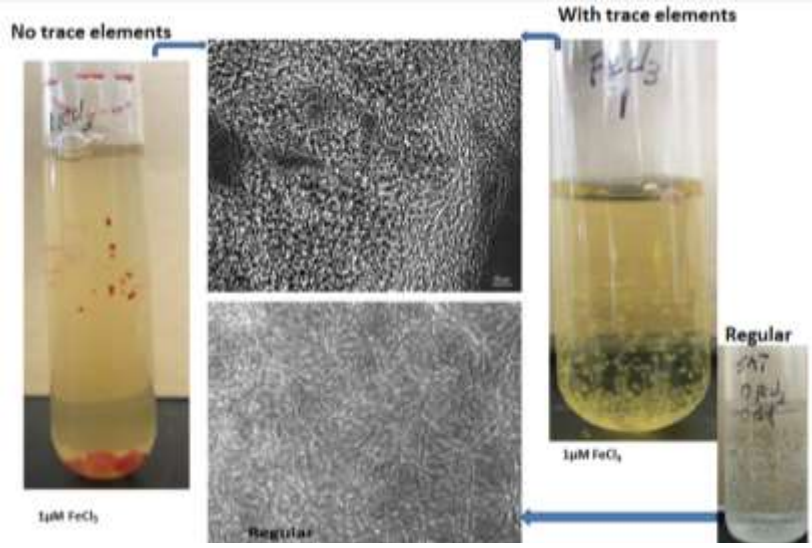


Fig. 7: Effect of iron in phenotypes

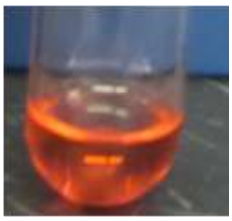
With or without trace elements, at 50µM Dip, there was no mycelia growth (Fig 8). This indicates that iron could be having a role in mycelia formation in actinomycetes as well as activation of adhesion. This findings support a previous study which indicated that high iron concentration could stimulate adhesion in fungi (Prevorovsky, Stanurova, Puta, & Folk, 2009).

Fig. 8: Effect of Dip on growth of mycelia

Lack of mycelia growth could be because cells treated with iron chelator 2,2 -dipyridyl (DIP) increase ROS generation (T. A. De Alencar et al., 2014). Increased production of ROS caused by Fe^{2+} chelation may potentiate the lethal effects (Bystrom, Guzman, & Rivella, 2014; T. A. M. De Alencar et al., 2014; Dixon & Stockwell, 2014).

Red Pigment spectral analysis

UV-vis spectroscopy was ascertained for the pigment extracted from 70% and 95% ethanol. The pigment wavelength corresponded with 536nm, with some shoulder (Fig.3).



