AN ABSTRACT OF THE THESIS OF

Danielle M. Stevens in Biochemistry and Biophysics

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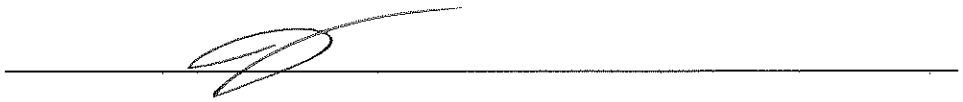

Jeff H. Chang

Bacteria are critical to the health of eukaryotes and the ecosystems in which they persist. Some symbiotic interactions are mutualistic in which both microbe and host benefit from the partnership. Other interactions are parasitic, and the microbe typically benefits at a cost to the host. *Rhodococcus* is a genus of bacteria in which members are frequently associated with plants. We sought to model the evolution of phytopathogenic lineages and mechanisms of virulence of *Rhodococcus*. Findings demonstrated that the acquisition of a plasmid carrying three loci implicated in virulence can transition *Rhodococcus* bacteria from being potential mutualistic bacteria to pathogenic bacteria. Findings also showed that the virulence *fasR*, carried by the plasmid is necessary but not sufficient for phytopathogenicity of *Rhodococcus*. Last, we report results from molecular genetic approaches, that are important for resolving conflict in the annotations of the coding sequence of *fasR*. The work described in this thesis highlights how the acquisition of a small number of genes can have a profound effect on symbiosis of plant-associated bacteria and provides a foundation for testing how FasR, a

predicted transcriptional regulator co-opts for pathogenicity the genes encoded on the chromosomes of *Rhodococcus*.

Characterizing the symbiotic interactions between *Rhodococcus* and plants

by

Danielle M. Stevens

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APPROVED:

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Danielle M. Stevens, Author

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Introduction

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Plants provide ecological niches where complex microbial communities can form. Plants can influence the composition of its microbiota and enrich for certain taxa of microorganisms, even those present in the soil rhizosphere (Berg, 2009; Finkel *et al.*, 2017). These microbial communities are critical to the health and growth of the plant host. The communities can promote plant health by enhancing nutrient availability, reducing environmental stress, influencing plant host metabolism, and/or antagonizing phytopathogens via antibiotic production (Nishiguchi *et al.,* 2008; Hamedi and Mohammadipanah, 2014). Microorganisms within these communities have often been suggested to influence plant growth and development through the secretion of phytohormones such as cytokinins, auxins, ethylene, and gibberellins (Berg, 2009).

Bacteria belonging to Actinobacteria, one of the largest taxa within the Bacteria domain, are frequently identified in microbial communities that are associated with plants (Bodenhausen *et al.,* 2013; Lewin *et al.,* 2016). Assigned to the Actinobacteria phylum is the *Rhodococcus* genus, whose members can inhabit both aquatic and terrestrial ecosystems. Within the latter systems, members of *Rhodococcus* can be abundant in soils or associated with terrestrial eukaryotic hosts. The plant-associated members have been suggested to improve plant fitness based on their ability to improve soil quality and potentially act as biofertilizers (Hamedi and Mohammadipanah, 2014). Likewise, Actinobacteria S*treptomyces olivaceoviridis* and *Streptomyces rochei* produce substantial amounts of phytohormones including auxins, cytokinins, and gibberellins, which increase shoot length and plant biomass (Hamedi and Mohammadipanah, 2014).

In addition to providing benefits to plants, some lineages of *Streptomyces, Clavibacter, and Rhodococcus*, within Actinobacteria, can cause disease to plants (Hogenhout *et al.,* 2008). Our understanding of the interactions between Gram-positive pathogenic Actinobacteria and their plant hosts is not well understood. Most models of interactions between plant and pathogenic bacteria are derived from studies of interactions that involve Gram-negative bacteria. Many Gram-negative bacteria rely on specialized secretion systems to deploy effector protein that perturb host immune signaling pathways. However, the structure of the cell envelope of Gram-positive bacteria is distinct from that of Gram-negative bacteria and thus result in reliance on different secretion systems (Silhavy *et al.,* 2010). In the *Rhodococcus* genus and other closely related taxa, the bacteria have an extracellular mycolic acid lipid wall but nevertheless still do not rely on the same types of secretion systems as those of Gram-negative bacteria (Gürtler *et al.,* 2004; Stes *et al.,* 2011). Gram-negative pathogens also synthesize small molecules that influence plant hormone signaling pathways (Ma and Ma, 2016). It is unclear whether Gram-positive pathogens have the potential to secrete small molecules to promote virulence.

The non-motile, non-sporulating, high G+C content, aerobic members of the *Rhodococcus* genus are predominantly benign environmental bacteria (Larkin *et al.,* 1998). But, there are two pathogenic groups, *Rhodococcus equi* and *Rhodococcus fascians* (and closely related sister taxa). *R. equi* is a coccobacillus bacterium that causes necrotic pneumonia in foals (Vázquez-Boland *et al.,* 2013). Characterization of the genome and transcriptome sequences of one pathogenic

isolate led to the development of a novel model for virulence gene evolution (Letek *et al.*, 2010). *R. equi* requires the *vap* virulence loci, located on a pathogenicity island to cause disease (Letek *et al.*, 2008). The acquisition of genes with new functions is often central to the change in development and metabolism of both simple and complex organisms (True and Carroll, 2002). But in the case of *R. equi*, one of the horizontally acquired genes is not predicted to confer an innovative function. Rather, the acquired gene contributes to virulence by co-opting chromosomal genes and misregulating them for virulence (Letek *et al.,* 2010).

The second group of pathogenic *Rhodococcus* are phytopathogens that cause leafy gall disease, witch's brooms, and other leaf deformations. In general, phytopathogenic *Rhodococcus* cause shoot proliferations at the base of the meristem but symptom development is dependent on multiple variables including the bacterial strain used for inoculation, the conditions for bacterial growth, the age and the genus of the plant (Vereecke *et al.,* 2000; Goethals *et al.,* 2001). When a seedling is infected with pathogenic *Rhodococcus,* true leaves do not form, growth is inhibited, and the hypocotyl thickens (Goethals *et al.,* 2001). This group of phytopathogens can affect a wide host range of plants, including monocots, dicots, herbaceous, and some woody plants (Putnam and Miller, 2007).

Given the grotesque nature of the symptoms caused by phytopathogenic *Rhodococcus*, economic costs can be high, particularly in the ornamental plant industry (Putnam and Miller, 2007). Compounding costs are the absence of universal preventative or curative treatments for plants infected with pathogenic *Rhodococcus* (Putnam and Miller, 2007). Last, pathogenic *Rhodococcus* is easily

propagated through infected plant materials, and the only action is to destroy infected plant material.

Understanding the epidemiology of phytopathogenic *Rhodococcus* is complicated by the genetic diversity of the bacteria. The analyses of genome sequences from 20 isolates of *Rhodococcus* cultured from symptomatic tissue of different plants, different times in plant development, and different geographical locations, have suggested plant-associated *Rhodococcus* represent multiple species (Creason *et al.*, 2014). A phylogenetic analysis showed the phytopathogenic isolates formed two distinct clades. However, not all isolates are pathogenic and were collected from various environments, including a glacial core.

Most phytopathogenic strains of *Rhodococcus* that have been analyzed carry a 200-kb conjugative virulence plasmid. Though, as described in a later section, two phytopathogenic strains lacking a plasmid have been identified (Creason *et al*., 2014). The plasmid, exemplified by pFID188 of *R. fascians* strain D188 has been the most intensively studied (Crespi *et al.,* 1992; Crespi *et al.,* 1994). When the strain D188 (D188-5) was cured of this plasmid, the strain lost the ability to cause disease; showing the plasmid is necessary for phytopathogenicity (Crespi *et al.,* 1992). However, D188-5 is still capable of colonizing plants, indicating that pFID188 is not necessary for survival of the bacterium.

Three loci, *fas, fasR,* and *att*, on pFID188 have been identified as necessary for full virulence of plasmid carrying-phytopathogenic *Rhodococcus* (Figure 1.1) (Francis *et al.,* 2012). Bioinformatic, genetic, and biochemical characterization of

these genes and their encoded products contributed to the development of the cytokinin mixture model (Pertry *et al.,* 2009; Pertry *et al.,* 2010). This model predicts phytopathogenic *Rhodococcus* synthesizes and secretes a mixture of the phytohormone cytokinins into plant cells (Stes *et al.,* 2013). The hypothesized increase in levels of cytokinins in plant cells is proposed to disrupt homeostatic levels, leading to unregulated growth and leafy galls or witches' brooms.

The *fas* locus, which consists of *fasA* through *fasF*, is key to the cytokinin mixture model. Four of its genes, *fasA*, *fasD, fasE,* and *fasF* were suggested to be necessary for pathogenicity, as mutants lacking functional genes were characterized as being compromised in virulence of plants (Pertry *et al.,* 2010). FasD is an adenylate isopentenyl transferase, which catalyzes the first step in cytokinin biosynthesis (Frébort *et al*., 2011). FasD uses *in vitro*, (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBDP) or dimethylallyl diphosphate (DMAPP) and ATP or ADP to synthesize isopentenyladenine ribotide (iPAMP), a cytokinin type that has no measurable biological activity in plants (Figure 1.2) (Pertry *et al.,* 2009; Frébort *et al.,* 2011). FasF is a phosphoribohydrolase and member of the Lonely Guy (LOG) family, a group of proteins that catalyzes the formation of free base forms of cytokinin (Figure 1.2) (Pertry *et al.,* 2009, Frebort *et al*., 2011). The;free base cytokinins are active and can influence the growth and development of plants (Frébort *et al*., 2011). Interestingly, *in vitro* both FasD and FasF preferentially react with DMAPP, which suggests iP cytokinin types would be predominant (Pertry *et al.,* 2010). FasE is a cytokinin dehydrogenase, which cleaves cytokinins into adenine and an aldehyde, irreversibly inactivating

cytokinins (Figure 1.2) (Creason *et al.,* 2014). FasA is predicted to be a P450 monooxygenase and has the potential to convert iP types to tZ types (Figure 1.2). (Crespi *et al.,* 1994). The functions conferred by *fasB* and *fasC* have not been experimentally tested but are nonetheless predicted to function in influencing cytokinin metabolism.

Studies have suggested the *fas* locus is necessary for the accumulation of three cytokinin types, isopentenyladenine (iP), *trans*-zeatin (tZ), and *cis*-zeatin (cZ), and their modified variants (Pertry *et al.,* 2010; Stes *et al.,* 2013). Consistent with predictions, the three cytokinin types and derivatives have been detected in extracts collected from culture-grown strain D188 alone or Arabidopsis inoculated with strain D188 (Pertry *et al.,* 2009). However, others have shown that most cytokinins do not accumulate in a *fas*-dependent manner and suggested that most cytokinins detected from extracts of *Rhodococcus*-infected plants are derived from the host (Eason *et al.,* 1996; Galis *et al.,* 2005; Creason *et al*., 2014). It is thus still unclear whether cytokinins synthesized in a *fas*-dependent manner function directly in plants to disrupt hormone signaling.

The *fasR* locus is also necessary for pathogenicity, as a deletion mutant of *fasR* is nonpathogenic on plants (Temmerman *et al.,* 2000). Previous findings have suggested that *fasR* is involved in the regulation of expression of the *fas* locus; however, there is little data to support this conclusion (Temmerman *et al.,* 2000). FasR is predicted to be an AraC-type transcriptional regulator. These regulators constituent one of the largest groups of regulatory proteins in bacterial (Yang *et al.,* 2011). The model that predicts how AraC-type transcriptional regulators

function is referred to as the “light switch” model (Figure 1.3) (Yang *et al.,* 2011). In the absence of a ligand, AraC-type regulators are predicted to bind as monomers to a specific DNA sequence and repress expression of target genes. Binding of the regulator to a cognate ligand stimulates a structural change, dimerization, and recruitment of RNA polymerase and the transcription of genes (Figure 1.3) (Yang *et al.,* 2011). A putative ligand binding domain and putative DNA binding domain have been identified in FasR (Yang *et al.,* 2011; Creason *et al*., 2014). But it is unclear what FasR regulates.

Last, the *att* operon is implicated in the virulence of phytopathogenic *Rhodococcus* (Figure 1.1). However, the *att* locus is not necessary for pathogenicity and is thus suggested to influence virulence, as mutants are reportedly attenuated in virulence (Maes *et al.,* 2001). In addition, the *in vitro* expression of *att* is induced by yet-to-be determined compounds extracted from leafy galls formed by pathogenic *Rhodococcus.* The *att* operon consists of nine genes including *attX*, *attA* through *attH*, as well as the putative transcriptional regulator-encoding gene *attR* (Figure 1.1) (Stes *et al.,* 2013). The latter gene is hypothesized to regulate the expression of the other *att* genes (Stes et al., 2013). The *att* genes are annotated with functions involved in antibiotic production and amino acid synthesis (Maes *et al.,* 2001).

Two strains, A21d2 and A25f, lack a virulence plasmid (Creason *et al.,* 2014). A25f is hypothesized to carry the *att*, *fasR*, and *fas* loci within its chromosome (Creason *et al.,* 2014). Similarly, the virulence loci A21d2 are also hypothesized to be encoded in the chromosome. However, A21d2 lacks the *fas*

operon and has a non-orthologous replacement by a single gene called *fasDF* (Figure 1.1). The *fasDF* gene is predicted to encode for only the protein domains of FasD (IPT) and FasF (LOG) (Creason *et al.,* 2014). The discovery of A21d2 raised questions regarding the cytokinin mixture model, as the strain lacks *fasA* and *fasE*, which were reported to be necessary for phytopathogenicity (Pertry *et al.,* 2010).

Analysis of A21d2 also yielded new insights into *fasR*. Its *fasR* allele is substantially more polymorphic, relative to comparisons between *att* alleles (Creason *et al.,* 2014). Moreover, there is evidence for a high degree of variation in the amino acid sequence corresponding to the putative ligand binding domain and a high level of conservation in the putative DNA binding domain (Creason *et al.,* 2014). Given that the adjacent *fas* loci are also polymorphic, we hypothesize there may be a genetic interaction between *fas* and *fasR* (Temmerman *et al.,* 2000, Creason *et al.,* 2014). Based on the virulence mechanism of the closely related species *R. equi*, we hypothesize that *fasR* may be critical for co-option of the chromosome of *Rhodococcus* for pathogenicity.

SUMMARY

Rhodococcus is a genetically diverse genus of Gram-positive, mycolic acid-containing bacteria that cause leafy gall disease on a broad range of plant species. The pathogenic lineages require three virulence loci, *fasR*, *fas* (or variants), and *att,* carried on a conjugative virulence plasmid. Here, we demonstrate that isolates lacking virulence genes promote beneficial plant growth, and that the acquisition

of the virulence plasmid is sufficient to transition beneficial symbionts to phytopathogens. This evolutionary transition, along with the distribution patterns of plasmids, reveals the impact of horizontal gene transfer in rapidly generating new pathogenic lineages and provides an alternative explanation for pathogen transmission patterns. Second, we characterized the *fasR* gene. The role of *fasR* in virulence is not well understood. We demonstrate that *fasR* is necessary, but not sufficient for phytopathogenicity of *Rhodococcus*. We also show that complementation of the *fasR* mutant with the wild-type allele from D188 or A21d2 restores pathogenicity, demonstrating that the two polymorphic *fasR* alleles are homologous in function. This suggests that *fasR* likely regulates the same set of genes between strains. Last, we used a genetic approach to infer the start codon of *fasR* of strain D188. This allele has three in-frame ATG codons. Results suggested the coding sequence may start at the second or third ATG. These findings will be used to inform on future tests regarding the role FasR has in phytopathogenic *Rhodococcus* and how it may function to co-opt *Rhodococcus* genome for virulence

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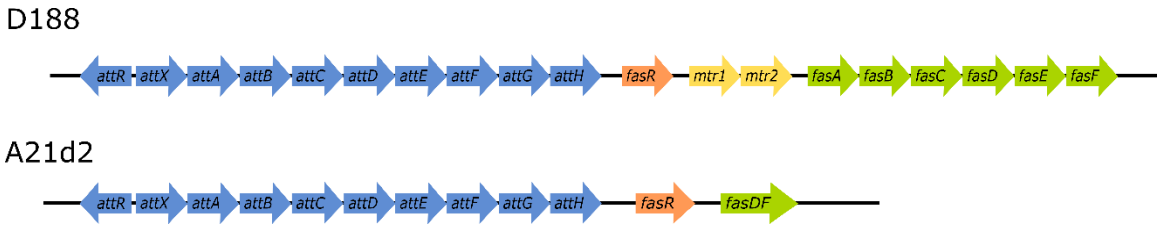

Figure 1.1. Schematic of virulence loci of two phytopathogenic strains of *Rhodococcus.* Top: three loci *att* (blue), *fasR* (orange), and *fas* (green) of strain D188 implicated in phytopathogenicity. The loci flank two genes, *mtr1* and *mtr2* (yellow), predicted to encode methyltransferases; these 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.

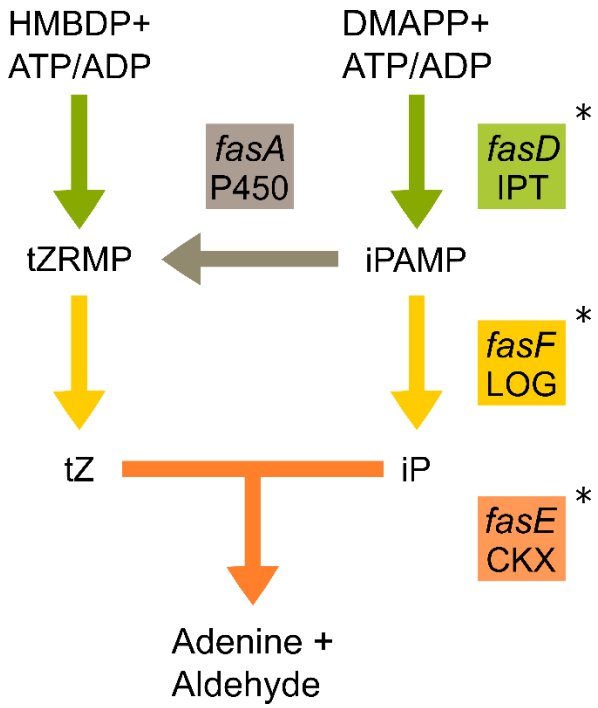

Figure 1.2. Proposed pathway of Fas-dependent biosynthesis of cytokinins in stain D188. *Rhodococcus* genes and their corresponding proteins are listed within colored boxes. FasD is an adenylate isopentenyl transferase (IPT) which catabolizes isopentenyladenine ribotide (iPAMP) from dimethylallyl diphosphate (DMAPP) or (E)-4-hydroxy-3-methyl-but-2-enyl diphosphate (HMBDP) and ATP/ADP respectively. FasF is a member of the "Lonely Guy" protein (LOG). These proteins activate cytokinin types. FasE is a cytokinin dehydrogenase (CKX) that degrades cytokinins into adenine and aldehyde. Finally, the predicted product of *fasA*, a P450 monooxygenase, has been hypothesized to covert iPAMP to tZRMP. * indicates proteins with *in vitro* activity consistent with predicted role in cytokinin metabolism (Pertry *et al.,* 2010).

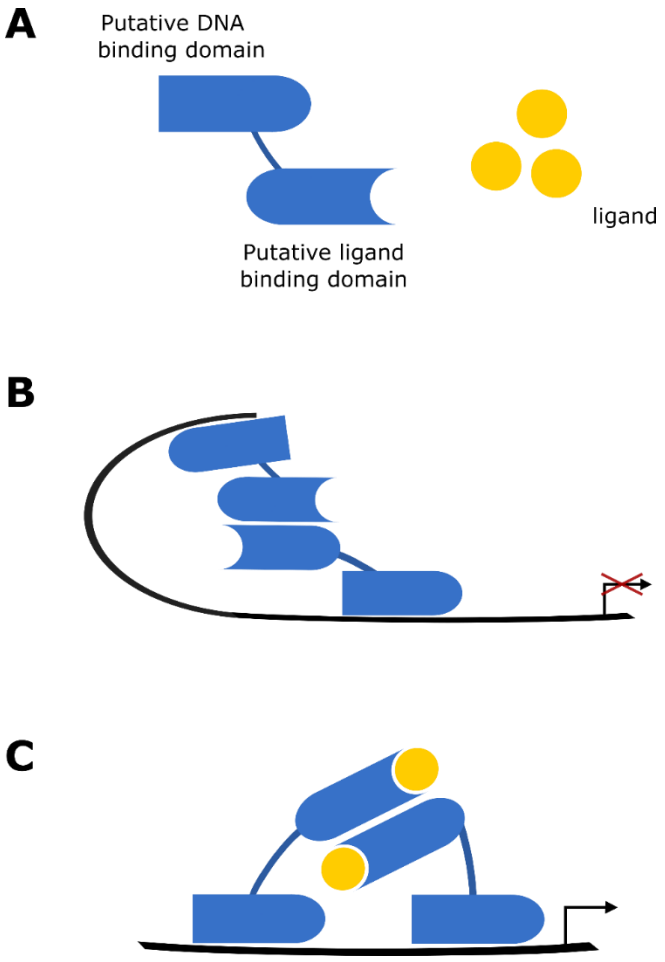

Figure 1.3. Schematic of AraC-type transcriptional regulator activation. (A) The monomeric unit of an AraC-type transcriptional regulator is separate from its potential cognate ligand. **(B)** Prior to binding its ligand, these regulators bind to DNA upstream of their regulon and represses expression. **(C)** The binding of its ligand causes a conformational change. This structural change results in a dimerization of the monomeric protein at the ligand binding domain and allows for expression of its target regulon through the DNA binding domain. Figure is adapted from Yang *et al.,* 2011.

Evolutionary Transitions between beneficial and phytopathogenic *Rhodococcus* challenge disease management.

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ABSTRACT

Understanding how bacteria affect plant health is crucial for developing sustainable crop production systems. We coupled ecological sampling and genome sequencing to characterize the population genetic history of *Rhodococcus* and the distribution patterns of virulence plasmids in isolates from nurseries. Analysis of chromosome sequences shows that plants host multiple lineages of *Rhodococcus*, and suggested that these bacteria are transmitted due to independent introductions, reservoir populations, and point source outbreaks. We demonstrate that isolates lacking virulence genes promote beneficial plant growth, and that the acquisition of a virulence plasmid is sufficient to transition beneficial symbionts to phytopathogens. This evolutionary transition, along with the distribution patterns of plasmids, reveals the impact of horizontal gene transfer in rapidly generating new pathogenic lineages and provides an alternative explanation for pathogen transmission patterns. Results also uncovered a misdiagnosed epidemic that implicated beneficial *Rhodococcus* bacteria as pathogens of pistachio. The misdiagnosis perpetuated the unnecessary removal of trees and exacerbated economic losses.

CONTRIBUTION TO THE PUBLISHED WORK

Danielle M. Stevens (DMS) contributed to the data presented in Figures 4, 6, 6-figure supplemental 1, 6-figure supplemental 3, 7, and 7-figure supplemental 1. Additionally, DMS developed D188ΔpFID188 isolate and measured its growth, presented in Figure 6-figure supplemental 2.

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

Danielle M. Stevens

ABSTRACT

Rhodococcus is a genetically diverse genus of Gram-positive, mycolic acid-containing bacteria. The genus circumscribes multiple species that can be pathogenic to a broad range of plant species. The pathogenic lineages require three virulence loci, *fasR*, *fas,* and *att,* to cause leafy gall disease. The current virulence model hypothesizes that the bacteria synthesizes, in a *fas*-dependent manner, cytokinins, which are a class of plant growth-promoting hormone. The cytokinin mixture is suggested to be secreted into the plant cell to cause an imbalance in hormone concentrations and upset homeostatic growth of the host. The *fasR* gene is predicted to encode an AraC-type transcriptional regulator. Its contribution to virulence is not well understood and its necessity and sufficiency has yet to be tested. Here, we demonstrate that *fasR* is necessary but not sufficient for phytopathogenicity of *Rhodococcus*. We also demonstrate that two polymorphic alleles of *fasR* can complement when swapped between mutants of strains D188 and A21d2, suggesting *fasR* are homologous and likely regulate in the strains the same sets of genes. Last, we used a genetic approach to infer the start codon of *fasR* of strain D188. This allele has three in-frame ATGs. Results suggested the coding sequence of *fasR* may begin from the second or third ATG codon. These findings contribute to a knowledgebase that will be used to inform on future tests on the hypotheses of the function of FasR in the phytopathogenicity of *Rhodococcus*.

INTRODUCTION

Members of the *Rhodococcus* genus are within the Actinobacteria class, one of the largest taxonomic units in the Bacteria domain. *Rhodococcus* are characterized as non-motile, mycolic acid-containing Gram-positive bacteria with a high G+C genome (Larkin *et al.,* 1998). The *Rhodococcus* genus is diverse, as members of *Rhodococcus* inhabit environments ranging from aquatic to terrestrial ecosystems and are frequently recovered from anthropogenically-disturbed environments (Bej *et al.,* 2000; Geize *et al.,* 2004). Most *Rhodococcus* are environmental bacteria or benignly associated with eukaryotic hosts (Larkin *et al.,* 1998). Several studies have identified *Rhodococcus* within plants and have suggested that *Rhodococcus* may confer benefit to their hosts (Cornelis *et al.,* 2001; Savory *et al.,* 2017).

Some *Rhodococcus* species are pathogens of mammals or plants. *Rhodococcus equi* is a pathogen of foals and an opportunistic pathogen of immunocompromised humans (Letek *et al.,* 2008). The evolution of virulence of *R. equi* was modeled as co-optive virulence (Letek *et al.,* 2010). This model predicts that horizontal gene transfer, such as the acquisition of the *vap* region in a plasmid, is a key step. But contrary to the evolution of other pathogenic lineages, *Rhodococcus* does not require additional gene gains or losses. Rather, the horizontally acquired *vap* genes co-opt and repurpose for virulence genes present in the chromosome (Letek *et al.,* 2010). The second group of pathogenic *Rhodococcus* are phytopathogens that can cause leafy gall disease, witch's broom, and other leaf deformities (Putnam and Miller, 2007). This disease is a

significant economic burden because *Rhodococcus* can infect over one hundred species of plants, many of which are produced by the US nursery industry, and growers have no available curatives or preventives.

Rhodococcus can grow as an epiphyte on the surface of plant leaves and plants can be asymptomatic. A yet-to-be characterized trigger causes a transition in some strains towards endophytic growth (Cornelis *et al.,* 2001). Some strains of *Rhodococcus* maintain an epiphytic life style and fail to transition to become endophytic (Dhandapani *et al.,* 2018). In the case of those that transition to endophytes, some lineages have the potential to cause disease.

Phytopathogenicity of *Rhodococcus* is dependent on a cluster of three virulence loci, *fas, fasR,* and *att*, most intensively studied in the model strain, D188 (Francis *et al.,* 2012). In this strain, the virulence loci are clustered on the conjugative plasmid pFID188 (Crespi *et al.,* 1992; Crespi *et al.,* 1994). The *fas* and *fasR* loci were concluded to be necessary on the basis of corresponding mutants in D188 of being non-pathogenic. The *fas* genes are predicted to be necessary for the synthesis of cytokinins (Pertry *et al.,* 2009; Pertry *et al.,* 2010). FasR is predicted to be a member of the AraC-type transcriptional regulator family that regulates the expression of the *fas* operon. But the data that support this are unclear (Temmerman *et al.,* 2000). AraC-type transcriptional regulators repress expression of target genes in the absence of its cognate ligand (Yang *et al.,* 2011). When the AraC-type transcriptional regulators undergo a structural change when binding to its cognate ligand leading to the recruitment of RNA polymerase and transcription of its target genes (Yang *et al.,* 2011). However, the *fasR* mutant that

was tested is also deleted of a portion of an adjacent gene and is potentially polar, so the necessity of *fasR* in pathogenicity has not been sufficiently tested (Temmerman *et al.,* 2000). A mutant with a deletion that encompasses most of the *att* locus was reportedly attenuated in virulence (Maes *et al.,* 2001). However, inconsistencies with the phenotype of the *att* mutant have been reported, raising questions regarding its role in pathogenicity (Savory *et al.,* 2017).

The *fas* locus is central to the virulence of *Rhodococcus*. The current virulence model predicts that *fas* is necessary for phytopathogenic *Rhodococcus* to secrete cytokinins into the plant tissue and the exogenously supplied hormones upset homeostatic levels, leading to the proliferation of shoots and disease (Stes *et al.,* 2013). Indeed, three of the Fas proteins have *in vitro* enzymatic functions in cytokinin synthesis and metabolism (Pertry *et al.,* 2010). FasD is an adenylate isopentenyl transferase that catalyzes the synthesis of the ribotide form of isopentenyladenine and *trans*-zeatin cytokinin types; ribotide cytokinins are not active in plants (Pertry *et al.,* 2008; Frébort *et al*., 2011). FasF is a phosphoribohydrolase that catalyzes the formation of free base forms of cytokinins, which are active and influence the growth of plants (Pertry *et al.,* 2009; Frébort *et al*., 2011). FasE is a cytokinin dehydrogenase, which cleaves cytokinins into adenine and an aldehyde, irreversibly inactivating cytokinins (Pertry *et al.,* 2010).

Findings from whole-genome sequencing of 64 isolates suggested that phytopathogenic *Rhodococcus* form two sister clades and potentially multiple species-level groups (Creason *et al.,* 2014). In all but two of the sequenced

pathogenic lineages, the three virulence loci are clustered on a conjugative virulence plasmid. However, strains A25f and A21d2 are unique in having the virulence loci located on the chromosome. In addition, the virulence loci of A21d2 are genetically distant (Creason *et al.,* 2014). Strain A21d2 has a non-orthologous replacement of the *fas* locus. In addition, its *fasR* allele is the most diverged among the 64 alleles that were sequenced. Analyses suggest *fasR*$_{A21d2}$ has an accumulation of non-synonymous substitutions in the region predicted to encode the ligand binding domain. On the other hand, analyses suggested the region predicted to encode the DNA-binding domain was biased towards synonymous substitutions.

The whole-genome sequence also revealed multiple sequencing errors in the original annotation of *fasR*$_{D188}$ (Creason *et al.,* 2014). The coding sequence of *fasR*$_{D188}$ was originally annotated as being 834 basepairs (bp) long (Temmerman *et al.,* 2000). However, subsequent sequencing efforts led to a shift in frames and as a consequence, the newly annotated *fasR* has three in-frame ATG codons and is predicted to be 1047 bp long (Creason *et al.,* 2014). In strain A21d2, the *fasR* was annotated as 993 bp in length (Creason *et al*., 2014).

Our overarching hypothesis is that FasR of phytopathogenic *Rhodococcus* co-opts genomes for virulence. Here, we generated new mutants of *fasR* and demonstrate it is indeed necessary but not sufficient for phytopathogenicity. We also reciprocally exchanged the *fasR* alleles between mutants of D188 and A21d2 and show the genetically distant alleles are homologous in function. Last, we constructed and characterized 30 different combinations of *fasR* and mutant

genotypes and show that the first in-frame ATG is not likely the start codon. Results point towards the second or third ATG codon for initiating translation of *fasR*.

RESULTS

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

The *fasR* mutant that was originally characterized in strain D188 has a deletion that spans from within the coding sequence of *attH* through the coding sequence of *fasR* (Figure 3.1A; Table 3.1; from hereafter this mutant will be referred to as rsΔfRD (Temmerman *et al.,* 2000)). To avoid any potential confounding effects, we generated a new mutant that was predicted to only affect *fasR* in the same strain, D188. We introduced a thymine nucleotide at position 303, which leads to a frameshift and a premature stop codon predicted to occur at position 423, truncating FasR to approximately one third of its original predicted size (Figure 3.1A). The resulting protein fragment is predicted to include only the putative ligand binding domain and lack the DNA binding domain (Creason *et al.,* 2014). The mutant allele was transformed into strain D188 and a recombinant, called fsΔfRD, was selected and confirmed via Sanger sequencing (Figure 3.2A).

The mutant fsΔfRD was assayed for its ability to inhibit root growth and form galls on *Nicotiana benthamiana*. The positive control, wildtype strain D188 caused a significant reduction in root elongation, relative to the length of roots of mock-inoculated seedlings and those inoculated with ΔpFD, the plasmid-lacking strain (Figure 3.3A and B, Table 3.1). In contrast, the roots of the seedlings inoculated

with fsΔfRD were not significantly different from those of the negative control treatments (Figure 3.3A and B). Mature plants infected with strain D188 formed galls approximately 18 days post-inoculation. Similar to the mock-inoculated plants, mature plants inoculated with fsΔfRD also failed to form galls (examined up to 24 days after inoculation) (Figure 3.3C).

To verify the loss of function was due to the mutation of *fasR*, we attempted to complement fsΔfRD. We cloned from strain D188 a region that spans the entire length of the newly annotated coding sequence of *fasR* (fDR1) and transformed it into fsΔfRD. Colonies were selected and verified via PCR. Inoculation of fsΔfRD + fDR1 on to seedlings and mature plants led to inhibited root growth and gall formation, respectively (Figure 3.3). Therefore, we concluded that *fasR* is necessary for pathogenicity of *Rhodococcus* strain D188.

Phytopathogenic *Rhodococcus* strain, A21d2, also causes leafy gall disease (Creason *et al.,* 2014). Its *fasR* and *fas* virulence loci are diverged from that of strain D188. To test if *fasR* is necessary for strain A21d2 to cause disease, we deleted the *fasR* coding sequence from A21d2. Approximately 1.5 kilobases of the regions flanking *fasR* were amplified and fused together. The plasmid construct was transformed into A21d2 and recombinants were selected and verified (Figure 3.2B). One recombinant, ΔfRA, was selected and assayed. The length of roots inoculated with ΔfRA was not significantly different than that of roots of mock-inoculated seedlings (Figure 3.3A and B). Likewise, galls did not form on mature plants inoculated with ΔfRA (Figure 3.3C). The mutant ΔfRA was complemented with the coding sequence of *fasR* from A21d2 (fRA) (Table 3.1). The

complemented strain inhibited root growth and caused galls to form on *N. benthamiana* plants (Figure 3.3). We concluded that *fasR* is necessary for strain A21d2 to cause disease.

Whether *fasR* is sufficient to mediate disease in phytopathogenic *Rhodococcus* has yet to be tested. Thus, we transformed fRD1 or fRA into ΔpFD (Table 3.1) and assayed the two strains for pathogenicity on *N. benthamiana* (Figure 3.3). The lengths of the roots of seedlings infected with ΔpFD + fRD1 or fRA were not significantly different relative to those of mock-inoculated seedlings (Figure 3.3A and B). Our results show that *fasR* is not sufficient for *Rhodococcus* strain D188 to cause disease.

The *fasR* alleles are homologous in function

The *fasR* allele of strain A21d2 is the most polymorphic of all *fasR* alleles (Creason *et al.*, 2014). To test if *fasR* alleles have homologous functions, we swapped the alleles between strains. Knockout mutants fsΔfRD and ΔfRA were transformed with the allele derived from the other genotype. The strains fsΔfRD + fRA and ΔfRA + fRD1 (Table 3.1) were tested in the root inhibition of seedlings and gall formation of mature plants. Both wildtype D188 and A21d2 inhibited roots and caused galls to form. Likewise, fsΔfRD + fRA and ΔfRA + fRD1 significantly inhibited the growth of roots, compared to mock-inoculated seedlings (Figure 3.3A and B). In addition, both strains caused galls to form on mature plants (Figure 3.3C). We also swapped alleles between wildtype genotypes. Strains D188 + fRA and A21d2 + fRD1 were tested for pathogenicity on *N. benthamiana* and the lengths of roots infected with either strain were similar in length compared to their

respective wildtype strain (data not shown). We concluded from these data that despite *fasR* being polymorphic, that they are homologous in function.

The *fasR* alleles have multiple in-frame ATG codons

The annotations for the coding sequences of *fasR* of strains D188 and A21d2 are unresolved (Creason *et al,* 2014). The *fasR* gene of D188 has three predicted in-frame ATG codons (Figure 3.4). The third ATG, leading to the shortest coding sequence was originally predicted to be the start codon; whereas the first ATG, leading to the longest coding sequence was later predicted to be the start codon (Temmerman *et al.,* 2000; Creason *et al.,* 2014). The *fasR* gene of A21d2 has two predicted in-frame ATG codons that correspond to the second and third ATG codons of the *fasR* gene of D188 (Figure 3.4).

To infer which ATG is the start codon, we amplified three fragments of *fasR* from strain D188, each starting at a different ATG and ending with the same stop codon (Figure 3.3). Each construct is referred to on the basis of the position of their respective ATG (e.g. fRD1 is $fasR_{D188\_ATG1}$) (Table 3.1). The constructs were verified by PCR and Sanger sequencing before being transformed into fsΔfRD and ΔfRA. Strains were assayed on *N. benthamiana* for their ability to inhibit root growth of seedlings and to form galls on mature plants. Roots of seedlings inoculated with fsΔfRD + fRD1 or ΔfRA + fRD1 were significantly inhibited in growth relative to mock-inoculated seedlings (Figure 3.5A and B). Similarly, mature plants infected with fsΔfRD + fRD1 or ΔfRA + fRD1 formed galls (Figure 3.5C). In contrast, fsΔfRD or ΔfRA carrying either fRD2 or fRD3 (Table 3.1) were unable to

inhibit root growth and root lengths were on average no different from those of mock-inoculated seedlings (Figure 3.5A and B). Gall assay data was consistent with, fsΔfRD or ΔfRA carrying either fRD2 or fRD3 failing to form galls (Figure 3.5C). These data suggested the first ATG is the likely start codon of *fasR*.

As another test, we used site-directed mutagenesis to introduce a nonsense substitution at position 13. This construct, fRD1s2, was transformed into fsΔfRD or ΔfRA and mutants were assayed on *N. benthamiana* seedlings and mature plants. Unexpectedly, both mutants carrying fRD1s2 significantly inhibited root growth of seedlings compared to mock-inoculated seedlings (Figure 3.5A and B). Similarly, mature plants infected with fsΔfRD + fRD1s2 formed galls; however, the galls of mature plant infected with ΔfRA + fRD1s2 were not as prominent compared to wildtype A21d2 (Table 3.1, Figure 3.5C). We observed a clear proliferation of shoot growth at the apical meristem, symptoms which are indicative of leafy gall formation, but the thickening at the apical meristem that is typically observed in a wildtype inoculated plants is absent (Figure 3.5C). We presume based on the proliferation of shoot growth, there is a delay in gall formation; however, this has yet to be tested.

The rsