Characterizing the role of *fasR* in phytopathogenic *Rhodococcus*

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Introduction

- Phytopathogenic *Rhodococcus* is a Gram-positive, mycolic acid containing bacteria that causes leafy gall disease (**Figure 1**).
- Broad host range and emerging threat to the nursery industry (Putnam and Miller, 2007).
- Virulence loci *fas* (or variant), *fasR,* and *att* are suggested to be necessary to cause leafy gall disease (**Figure 2**).
- Isolates D188 and A21d2 show notable difference in structure of their virulence loci (**Figure 2** and **Figure 3**).

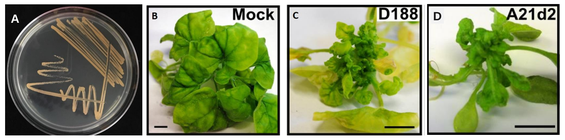

Figure 1. Phytopathogenic *Rhodococcus* causes leafy galls in plants. (A) Streaked plate of phytopathogenic *Rhodococcus* strain D188. **(B)** Mock (water) inoculated *Nicotiana benthamiana.* **(C)** *N. benthamiana* inoculated with strain D188. **(D)** *N. benthamiana* inoculated with isolate A21d2.

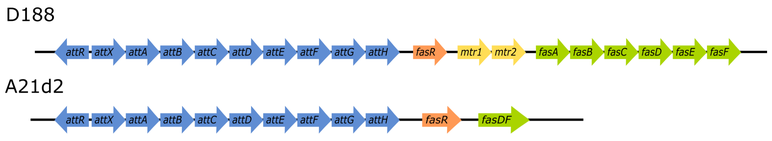

Figure 2: Schematic of virulence loci of two phytopathogenic isolates of *Rhodococcus*. Top: three loci: *att* (blue), *fasR* (orange), and *fas* (green) of strain D188 implicated in phytopathogenicity. The loci flank two genes, *mtr1* and *mtr2* (yellow), predicted to encode methyltransferases; these have not been implicated in virulence. The virulence loci are carried on the plasmid pFiD188. Bottom: three virulence loci: *att* (blue), *fasR* (orange), and *fasDF* (green) of strain A21d2. FasDF is predicted to have the functional domains of FasD and FasF of D188. These three loci are hypothesized to be present within the chromosome of A21d2.

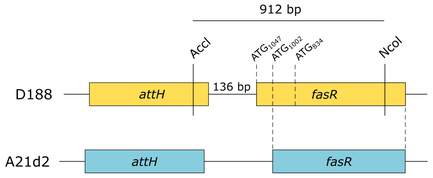

Figure 3. Schematic of *fasR* in D188 and A21d2. Comparison of the *fasR* coding sequence of D188 (top) to *fasR* of A21d2 (bottom). In D188, the *fasR* allele has three in frame ATG codons. The *Acc*I and *Nco*I restriction sites are also depicted; these demark the region that was deleted in the mutant strain developed by Temmerman *et al.*, 2000. The *fasR* allele of A21d2 has only a single ATG start codon. Boxes = coding sequences; lines = non-coding regions.

Hypotheses

- The *fasR* gene is necessary for phytopathogenicity of *Rhodococcus.*
- *fasR* of D188 and A21d2 are homologous in function.

