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Screening of Siderophore Producers from Soil

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By

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ABSTRACT

Iron is a vital nutrient for the maintenance of enzymatic function, electron transport, DNA synthesis, and other metabolic processes. Thus, bacteria and other microorganisms cope with iron deficiency by secreting high-affinity iron chelators called siderophores. This investigation aimed to characterize siderophore-producing soil bacteria. The soil provides the most complex ecological environment where one can look for promising siderophores to be used for treating either iron overload conditions or to be used as an antibiotic carrier.

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CONTENTS

INTRODUCTION

1. Iron and bacteria

As science illuminates greater understanding of the microbial world, identification of regulatory bioactive compounds uncovers new targets for antibiotic drugs and allows us to tackle the greatest challenges in infectious diseases. Better understanding of how bacteria obtain and process essential nutrients is central to this cause. Iron, essential for all living organisms, except for the genus *Lactobacilli* and *Streptococcus sanguis*, is among such vital nutrients and is especially critical to the growth of microorganisms in which iron deficiency is certainly lethal [1]. Iron is necessary for the maintenance of enzymatic function and many metabolic processes. Specifically, iron serves as a component of iron-sulfur centers of cytochromes in the electron transport chain. In anaerobic bacteria, iron may also serve as a terminal electron acceptor in energy producing pathways [2]. Iron is also an integral cofactor in many enzymes such as nitrogenases of nitrogen fixing bacteria and in reductases that convert ribonucleotides to deoxyribonucleotides, and thus is required for DNA synthesis [3].

Iron is the fourth most abundant element on the earth's crust, following oxygen, silicone, and aluminum. Despite its abundance in nature, iron is not readily available in the reduced ferrous form (Fe^{2+}). In aerobic conditions and physiological pH, ferrous iron is oxidized to its ferric form (Fe^{3+}) which hydroxylates to form insoluble $Fe(OH)_3$ polymers, rendering it inaccessible for acquisition by microbes [4]. The concentration of ferric iron in the environment is about 10^{-18} M, meanwhile, bacteria maintain an internal iron concentration near 10^{-6} M [5]. Therefore, microorganisms in iron-limiting conditions must utilize a mechanism to maintain a sufficient supply of usable iron. To circumvent this limitation, bacteria produce and secrete low-molecular-weight iron chelators known as *siderophores*. Greek for "iron bearer," siderophores

have a high affinity for Fe^{3+} and thus capture extracellular ferric iron and shuttle it across the cellular membrane for productive use. In fact, the association constant of siderophores for ferric iron lies between 10^{12} - 10^{52} [6]. Siderophore-mediated iron transport involves a complex system of membrane proteins and transporters. The action of these systems is regulated by external iron concentration and expressed only when bacteria are deprived of iron [7]. Most commonly, siderophore biosynthesis is negatively controlled by intracellular iron concentrations. In particular, the *Fur* (ferric uptake regulator) repressor protein is responsible for regulating synthesis [8].

2. Siderophores

Siderophores are as diverse as the microorganisms which produce them. This diversity is thought to be due to evolutionary pressures of bacteria that facilitated the development of siderophores which could not be utilized by other organisms [9]. Although over 500 different compounds provide iron transporting functions, four major classes chemically characterize siderophores in relation to metal binding sites [4]. These iron-binding functional groups, derived from modified amino acids, include catecholates, hydroxymates, carboxylates, and mixed types. Catecholates contain phenolate groups and are commonly produced by organisms such as *Vibrio cholerae, Streptomyces,* and most *Enterobacteriacae*. In contrast, hydoxymates are classified by the presence of hydroxamic acids and are widely found in opportunistic pathogens and most fungi. In fact, pyoverdine, the most complex siderophore known to date, is a hydroxymate which gives fluorescence to *Pseudomonads*. Hydroxymate siderophores are more prevalent in lower pH while catecholates are found in more neutral or alkaline pH [10]. Additionally, the mixed class of siderophores are hybrids of hydroxymates and catecholates. A complete siderophore constitutes a

large multi-enzyme structure containing a peptide backbone including one of three iron-coordinating ligands which surround Fe^{3+} in an octahedral configuration [11].

