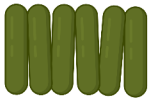
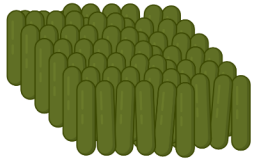
Growing Microbial Strain & Isolating and Sequencing Model Phages

Growing Model Pathogen



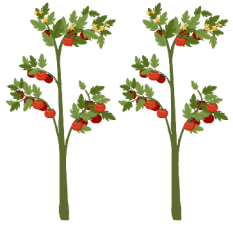
R. solanacearum strain QL-Rs1115 served as model plant pathogen throughout experiment

Grow *R. solanacearum*: 24h at 30°C in nutrient broth, shaking (170 rpm) to avoid bacteria settling at the bottom, creating samples for subsequent experiments

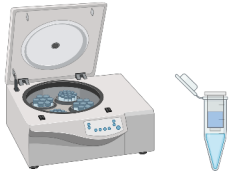


Results in many model plant pathogens to be used for different investigations and phage combinations/treatments

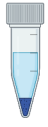
Isolating and Preparing Model Phages



Four lytic phages: NJ-P3, NB-P21, NC-P34, NN-P42 were selected as model phages against *R. solnacearum*



Grow each phage with stock *R. solanacearum* for 24h and centrifuge and filter to isolate and purify phages from bacteria



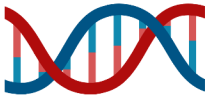
Contains one phage type (100%)

Contains two phage types (50%) each

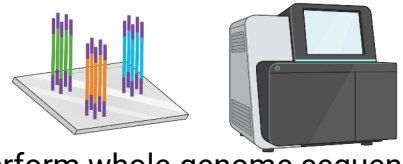
Contains three phage types (33%) each

Assemble phages into communities with 1, 2, or 3 phage combinations (100%, 50% or 33%) for experiments comparing number of phage types used with disease incidence (Figure 1); do this to create all possible combinations with four phages

Sequencing Model Phages



Isolate phage chromosomal DNA of each phagefollowing ABigen DNA purification kit instructions



Perform whole-genome sequencing using Illumina Hiseq 400 to obtain genomes of all four phage types

Revealed NJ-P3, NB-P21, NC-P34, NN-P42 have greater than 99.3% genome similarity; uploaded to NCBI database so further investigation of these phages may be performed

Collection of Soil Samples to Analyze Phage Combination Efficacy in Greenhouse and Field Experiments

In Greenhouse:

Surface-sterilize tomato seeds and germinate for 2d on water-agar plates to prevent presence of microbial disease before planting

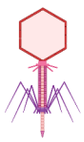
Sow seeds into seedling plate to allow tomato plant to grow further

At three-leaf stage: transfer tomato plant to eight-cell seeding tray with soil devoid of *R. solanacearum* to allow further growth

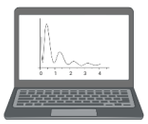
After 7d: inoculate all roots with *R. solanacearum* using a pipette to introduce model pathogen to rhizosphere

After 2d

Control group containing only pathogen and no additional phages



Experimental: Inoculate plants with different phage combinations (1, 2, or 3 phage types)



After 38d: record disease incidence of plants, collect rhizosphere samples from two randomly chosen healthy plants from each respective treatment (slightly diseased if none were healthy) for further phage and microbiome analyses

Mix 1g of each soil sample with water, filtering and storing to isolate phages

Take other 2g of each soil sample and store in 15% glycerol to freeze for further analysis of *R. solanacearum* population densities and bacterial community

In Field:

Grow tomato seedlings for 30d to allow initial growth before placing in field

Field was naturally infected with *R. solanacearum* for the last decade; added phages after 7d to allow tomatoes to adjust to field rhizosphere

Control: only phage buffer

Experimental: single phage treatment

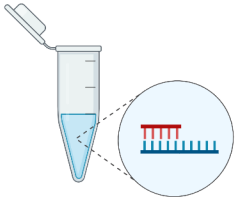
Experimental: all four phage-type treatment

After 45d

Record bacterial wilt disease severity on a 4 point scale for all treatment groups

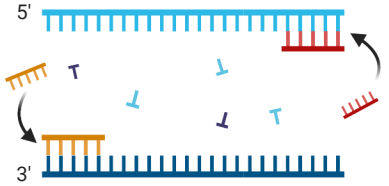
0	1	0	0	4
no leaf area wilting	1-25% leaf area wilting	26%-50%...	51%-75%...	76%-100%...

Quantification of *R. solanacearum* and Phage Densities

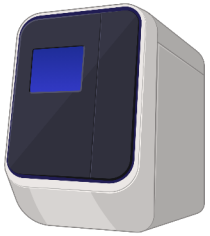


Take previous rhizosphere samples from greenhouse experiment and extract DNA using Power Soil DNA isolation kit to isolate DNA from sample

Use spectrophotometer on isolated DNA to determine DNA concentration

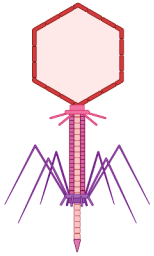


Use primers targeting fliC gene on pathogenic DNA bacteria for density measurements

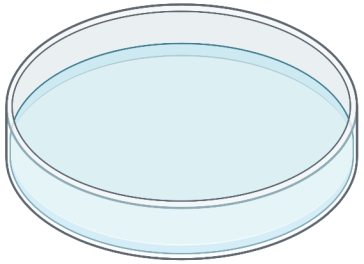


Run qPCR analysis to quantify density

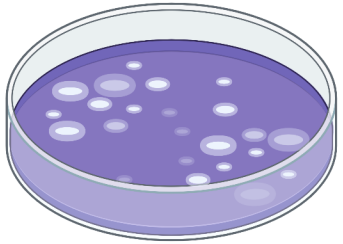
Phage Density Quantification



Take isolated phage samples from greenhouse experiment

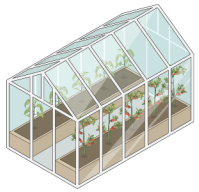


Conduct spotting assay to quantify phage density



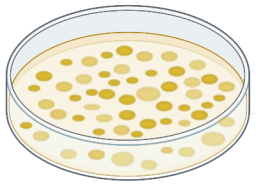
Allow growth for 24h in order for plaques to generate, allowing for phage density calculation through PFU

Quantification of Phage Resistance in *R. solanacearum* & Cost of Resistance

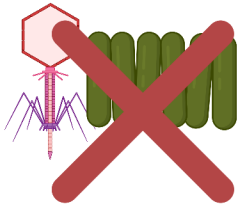


Refer to greenhouse rhizosphere samples

Perform serial dilutions on cryopreserved rhizosphere samples and plate on semiselective agar medium to isolate *R. solanacearum*



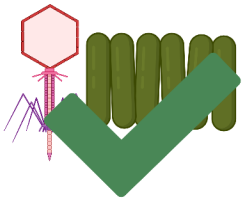
Let grow for 48h to allow colony formation then isolate eight random colonies from each phage combination therapy (separate experimental conditions)



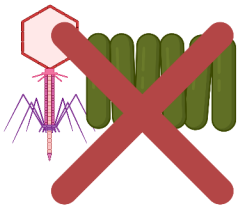
Control: measure growth of ancestral (unexposed) *R. solanacearum* in presence and absence of ancestral and evolved phages

Experimental: measure growth of evolved (exposed) *R. solanacearum* in presence and absence of ancestral and evolved phages

Use spectrophotometer after 24h of introducing phage to quantify growth



Experimental: Add exposed coevolved strain to model root with essential nutrients typical of tomato roots



Control: Add ancestral strain to same environmental condition as ancestral strain

Analyze cost of resistance as reduction in bacterial maximum density from spectrometer when compared to ancestral strain: Measuring bacterial density every 4h for 24h

Comparing Competitive Ability of Evolved *R. solanacearum* with Ancestral *R. solanacearum* (Sup. Fig 7 fits in with Fig 2)

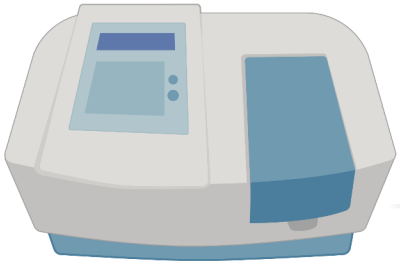
Flourescently label ancestral, suceptiple pathogen red to measure growth using optical density

Plate on competition assay to coculture the strains -->

Control: absence of evolved pathogen (*R. solanacearum*)

Experimental: presence of evolved pathogen (*R. solanacearum*)

24h



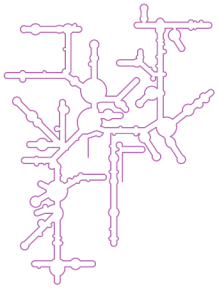
Measure red fluorescence signal to calculate growth of two groups

Equation used once OD600 measured

Competitive ability = (flourescence in control - fluorescence in experimental) / (fluorescence in control) * 100

Changes in Rhizosphere Microbiome Composition (uses Illumina MiSeq)

Take rhizosphere soil samples



Amplify 16S rRNA gene to identify different types of bacteria present in the sample's microbiome

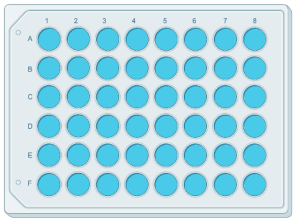
OTU = groups of closely related individuals

Assign OTU (operatuonal taxonomic unit) cutoff at 97% identity to compare sequences with USEARCH and Ribosomal Database Project database

Upload sequence data to NCBI and microbiome list accession numbers for further analysis and comparisons between soil samples and phage therapies

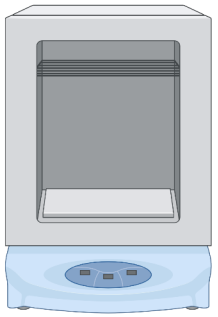
Testing for Direct Effects of Phage on Bacterial Community Composition and Diversity

Prepare rhizosphere microbiome inoculum using soil-sterile water mixture with same soil used in greenhouse

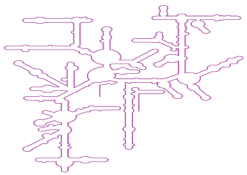


Inoculate this into 48-well microplates with nutrient broth medium to allow growth of bacteria

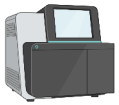
- Control: no additions; just rhizosphere inoculum
- Experimental: add *R. solanacearum* alone to samples
- Experimental: add three-phage combinations to sample
- Experimental: add three-phage combinations and *R. solanacearum* to sample



48h of culturing with shaking to ensure nutrient availability



Extract DNA to measure bacterial diversity and target 16S rRNA gene to characterize microbiome



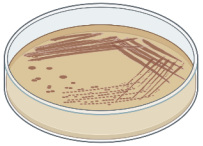
Refer to Illumina MiSeq Methods Diagram

Testing *R. solanacearum* infecting phages against nonpathogenic bacteria

Isolate random selection of culturable bacteria from soil used forgreenhouse experiment by plating on nonselective agar media

Serial dilute soil and incubate to generate bacterial colonies

Randomly pick colonies and identify using 16S rRNA sequencing



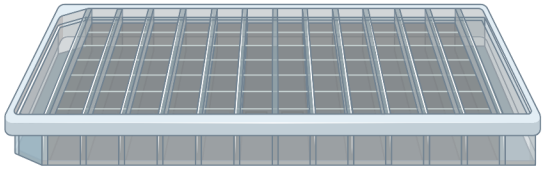
Conduct streak assay with phages to see if bacterial growth was inhibited by streaked phage

Control: streak assay with no phages added to bacterial sample

Measuring *R. solanacearum* inhibition by Nonpathogenic Bacteria

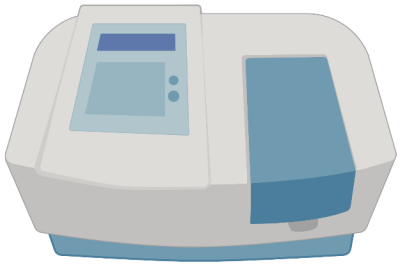
Fluorescently tag *R. solanacearum* with mCherry so growth can be tracked

Control: Grow pathogen alone; measure everything in triplicates to check for procedural error



Experimental: Co-culture with even starting volumes in 96 well plate with nonpathogenic bacteria and *R. solanacearum*

48h



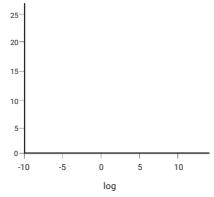
Measure bacterial densities using optical density and spectrophotometer

Calculation for Relative *R. solanacearum Density*

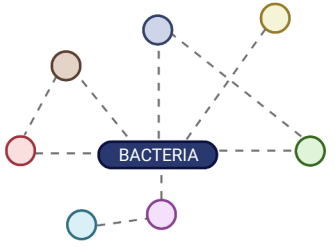
AmCherry (intensity of red fluorescence) / OD (optical density) –>

pathogen inhibition = percentage reduction in pathogen growth relative to Control group growth

Various Statistical Analysis Used with Data and Model Creation (Mentioned Programs)

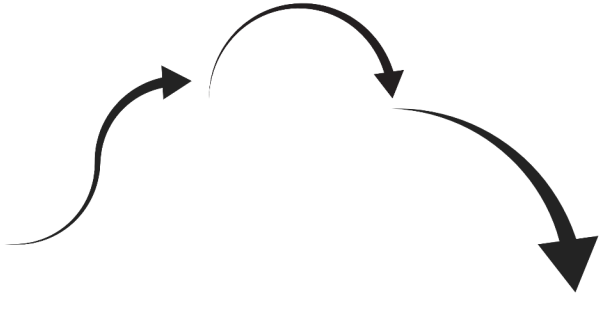


Before numerical analyses, transform density data into log form to make programs easier to work with



Networks (Figure 3) drawn using Gephi and NetShift which identify driver taxa

Used NMDS (non-metric multidiminesional scaling) in R to observe patterns of similarity between rhizosphre microbiome samples shown in Figure 3 as composition



PLS-SEM (partial least squares-structural equation modeling) used for Figure 5 to create path model in quantifying ecological and evolutionary effects of phage therapy on disease incidence

Figure Summaries

Figure 1: Demonstrates usage of more than one phage type leads to lower disease incidence and lower pathogen density.

Figure 2: Highlights that, as phage therapy selects for more resistant pathogen, there is a fitness trade-off as phage-resistant pathogen experience lower carrying capacity in the absence of phage.

Figure 3: Demonstrates the effects phages may have on the rhizosphere, including effects on the diversity and makeup of the microbiome based on OTUs and presence of driver taxa.

Figure 4: Shows that phage effects on the rhizosphere are more indirect, and may work by limiting the effects to the community brought about by the pathogenic bacteria, *R. solanacearum*.

Figure 5: Shows a model/schematic that attempts to highlight the ecological and evolutionary pathways stemming from phage therapy that relate to disease incidence. (Used PLS-SEM modeling)