70% EtOh extract

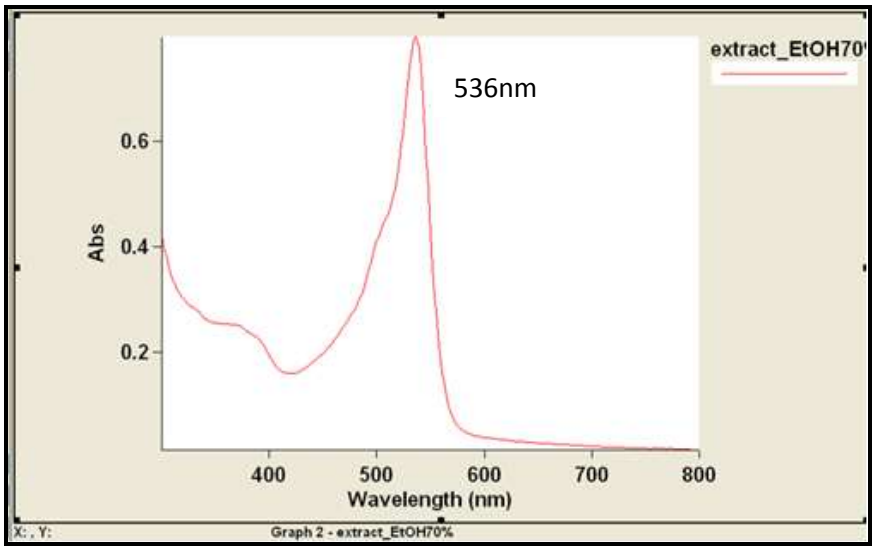


Fig. 9: Spectral analysis pink/red extract from the colonies

Conclusions

The genome could be having two obligate symbionts belonging to family *Actinomycetaceae*. Dip, an iron chelator limits growth of actinomycetes at certain concentrations. Iron, as a macronutrient has an effect on phenotypes, including mycelia formation. Molecular and biochemical characterization is ongoing.

Acknowledgements

I want to thank my PhD adviser, Dr. Anthony Moss who strongly encouraged me to apply for the course. My Attendance and participation in the course was supported by: Selman A. Waksman Endowed Scholarship in Microbial Diversity, Bernard Davis Endowed Scholarship Fund, Auburn Graduate School Travel Award, Farrington Travel award, AU-CMB Program Summer 2014 Fellowship. Many thanks to the entire MBL-MD2014 course directors, faculty, Group 1 and the entire 2014 Microbial Diversity students. I am very grateful especially to Dr. Kurt Hanselmann (ETH Zurich), Dr. Dianne Newman (Caltech), Dr. Scott Dawson (UC-Davis), Dr. George O'Toole (Dartmouth), Dr. Jared Leadbetter (Caltech) and Dr. Arpita Bose (Washington University in St. Louis) for all the fruitful discussions and guidance. I am thankful to all the wonderful TA's and staff for making the course the most wonderful yet scientifically challenging experience of my life. To Dr. Alison Butler (UC-Santa Barbara) and Dr. Roberto Kolter (Harvard), thanks for the thought provoking discussions during your visits.

Appendix 1

a. MR_MD2014

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1         10         20         30         40         50
|         |          |          |          |          |
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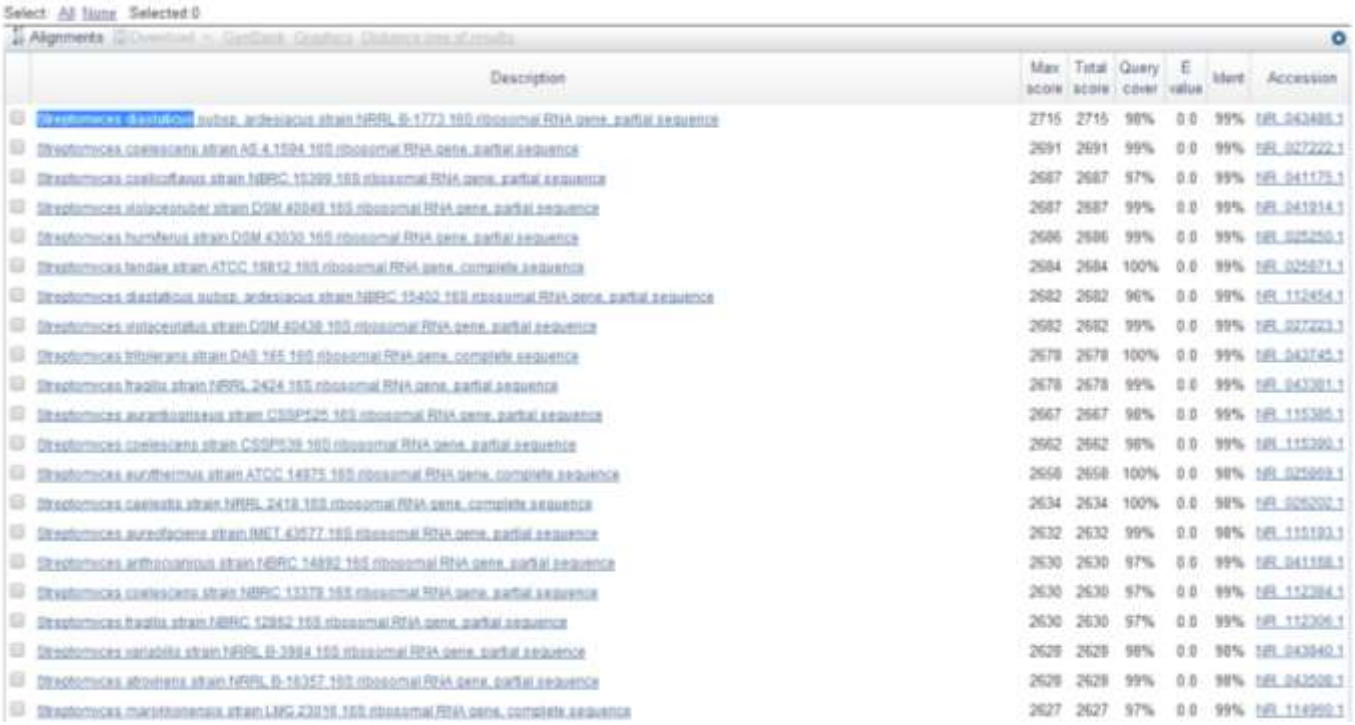