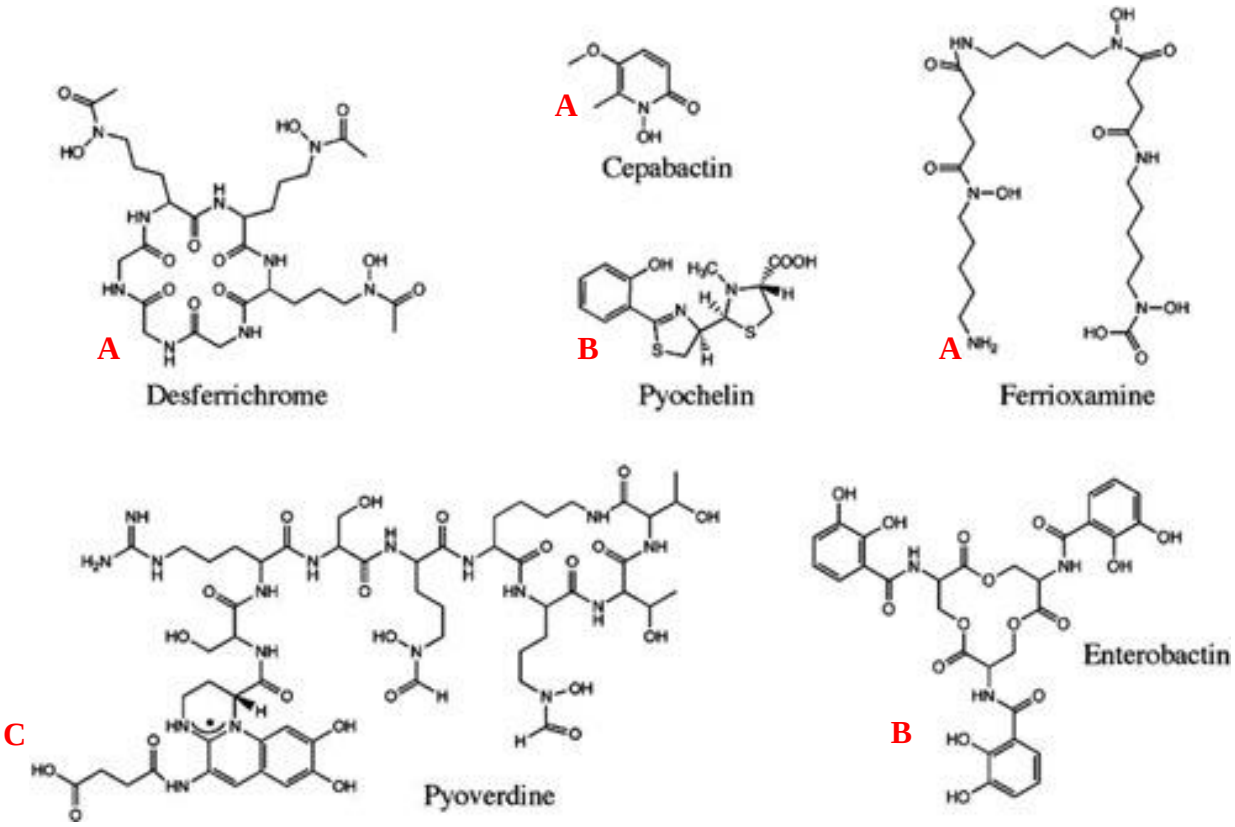

Figure 1. Examples of siderophore structure: hydroxymate (A), catecholate (B), and mixed type (C) siderophores

3. Transport

In low-iron states, newly synthesized siderophores are exported from the cell via transport pumps to scavenge for ferric iron. The siderophore-Fe^{3+} complex is too large to enter the cell through porins; therefore, internalization of the complex requires special outer membrane receptor proteins. The crystal structures of a few such receptors, FhuA, FecA, and FepA, have been identified [12]. Many of these share common structural components including a 22 antiparallel ß-barrel domain and an N-terminal globular domain which acts as a plug to occlude

the ß-barrel. Furthermore, a conformational change in these domains leads to the passage of siderophore-Fe^{3+} [13]. These receptors require an energy source to transport the large complex through the periplasm and inner membrane. In gram-negative bacteria, the energy required for this function is provided by the coordination of an outer membrane protein, TonB, with two inner membrane proteins, ExbB, and ExbD, which presumably together catalyze active transport of the siderophore complex by creating proton-motive force [14]. The role of TonB, ExbB, and ExbD in energizing the siderophore uptake process has been extensively studied in *E. coli* [6]. After reaching the periplasm, the complex attaches to a periplasmic binding protein, then passes through the inner membrane via ABC transporters or permeases. In contrast, gram-positive bacteria which lack an outer membrane, instead have lipoproteins attached to the external side of the cytoplasmic membrane which channel the complex to ABC transporters. Cytoplasmic subunits of the ABC transporter undergo conformational change due to NTP hydrolysis, ultimately providing the energy for this process [15]. Once the siderophore-iron complex is delivered to the cell's interior, Fe^{3+} is reduced to soluble Fe^{2+} after which it is released from the siderophore and made available for metabolic use. The large difference in binding constants for Fe^{2+} and Fe^{3+} is what drives the oxidation-reduction potential, facilitating the efficient release of Fe^{2+} inside the cell [16]. Meanwhile, the unbound siderophore is either degraded or recycled by efflux pumps into the external medium, as is the case with pyoverdine [17].

4. Soil Bacteria

The study of siderophores has been advanced in part due to the successful isolation of siderophores from plant bacteria. Nitrogen-fixing microorganisms found in soil, such as the genus *Rhizobacterium,* must effectively compete with their plant hosts and other soil inhabitants for iron. *Rhizobacterium* produce rhizobactin, a mixed type of siderophore, among other iron

chelators with varying specificities [18]. Consequently, soil bacteria utilize efficient iron-transport systems and are known to secrete siderophores to satisfy their iron needs. In soil bacteria, iron is also involved in the nitrification process and has immense implications for the symbiotic interaction between nodular bacteria and their plant hosts. For these reasons, plant bacteria are especially useful in the analysis of siderophore mechanisms.