Development of knockout mutants of *fasR* and DNA constructs

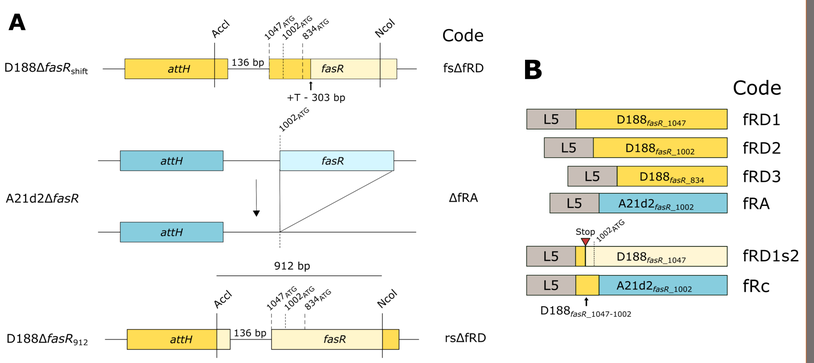

Figure 4. Development of knockout mutants of *fasR* and DNA constructs. (A) Schematic shows three knockout mutants. D188Δ*fasR*$_{shift}$, a frameshift mutant with a thymine inserted at 303 base pairs downstream of 1047 start site. D188Δ*fasR*$_{912}$, a deletion mutant made with restriction sites *Acc*I and *Nco*I (Temmerman *et al.*, 2000) A21d2Δ*fasR*, a non-polar deletion mutant developed by homologous recombination. **(B)** Constructs developed with an L5 constitutive promotor and different mutant alleles of *fasR* including different in-frame ATG variants, site-directed mutagenesis, and a chimera.

Functionally similar polymorphic *fasR* is necessary in phytopathogenic *Rhodococcus*

Polymorphic *fasR* functions similarly in *Rhodococcus* isolates D188 and A21d2

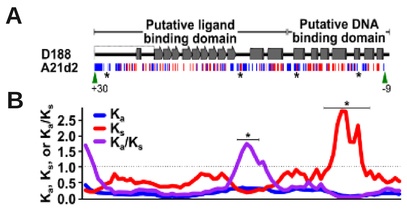

Figure 5: *R. fascians fasR* alleles are polymorphic (Creason *et al.*, 2014). (A) Predicted domains of *fasR*. Synonymous (red), non-synonymous (blue), and INDELs (green arrows with the number of nucleotide differences). Clusters of non-synonymous substitutions in A21d2 *fasR* are denoted with "*". **(B)** Sliding-window analysis of synonymous and non-synonymous substitutions in *fasR* of A21d2 paired with *fasR* of D188.

Three in-frame ATG codon found in D188 *fasR*

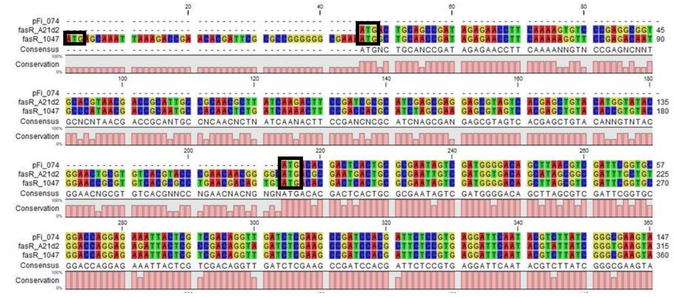

Figure 6. Nucleotide alignment of *fasR* sequences show three ATG codons in-frame in *fasR* from strain D188. Each start site is highlighted by a black box. Alignment was developed using CLC Sequence Viewer (Qiagen Company, Hilden, Germany).

An engineered nonsense substitution after the first ATG suggests sequences upstream of the other ATG codons are necessary for expression

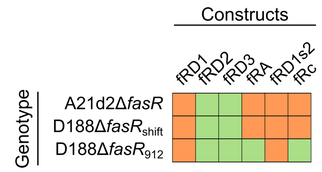

Figure 7: Heat map of pathogenicity phenotype of mutant 18 construct combinations. Orange designates mutant as pathogenic and green designates mutant as nonpathogenic. Pathogenicity is based on the ability to inhibit root growth of *N. benthamiana* seedlings similar to wildtype.

fasR is necessary but not sufficient for virulence of isolates of phytopathogenic *Rhodococcus*

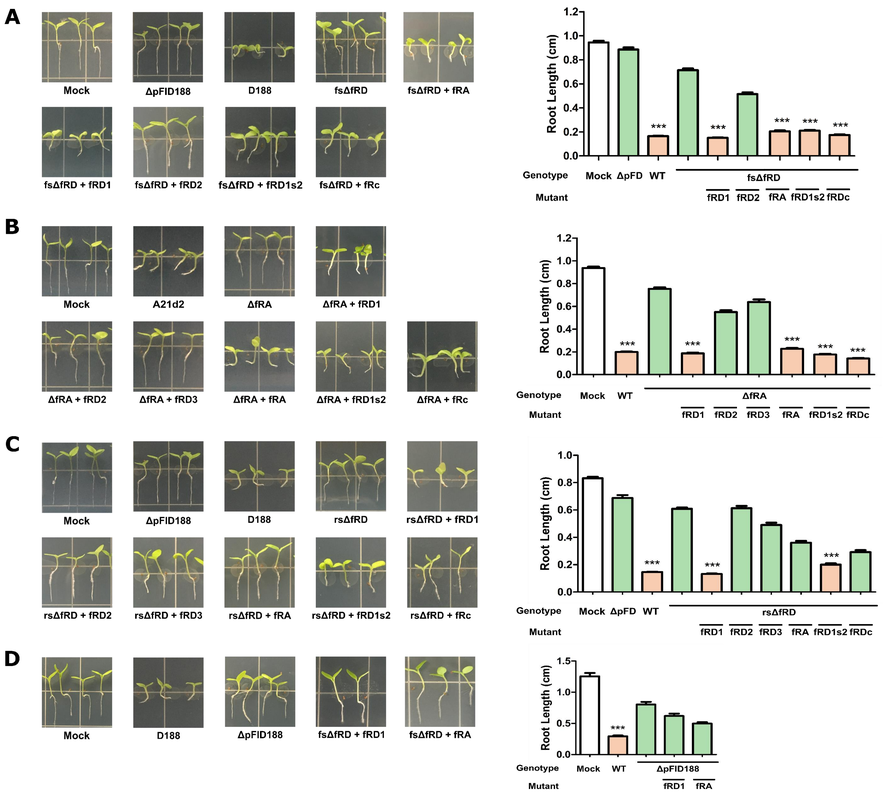

Figure 8. Functionally similar polymorphic *fasR* is necessary but not sufficient for phytopathogenicity of *Rhodococcus*. (A) *fasR* gene is necessary for pathogenicity of D188. (B) *fasR* gene is necessary for pathogenicity of A21d2. (C) The D188Δ*fasR*$_{912}$ is polar and cannot be used to inform on necessity of *fasR* in pathogenicity. (D) The *fasR* gene is not sufficient for phytopathogenicity. For A, B, C, and D, the following is the same. On the left, three-day old *N. benthamiana* seedlings inoculated with indicated isolates or water (mock). Photos were taken at 7 dpi. At least three replicates were performed with 40 individuals per experiment. On the right, quantification of root length of part A. Error bars indicate standard error of the mean (SEM); *** represents a significant difference (p-value < 0.001) between treated plants and water (mock). Mutants were additionally tested on mature plants for the formation of galls. Mutants that were phytopathogenic by the ability to inhibit root growth on *N. benthamiana* seedlings showed consistent pathogenicity results by the ability to form galls on mature *N. benthamiana* plants (data not shown).

Summary

- *fasR* is necessary for phytopathogenicity.
- The two *fasR* variants are functionally homologous and potentially regulate the same genes.
- Potential 5' untranslated region involved in regulating *fasR* expression.
- FasR is key to the co-option model by potentially misregulating the expression of other genes and reprogramming the genome for pathogenicity.

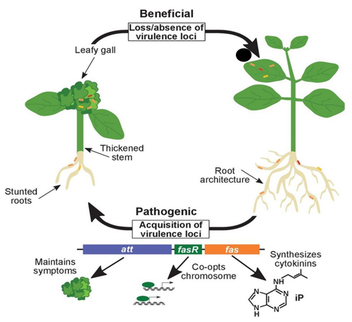

Figure 9: Virulence loci *fasR, fas*, and *att* co-opt the *Rhodococcus* genome for pathogenicity (Savory *et al.*, 2017). Model shows evolutionary transition in *Rhodococcus* between mutualist and pathogen.

Future Directions

- Differentiate ATG codons in D188 *fasR* (2nd vs 3rd) and ATG codons in A21d2 *fasR.*
- Assess transcriptional and translational expression of *fasR* mutants via qRT-PCR and western blots respectively.
- Determine if *fasR* is regulated via a post-transcriptional mechanism.

References

1. Putnam ML and Miller ML. (2007) *Rhodococcus fascians* in Herbaceous Perennials. Plant Disease. 91, 1064-1076.
2. Temmerman W, Vereecke D, Dreesen R, Montagu MV, Holsters M, and Goethals K. (2000) Leafy Gall Formation is Controlled by *fasR*, an AraC-Type Regulatory Gene in *Rhodococcus fascians*. Journal of Bacteriology. 182, 5832-5840.
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Acknowledgements

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