Appendix 1

b. CCM_MD2014

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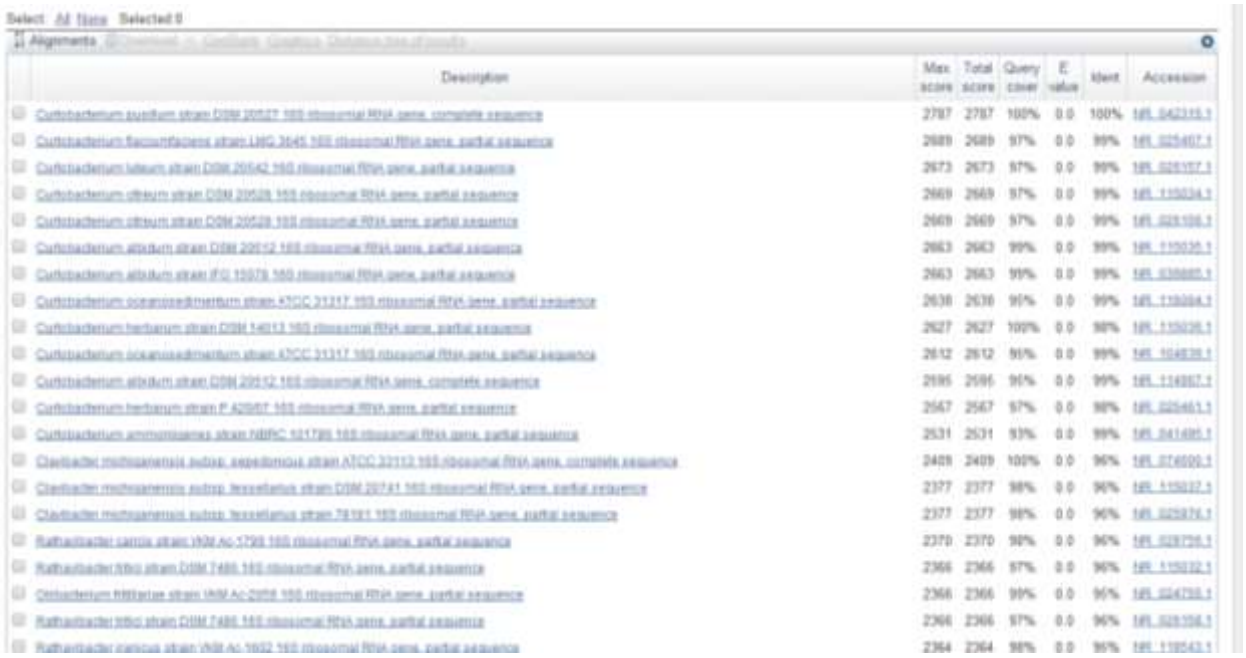

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