5. Applications

Because iron is an essential nutrient for the survival of bacteria, their uptake mechanisms contribute an important piece of the puzzle in bacterial physiology. Growing knowledge of iron chelators also presents many productive applications in human and environmental health. First, in mammalian hosts, siderophores secreted by pathogens serve as virulence factors. Siderophores can sequester iron from host lactoferrin and transferrin in order to proliferate throughout the body. It has also been shown that siderophores are critical to the full maturation of biofilms, an important virulence factor for organisms like *Psuedomonas.* This is thought to be due to the role of intracellular iron concentration in the formation of biofilms. Conversely, the iron binding action of molecules like transferrin can limit the availability of iron in the blood for pathogenic bacteria and thus contribute to innate immunity [19]. Another important characteristic of siderophores is their capacity to chelate metals other than iron. This ability, coupled with high affinity of specific ions, make siderophores an effective alternative for phytoextraction of toxic substances from the environment that may be harmful to crops. Similarly, siderophores that are secreted near roots can suppress plant diseases by limiting iron supplies for surrounding pathogens [20]. Siderophores are also being explored as drug delivery agents in the battle against infectious diseases. Antimicrobial resistance is a serious problem plaguing the medical community. In the United States, each year two million people are infected with antibiotic

resistant bacteria and, of that number, 23,000 people die [21]. To facilitate evolutionary advantage, some bacteria can recognize and internalize the siderophore-iron complexes produced by other species. This "thievery" is further manipulated by some bacteria to attach toxic compounds to siderophores. This form of natural antibiotic delivery is mediated by siderophore-drug conjugates known as *sideromycins*. For example, *Klebsiella pneumoniae* produce microcins which are the first members of a class of antimicrobial peptides with siderophore-like post-transcriptional modification [22]. This model of natural antibiotic delivery is being used to design synthetic drugs through the "Trojan Horse" method. In this process, biomimetic siderophore moieties with optimal iron complexing capacity are synthesized, then conjugated to drugs like ß-lactams, cephalosporins, and carbapenems. Eventually, a drug that is unable to permeate the membrane barrier can gain access to the cell by being recognized by a receptor as a part of a siderophore complex. These conjugates serve a dual purpose as well: providing needed iron and transporting the antibiotic that is attached [23]. In addition, the mechanism of efficient iron acquisition guided by siderophores offers a hopeful cure for human iron overload diseases. Currently, the siderophore desferrioxamine (DFO) is used clinically in the treatment of iron overload diseases such as haemochromatosis and β-thalassemia. However, DFO has multiple disadvantages; it is expensive, orally inactive, and has a short serum half-life. As a result, treatments using DFO require long periods of subcutaneous infusion which often cause swelling and pain in patients. Therefore, more efficient, novel iron chelators that are economical, orally active, and better absorbed through the gastrointestinal tract are being sought as alternatives to DFO for the treatment of iron overload diseases [24].

Research goals

With all of these valuable applications, greater clarity of how siderophores operate in the natural environment is necessary. Although many siderophores have been isolated from various bacterial species, little has been done to find trends and unifying characteristics that bind siderophores from bacteria of similar origin. We do know that in addition to external iron concentrations, environmental factors such as pH, temperature, carbon source, and the presence of other metals, affect siderophore synthesis [10]. Further studies may elucidate factors which explain why some microbes from similar environments produce more siderophores than others. In the study of soil bacteria, in particular, depth, moisture, salinity, and the concentration of other metals in the soil may impact accessibility of iron and, ultimately, the siderophores employed for iron acquisition. Furthermore, geographical and chemical differences in the environment can impact the diversity of microorganisms. By identifying the dominance of certain bacteria in unique environments and analyzing their iron uptake systems, we may increase our ability to predict the nature of siderophores based on the biological identities of the bacteria which secrete them. For instance, gram negative and gram positive organisms differ widely in their outer membrane structures. Because siderophore function significantly depends on the transmembrane shuttling mechanism, such as that afforded by the TonB/ExbB/ExbD system, variations in surface structure directly impact the efficiency and biological nature of iron uptake.

With the goal of better characterizing siderophores from various microbes, an exploratory study will be conducted for analysis of different soil samples with the goal of screening for siderophore producers. Due to the geochemical complexity and the competitive nature of its biosphere, soil presents one of the best sources for siderophore-producing organisms. First, a variety of soil samples will be collected from the region and the population of microbes will be

cultured. Among those which produce siderophores, the second objective is to genetically identify the organisms. Lastly, these findings will be reevaluated along with consideration for the origin of the soil sample, and to potentially identify novel iron uptake systems.

MATERIALS AND METHODS

1. Soil collection

With help from the Geosciences Department at East Tennessee State University, soil was collected from two separate sites following a day of rain at the ETSU Eastman/Valleybrook Campus. The sites were assessed to ensure distinctions in geographical and chemical properties. The two varieties of soil included bare and forest soil. Bare soil is eroded and drained, has been removed of aluminum and silica, and has little to no organic matter. This soil is dry and orange in color. In sharp contrast, forest soil is rich and dense in organic content, retains more moisture, and is characterized with a dark earthy color. Three samples were taken from each site for a total of six soil samples.

