Isolation and Classification of Nitrogen Fixing and Phosphate Solubilizing Bacteria

Rattanah Mahal[1], Marcus Schicklberger[2], Romy Chakraborty[2]

[1] Reedley College [2] Lawrence Berkeley National Labaroatory

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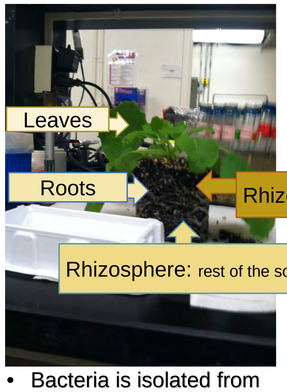
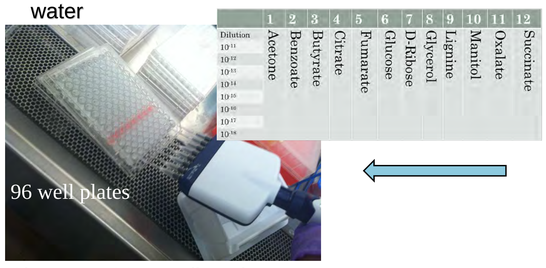
Abstract

The elements N and P are essential elements for the growth and survival of plants. Yet plants are limited in their ability to fix elemental N from the atmosphere, as well as hydrolyze organic and inorganic phosphorous from insoluble compounds. To compensate for this shortcoming, plants form a mutualistic relationship with bacteria to obtain usable nitrogen and phosphate. Although the majority of plants that form nitrogen-fixing root nodules are in the legume family, new species of N_2-fixing bacteria have been discovered in association with non-nodulating crops. The goal of this research lies in the identification of beneficial bacteria capable of fixing nitrogen and solubilizing phosphate . In this study, High-Throughput Isolation (HTI) was used to identify N fixing and/or P solubilizing bacteria from tobacco (Nicotiana tabacum). Overall five different phylogenetic orders were identified: *Enterobacteriales, Bacillales, Actinomycetales, Rhizobiales*, and *Sphingobacteriales*. The strain Kosakonia Oryzae ola 51 from the order *Enterobacteriales,* in particular, was identified as a nitrogen fixers by amplifying the *nifH* gene; those results were then confirmed by the acetylene reduction assay. Ultimately, the goal of this research is to decrease fertilizer dependency by engineering plants to attract diazotrophic bacteria.

Bacteria Extraction



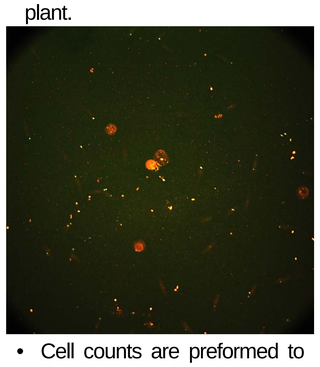
- Bacteria is isolated from Nicotiana tabacum: in four sources: leaves (including the stems), rhizosphere, rhizoplane, and roots.
- The roots and leaves were sterilized using 1.5% bleach solution and washed with water



Sixteen 96 well plates were prepared with carbon sources, agar, dilution and medium:

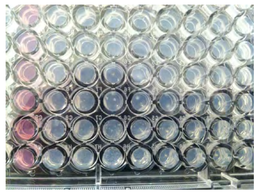
- Pikovskaya(phosphate screener)
- NM8 (phosphate screener)
- HGB (nitrogen deficient)
- NFB (nitrogen deficient)

Bacteria and O_2 were dislodged from the leaf surface by submerging the leaves in a 5 mM of Na-pyrophosphate solution. Bacteria from inside the leaves was obtained by mechanical breakdown of the plant.



