Investigation of Antibiotic Production by Actinomycetes from Soil
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Introduction

There has been extensive research done by many scientists with bacteria in the genus Streptomyces. They are a good source of useful antibiotics such as streptomycin and bleomycin. Our lab has amassed a large (over 300 isolates) collection of soil bacteria which secrete compounds that inhibit other bacteria. The collection of these "candidates" has been biased against including Streptomyces, to deliberately avoid this popular group. However, about two dozen of our candidates have the colony morphology (burrowing, brown) and microscopic appearance (Gram variable, filamentous) of actinomycetes. We have recently undertaken investigations of these candidates.

Methods

When we started to research the filamentous bacteria that we had in our soil samples we needed to narrow our selection down as not to have a purely filamentous species only to find out later that there were duplicates that could have been eliminated earlier on. The experiment that we did to eliminate any duplicates is called the "stale cage method". This experiment takes advantage of the way that the bacteria thrive. They secrete out a fluid which scientists use as antibiotics, that will kill anything that comes into contact with it enabling the bacteria that are secreting the fluid to consume any food sources available. As the old in the colonies die and the food source in the colony because scarce from earlier populations using it up, the young are able to feed on the carcasses of the previous generations. The fluids secreted by one type of bacteria will never kill that type of bacteria but will kill other types. Therefore we deduced that if we put a stripe of bacteria on an agar plate and let it grow and allow it to secrete its fluid into the agar then when we streak other bacteria either the growth will be inhibited by the fluid or it will not. If the growth of the second bacteria is inhibited by the fluid secreting agar then those two bacteria are not making the same compound and are not the same. If the growth is not inhibited than we compare the action of the bacteria to the action of all the other bacteria and look for similarities. Based on the reaction to and from other bacteria we can decide that two bacteria are either the same or so similar that we are able to eliminate one of them.

Results

In Table 1, we see the results of pairwise comparisons between each of the 24 strains studied. The columns ("Killers") indicate the strain inoculated first (see PP56 in Figure 1), and the rows the strain inoculated second. Analysis shows that some pairs of candidates fail to inhibit each other, and in addition show nearly identical effects on the remaining candidates. We developed a set of "Strict Criteria" for identity, based on the unplanned inclusion of two candidates (P10 and P27) that were actually duplicates saved from a single purified soil bacterium. These criteria have been used to attempt to identify other identical isolates, and suggest that none of the other candidates are identical to each other. We have also developed a set of "Suggestive Criteria", used to identify other potentially identical (or highly similar) candidates. For example, these Suggestive Criteria indicate behavioral similarity among Candidate #24, #22, #21, and #1 (Table 2).

Future Directions

Though these results support the continued efforts to isolate and identify the antibacterial compounds produced by these eight candidates, the approach is only of limited general utility in eliminating candidates from further consideration. There are hundreds of species of Streptomyces, and far more antibacterial compounds are produced than the four tested here. The demonstration that one candidate produces a specific antibiotic will only come with a bit of luck (see Candidates #10 and #27 below). However, our full set of Streptomyces candidates will be screened in this manner. We will also increase our test bank of E.coli variants resistant to known antibiotics.

Identification of unknown bacterial candidates using sequential gene restriction patterns.

The misuse and overuse of antibiotics has led to a rapid evolution of antibiotic resistant pathogens. This evolution of resistance has also led to other antibiotics becoming ineffective. Many of these antibiotics that are used today are chemicals produced by bacteria to kill competing species in their natural environments. The genus of bacteria in question produces approximately 85% of the antibiotics utilized in the medical field today. These bacteria are widely distributed in terrestrial environments and have long been a source for antibiotics. The genus of bacteria encompasses a large number of species; therefore classifying these bacteria becomes progressively more difficult. Phenotypic tests are already being utilized to separate these bacteria in an attempt to more closely related subtypes. At this point genotypic testing would be a helpful addition to the identification process.

The compilation of genetic material that is used for the identification process is acquired by using PCR to amplify 16S rRNA, a gene common to all cells, including actinomycetes. Polymerase Chain Reaction is performed using specific primers to amplify the gene of interest. To perpetuate the identification process, restriction endonuclease digests are performed on the PCR product. The protocol being utilized as reference for the identification of the unknown candidates is a sequential set of digests of the amplified PCR product using the restriction enzymes Kpn I and Sph I, respectively (Cook and Meyers, 2003). According to Cook and Meyers this sequential digest would be able to identify if the unknown candidate to the genus level, for our needs, determining if the bacteria in question were specifically Streptomyces.

If the fragment pattern follows the procedure outlined in Table 1 below (to determine genus), and the candidate is identified as a Streptomyces, then the DNA can be sent out to be sequenced. Once the DNA is sequenced, the results are then compared to published databases containing gene sequences of known actinomycetes. The comparison will provide the information needed to determine if the candidate is already identified.

Furthermore, the initial phenotypic tests that were performed on the unknown bacterial candidates produced data that has proven very useful and has been a source of direction for the implementation of genotypic similarities may be indicative of genotypic similarities as well. Similarities at the genus level are extremely in further determination of the species of the unknown candidates. So, instead of blindly testing many candidates, a more specific approach to eliminating "multiples" of candidates or already "known" candidates from the large stock of samples is a perfect way to sharpen the focus of the experiment. This will further allow this genotypic approach to then be utilized in the identification of novel bacterial candidates in a more efficient manner.

The bacteria of immediate interest are shown in Table 4. The inhibition testing provided significant data that suggests candidates TAS 27 and TAS 10 may be in fact the same bacteria, and additionally may be a known species, Streptomyces venezuelae.

Preliminary Assignment of Candidate #10 (#27) as Streptomyces venezuelae

The work presented in Table 1 indicated that Candidate #10 (#27) was very similar to Streptomyces venezuelae in its pattern of inhibition of the other candidates studied. This shows a portion of the table, and it is apparent that there is agreement at the level of the "Streptomyces". We have begun work designed to confirm that Candidate #10 (#27) produces the antibiotic chloramphenicol, and are in fact re-isolating this organism. Preliminary genotypic analysis of this particular candidate #10 (#27) grows well on plates media containing 5 µg/ml of chloramphenicol. Second, Candidate #10 (#27) does not inhibit the growth of E.coli CMR (see above). Additional experiments (based on genomic comparisons) are underway to confirm this assignment.

<table>
<thead>
<tr>
<th>Candidate</th>
<th>Imp 1</th>
<th>Imp 2</th>
<th>Imp 3</th>
<th>Imp 4</th>
<th>Imp 5</th>
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<tbody>
<tr>
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</table>

Introduction

The candidates that show filamentous growth are likely to be Streptomyces spp. We have screened some of these isolates for production of well-characterized antibiotics known to be produced by organisms in this genus. Every strain of E.coli that we screened did produce a clear band of inhibition against these bacteria, and 6 of these candidates have the colony morphology (burrowing, brown) and microscopic appearance (Gram variable, filamentous) of actinomycetes. We have recently undertaken investigations of these candidates.

Methods

The E.coli strains used are WT (K-12) and derivatives of them carrying different antibiotic resistance plasmids. The candidates were grown on 24 plates of agar with each of the four test strains listed above. Side striping tests were performed as previously described. Each candidate was inoculated in a broad strip across a nutrient agar plate, incubated at room temperature for three days, and then inoculated again in perpendicular strips with E.coli test strains. Four of the antibiotics investigated are produced by Streptomyces: Cin (chloramphenicol), Tc (tetracycline), Km (kanamycin), and Sp (spectinomycin). The fifth, Amp (amoxicillin), is derived from penicillin, a compound produced by a fungus (Penicillum).