2. Culturing organisms from soil

In order to sample the microorganisms contained in the bare and forest soil, for each of the 6 samples, 0.2g of soil was weighed and suspended in distilled water. For each liquid soil suspension, 10 µL of supernatant was used to inoculate 5 ml of nutrient broth. The nutrient broths were incubated for 24 hours at 30°C. After 24 hours, turbidity was examined to analyze growth using a spectrophotometer to measure optical density of 1 ml from each nutrient broth at 600 nm. The six samples were then returned to the incubator for another 24 hours. Optical density was measured again after 48 hours. Unused soil was frozen in -20°C for future use.

3. Siderophore detection

The Chrome Azurol S (CAS) assay was used for detection of siderophore production throughout experimentation. The CAS assay is a universal chemical test that detects siderophore production. The assay is based on siderophore ability to bind to ferric iron with high affinity. The agar contains Chrome Azurol S (CAS) dye which, when complexed with Fe^{3+}, is blue in color. If the inoculated organisms secrete siderophores, ferric iron is stripped from the dye, causing the media to change colors from blue to orange or yellow. Therefore, the presence of siderophores is indicated by coloration surrounding the inoculation site.

$$Fe^{3+}\text{-dye (blue)} + \text{siderophore} \rightarrow Fe^{3+}\text{-siderophore} + \text{dye (orange)}$$

To identify siderophore producers in the six soil samples, 10 µl of supernatant from the initial soil suspension was inoculated onto a CAS plate by spreading the liquid evenly over the surface of the media. The CAS plates were kept in a 30°C incubator for 48 hours.

4. Isolation of siderophore producers

After 48 hours of growth, the CAS plates were examined for any areas of coloration. Areas of growth on the plates, which were surrounded by orange halos, were drawn with a sterile loop and streaked on a Luria Broth (LB) agar plate for isolation of the organism using the streak plate method. This step allows for isolation of a pure strain for further identification. The LB agar plates were then incubated at 30°C for 24 hours. After growth, isolated samples were given a number for tracking in later analysis. Purified colonies were also used to determine the organisms' gram reaction and physical morphology by traditional gram staining technique. This allows for distinction between gram positive and gram negative bacteria, as well as bacilli and cocci.

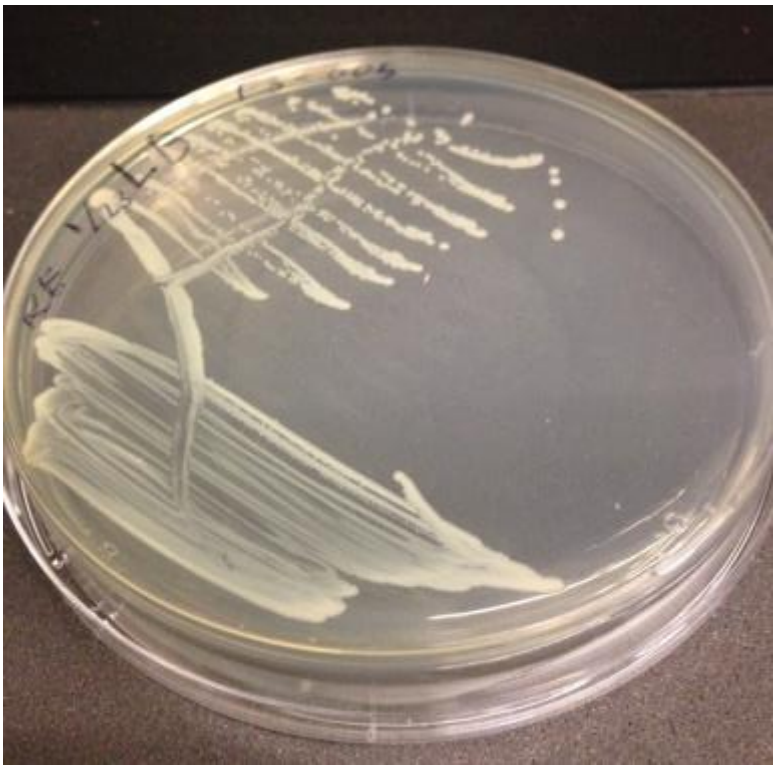

Figure 2. Streak plate isolation on LB plate

5. Confirmation and comparative analysis of siderophore production

In order to confirm siderophore production and identify the single organism responsible for the color change observed in the initial CAS assay, an additional CAS plate was inoculated from the streak plates. Using aseptic technique, a loop was used to pick up a single colony from the LB plate. Subsequently, the inoculum was spotted onto a sectioned CAS plate and the section was labeled accordingly with the sample number. A total of 34 samples were inoculated. After 48 hours in 30°C, the sectioned and labeled CAS plates were removed from the incubator and the zone of coloration was analyzed. The diameter (in mm) of the halo surrounding growth, as well as the degree of clarity were noted. The size of coloration is an indication of the amount of siderophore secreted, while the clarity is related to the efficiency and strength of iron chelation. Samples were given a score of 1, 2, or 3, depending on the clarity of its halo with 1 being

"transparent" and 3 being "opaque." Siderophore-positive samples were inoculated in cryotubes and frozen as stock cultures for future use.