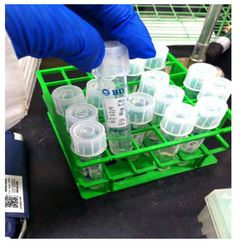
- Cell counts are preformed to determine dilutions ($5x10^7$)
- Dilutions are done to increases the probability of growing a single bacterial colony in one well

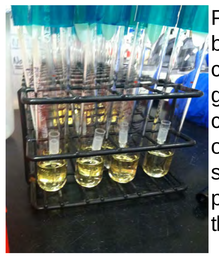
The Four Stages of Isolation



Single bacteria colonies are picked from the 96 well plate

Bacteria are grown in cultures containing medium and carbon source.



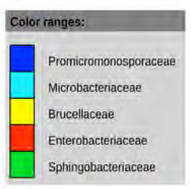


Pure bacteria cultures are grown consisting of a single strain picked from the LB plate

Bacteria are streaked out on LB agar plates to further isolate a single strain

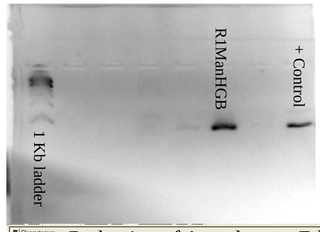
Results

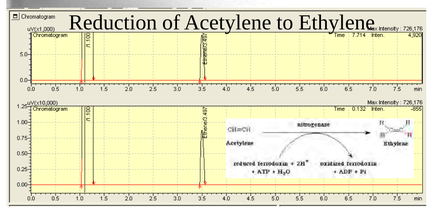
Isolate	Order	Closest Relative in the NCBI database	Similarity
R1ManHGB	Enterobacteriales	Kosakonia Oryzae ola 51	99%
RS7OxaP1	Bacillales	Paenibacillus amylolyticus strain JCM 9906	99%
R4ManHGB	Actinomycetale	Isoptericola variabilis strain 225	99%
RS3FumNFB	Rhizobiales	Ochrobactrum haematophilum strain CCUG 38531	99%
RP3GluNFB	Sphingobacteriales	Sphingobacterium sp. 21 strain 21	99%



- After DNA was extracted from the isolates five different orders were identified.
- The phylogenetic tree breaks these orders down to the family level with the closest related strain.
- From these isolates R1ManHGB was identified as a nitrogen fixer by amplifying the *nifH* gene and detecting ethylene by using the Acetylene Reduction Assay.

Screening for Nitrogen Fixers





- Since the nifH gene is highly conserved, it was used as a screen to identify nitrogen fixing bacteria by amplifying the *nifH* gene in the extracted DNA by PCR.
- We used Pol F and Pol R primers as well as Adiosella as the positive control and DEPC water as the negative control
- One assay used was the Acetylene Reduction Assay which takes advantage of the dinitrogenase's property to reduce acetylene to ethylene.
- This graph shows the production of ethylene by Gass Chromatography analysis of the strain R1ManHGB

Screening for Phosphorous Solubilizers

- Phosphorous solubilizing bacteria are known to release organic acid to solubilize phosphorous. That property was used to screen for phosphorous solubilizing bacteria by using phosphorous selective medium.
- Pikovskaya medium contains tricalcium phosphate and bromophenol blue (an indicator).
- Another medium used was the NM8 medium which contains hydroxyapatite [Ca_5 (PO_4)$_3$ OH] and bromophenol blue.
- The starting pH of both medium is 7 and a change in color from blue to yellow indicates drop in pH which also indicates the presence of potential phosphorous solubilizing bacteria.

Conclusion

We were able to successfully isolate diazotrophic bacteria from Nicotiana tabacum. Most of the bacteria were from the strain Kosakonia Oryzae ola 51 which can fix nitrogen as evidenced by the *nifH* PCR and the acetylene assay. An assay for screening for phosphate solubilizing bacteria is still in development.

Future Projects

- Measuring and comparing growth of re-inoculating hydroponic plants with the isolated bacteria
- SDS-PAGE analysis on the isolated bacteria
- IC of prospective phosphorous solubilizing bacteria to detect solubilization activity.

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Contact Information:
Rattanah Mahal
Reedley College
Email: rattna.mahal15@gmail.com

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