Preparation of the CAS medium is described as follows:

A. CAS indicator solution:

First, 60.5 mg of chrome azurol S was dissolved in 50 ml of ddH_2O. Then, 10 ml of Fe(III) solution (27 mg $FeCl_3$-$6H_2O$ and 83.3 µl of concentrated HCl in 100 ml ddH_2O) along with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) were dissolved in 40 ml ddH_2O, resulting in a dark blue solution. The mixture was then autoclaved for sterilization.

B. Basal agar medium

In a 250 ml flask, 3 g of 0.1 M 3-(N-morpholino) propane sulfonic acid (MOPS), 0.05 g NaCl, 0.03 g KH_2PO_4, 0.01 g NH_3Cl, and 0.05 g L-asparagine were combined in 83 ml ddH_2O. Using 6 M NaOH, the pH of the solution was then brought to 6.8. To reach a total volume of 88 ml, ddH_2O was added. Finally, 1.5 g agar was added while stirring the solution over heat. The solution was then autoclaved for sterilization.

C. Preparation of CAS agar plates

The autoclaved basal agar medium and CAS indicator solution were both cooled in a 50°C water bath. Then, 2 ml of 50% glucose solution was added to the basal agar medium while stirring at low speed, along with 10 ml of the CAS indicator solution. After sufficient mixing, the 100 ml resulting solution was poured carefully into sterile petri plates, with approximately 21-25 ml of blue medium in each plate.

6. Identification of siderophore producing soil isolates

Colony PCR was used as the first step towards elucidation of biological identity of siderophore-producing soil isolates, as determined by a positive result on the CAS assay. Of the

32 positive samples, the 10 organisms with the greatest capacity to chelate iron were pursued further for identification. From the frozen stock cultures, selected samples were re-streaked on fresh LB agar plates in order to provide a fresh and pure colony for the PCR procedure. These plates were incubated for 24 hours at 37°C. The colony PCR protocol is described as follows with Table 1 showing the composition of each PCR reaction.

Table 1. Composition of PCR reaction

PCR Reaction	
Reagent	Volume (µl)
dH_2O	22
10X PCR buffer (Go Taq Flexi)	10
25 mM $MgCl_2$ (Promega)	3
10 mM deoxynucleotide triphosphate mix (Promega)	1
10X Enhancer (Eppendorf)	10
DNA template (from single colony)	1
20 µM Forward primer (63f)*	1.25
20 µM Reverse primer (1387r)*	1.25
Taq polymerase (Go Taq Flexi)	0.5
Total volume	50

*: Forward primer 63f was used with 1387r [25]

To prepare the DNA template, a single colony was picked using a sterile toothpick from the fresh streak plate and dispersed in 10 µl of dH_2O by rotating the tip in the bottom of a 1.5 ml Eppendorf tube. The sequences of the primers are listed below [25].

63f: 5'-CAGGC CTAACACATGCAAGTC-3'

1387r: 5'-GGGCGGWGTGTACA AGGC-3'

PCR reactions were then cycled using the following parameters:

1. 95°C for 3 minutes
2. 95°C for 1 minute
3. 55°C for 1 minute
4. 72°C for 2 minutes
5. repeat steps 2-4 for 29 cycles
6. 72°C for 5 minutes

To confirm DNA amplification, the PCR reactions (5 µl of each reaction) were checked on agarose gels using gel electrophoresis. The ladder used in this step was Lambda DNA/HindIII Marker (Thermofisher). The marker is used for sizing as well as approximate quantification of the DNA fragments.

Figure 3. Gel purification kit from MP Biomedicals

To purify the DNA, gel electrophoresis was repeated, but with 20 µl of each successful PCR reaction. Then, gel purification was carried out using the FastDNA Spit Kit (MP Biomedicals). Purified samples were sent to the East Tennessee State University Molecular Biology Core Facility for DNA sequencing. The chromatograms received will be examined using Chromas, a software used to view sequence data. To identify the organisms to the genus level, corresponding DNA sequences along with primer codes are submitted to Ribosomal Database Project for detection of a "sequence match." This comparison of test sequences to the online database of known 16S rRNA, will allow identification of the siderophore-producer up to the genus level by providing a list of possible candidates based on the degree of homology between the test and known sequences. The use of 16S rRNA gene sequences has become a common genetic marker in the study of bacterial phylogeny. Amplification of genes for bacterial 16S rRNA is desirable

for several reasons. It is found in almost all bacteria, random genetic changes are less common, and this gene sequence is often found in operons. In addition, 16S rRNA can provide genus identification in over 90% of cases [26].

RESULTS AND DISCUSSION

1. Culturing soil samples for microorganisms

Due to the fact that Bare and Forest soil differ significantly in composition and moisture, the presence of microorganisms from the respective environments also differed greatly. After initial inoculations from soil suspensions of the three Bare soil samples (B1, B2, B3) and three Forest soil samples (F1, F2, F3), only nutrient broths inoculated with Forest soil grew a sufficient density of organisms, as indicated by visible turbidity. The optical density of the six samples, after 24 and 48 hours of growth in 30°C are listed in Table 2 below.

Table 2. Optical density of nutrient broth samples: samples inoculated from initial soil suspensions

Sample	OD at 24 hours	OD at 48 hours
Blank	0.000	0.000
B1	0.190	0.307
B2	0.015	0.139
B3	0.015	0.117
F1	0.400	0.821
F2	0.371	0.653
F3	0.283	0.870

Figure 4. CAS plate results: (left) Bare soil CAS assay, a negative result; (right) Forest soil CAS assay, a positive result with colonies surrounded by yellow colored halos.

2. Preliminary CAS Assay

Of the CAS plates inoculated from 48-hour nutrient broth culture, only Forest (F1, F2, F3) plates present growth and coloration. In contrast, Bare soil (B1, B2, B3) plates show no growth or coloration (Figure 4). The analysis of colonies on CAS plates is shown in Table 3 below. This result is consistent with the nature of the distinct soil compositions. Organisms found in complex, and resource-rich environments such as Forest soil tend to face competition for nutrients like iron and therefore have adapted mechanisms to acquire nutritional needs. For this reason, the forest soil samples grew many siderophore-secreting organisms. A comparison of CAS plate results between bare and forest soil samples is shown in Figure 4. It is important to keep in mind that the CAS media is limited and may not support the growth of all organisms that are naturally found in the soil. Therefore, the detection of colonies on CAS media does not exhaust the entire microbial population from these environments. This also explains why liquid

cultures from bare soil showed growth while the corresponding CAS plates did not. HDTMA, a crucial ingredient in the blue agar, can be toxic for some bacteria and therefore limits the culturability of many organisms. Furthermore, Fungi and gram-positive bacteria do not grow well in higher concentrations of HDTMA [27].

Table 3. Results of CAS plates: growth and coloration after 24 and 48 hours

	No. Total Colonies		No. Colonies with halos	
Plate	24 hrs	48 hrs	24 hrs	48 hrs
B1	0	0	0	0
B2	0	0	0	0
B3	0	0	0	0
F1	42	59	4	20
F2	18	40	2	14
F3	23	63	1	20

3. Isolation of siderophore producers

After colonies from preliminary CAS assays were isolated using the streak plate method (Figure 2), individual colonies were spotted on new, sectioned CAS plates in order to compare the extent of siderophore production between organisms on iron-limiting media. The results of those plates are shown below in Figure 5. The majority of sampled colonies confirmed the secretion of siderophore, yielding a total of 32 siderophore-positive isolates, as determined by the presence of yellow and orange-yellow halos surrounding growth. However, not all zones are equal in size and clarity. Measurements of halo diameters and relative assessment of zone clarity is provided in Table 4. Ten of the 32 siderophore-producing isolates with the largest halo

diameters and high degrees of clarity, were noted as those of special interest for further identification.

Although both orange and orange-yellow zones around growth indicate iron chelation, the distinction in color may indicate differences in the chemical nature of siderophores. More understood is the clarity of the zone of coloration and its relation to the strength of iron chelation. Therefore, samples with transparent halos are of particular interest because this is evidence of highly efficient siderophore activity. Meanwhile, zones with large diameters correlate with the relative amount of siderophore excretion. However, the diameter alone cannot suffice in the quantification of siderophore production because variations in size of the zone may also be due to differences in growth rates of the tested organisms. It has been indicated that slower growing cultures make smaller zones than faster growing cultures [28]. Therefore, further chemical analysis is required for precise measurement of siderophore production.

Although the CAS assay is a great tool for the detection of siderophores, the chemical class of the siderophores cannot be confirmed without characterization using colorimetric assays. To determine whether the siderophore being secreted is a hydroxamate or catechol type, the Atkins [29] and Arnows [30] tests can be used.

Growth conditions are also important considerations when trying to optimize siderophore production. With the goal of assessing the efficiency of different iron-uptake systems, bacteria should be studied after being cultured in iron-limited media. These may include Fiss Minimal Media or nutrient agar mixed with dipyridyl to scavenge free iron. Doing so ensures that if an organism does indeed produce siderophores, their expression and secretion is maximized for accurate detection and quantification.

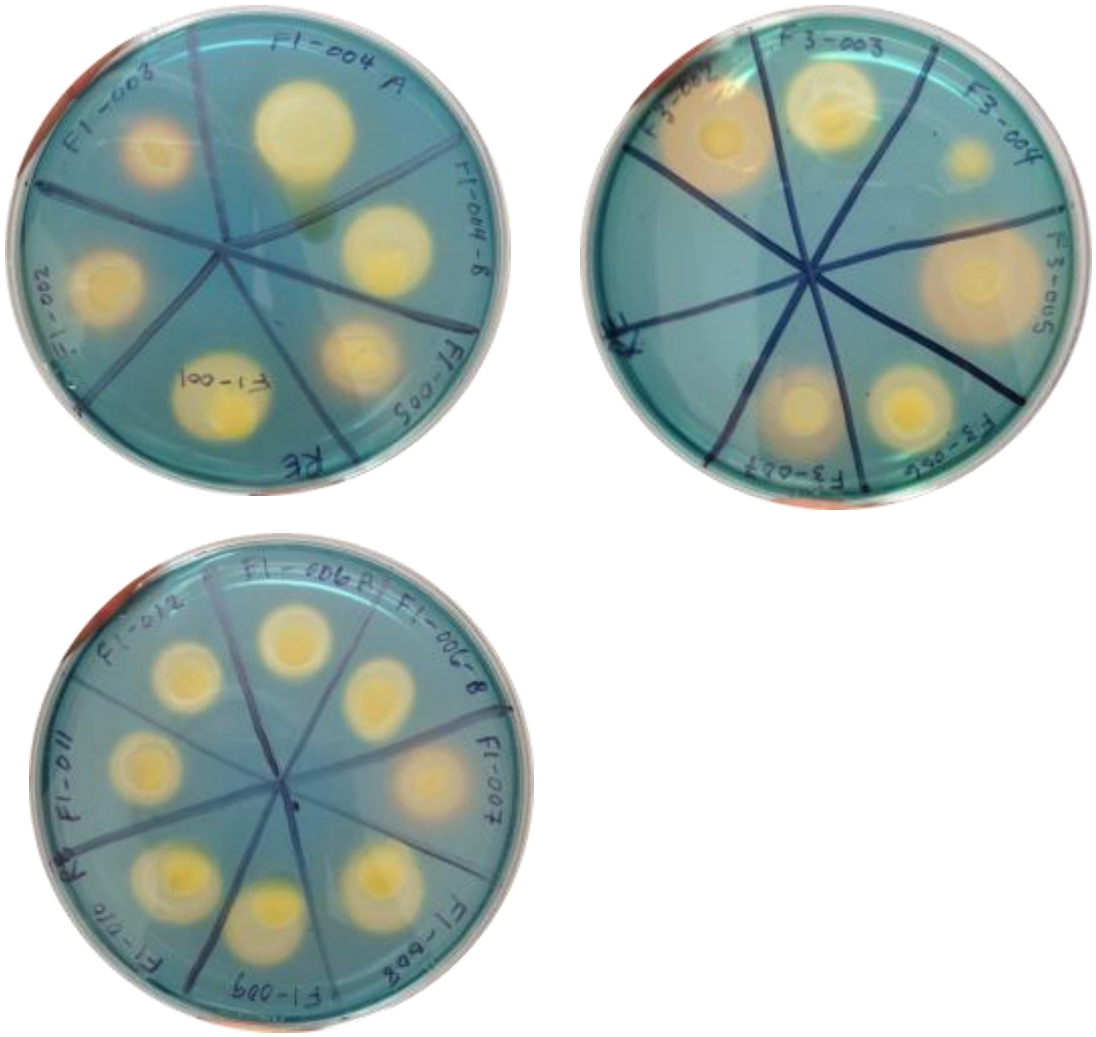

Figure 5. Comparative and confirmatory CAS plates: results of CAS assay to confirm and compare siderophore production after colony isolation from streak plates.

Table 4. Assessment of CAS assay zones of chelation from isolated colonies: A, B, C, isolates from F1, F2, F2, forest soil samples respectively. Key: Color—O=orange-yellow, Y=yellow, Clarity—1=transparent, 2=moderately transparent, 3=opaque. Table cells highlighted in yellow are to distinguish the 10 colonies of greatest interest due to their large halo diameter or high degree of clarity.

A

F1 Colony	Color	Clarity	Diameter (mm)
001	Y	3	16
002	O	1	13
003	O	1	11
004A	Y	3	17
004B	Y	3	16
005	O	1	14
006A	Y	2	12
006B	Y	3	12
007	O	1	11
008	Y	3	16
009	Y	3	14
010	Y	3	14
011	Y	2	11
012	Y	2	11
013A	Y	3	15
013B	Y	3	16

B

F2 Colony	Color	Clarity	Diameter (mm)
001	O	1	21
002	O	2	19
003	O	1	13
004A	Y	3	17
004B	Y	3	15
005A	Y	3	16
005B	Y	3	17
006	Y	2	14
007	Y	1	16
008A	O	1	20
008B	O	1	23

C

F3 Colony	Color	Clarity	Diameter (mm)
002	O	1	20
003	Y	2	16
005	O	1	21
006	Y	2	15
007	O	1	16

4. Identification of strong siderophore producers

Gram staining revealed that samples F2-001, F2-002, and F3-002 are all gram-negative bacilli. However, for further elucidation of biological identification to the genus level, Colony PCR and subsequent gel purification were used to isolate and purify DNA from samples of greatest interest. Of the attempted PCR reactions, only samples F2-001, F2-002, and F3-002 resulted in amplification of DNA when observed under gel electrophoresis. The result of the colony PCR and gel is shown in Figure 6. The location of the bands matches to approximately 1500bp in size as corresponding to the HindIII ladder that was used.

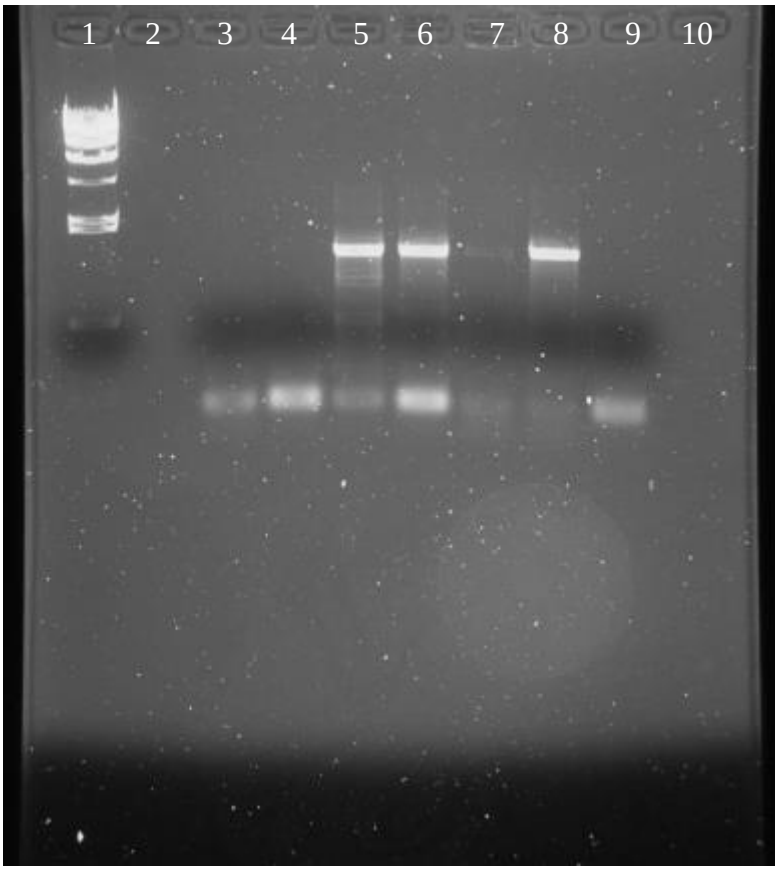

Figure 6. Gel electrophoresis of colony PCR: results from 7 different colonies, only three of which sufficiently amplified DNA. Clear bands are seen in wells 5, 6, and 8 (from left) corresponding with samples F2-001, F2-002, and F3-002 respectively. HindIII ladder is in the first well. The location of the three bands with respect to the ladder suggest the approximate size of the genome around 1500 bp.

These three successful PCR reactions were re-run on 1% agarose gel for gel purification using the FastDNA Spin Kit (MP Biomedicals) to purify the DNA. After this step, a confirmatory gel was used to verify the presence of DNA. The result of that gel is shown in Figure 7. Although the approximate size of the DNA remains the same, the faintness of the bands indicates low concentration of purified DNA. Low concentrations likely explain why genomic

sequencing was unsuccessful and did not yield readable sequences. Unfortunately, for this reason, sequence matching of 16S rRNA genes could not be pursued for identification to the genus level. The quality of the sequencing result depends on the quality of the starting DNA. Thus, the method for purification is an important process that underlines DNA purity and successive genomic sequencing. Obtaining pure DNA at a sufficient concentration is also critical to successful sequencing procedure. There is also diversity in DNA isolation kits and different kits can yield different results with regard to concentration and degree of purity.

Figure 7. Gel electrophoresis of purified DNA: Very faint bands are visible in wells 3, 5, and 7. These correspond to DNA purified from samples F2-001, F2-002, and F3-002 respectively.

CONCLUSION

A more complete understanding of siderophore structure and function provides opportunities to manipulate iron uptake systems for therapeutic use. Already, new drug delivery methods using conjugated siderophores are being investigated as a means of delivering antibiotic drugs. As the medical community struggles to control the dramatic rise of membrane-mediated antibiotic resistance, such developments may offer a promising alternative. In the "Trojan Horse" strategy, siderophores are used as drug delivery agents; siderophore moieties are synthesized to resemble the natural iron-binding capacity, then subsequently conjugated to desired antibiotics which cannot independently cross the microbial membrane [31]. However, the efficacy of these drug delivery systems depends on siderophore analogs which are closely identical in structure and bioactivity to natural siderophores, therefore necessitating more work to identify such characteristics necessary to optimize siderophore-like properties. In addition to new antibiotic alternatives, a compound which effectively binds iron may offer treatment prospects for iron overload disorders like hereditary haemochromatosis or complications which arise from red blood cell transfusions. Iron chelating compounds which mimic siderophores can scavenge and remove excess iron from the body [32]. These methods can also be extended to treat phytopathologies. Siderophores like pyoverdine, produced by Pseudomonads, can be used to capture iron in the soil and subsequently limit the amount of iron available for plant pathogens to prevent soil-borne plant diseases [33]. The applications of siderophores are vast and promising, but the success of iron chelators for medical and plant therapies depends on more precise knowledge about the production and nature of iron uptake systems in relation to the microbial populations which produce them. This quest however, will require the continued biological identification of diverse siderophore producers and the environments that foster them.

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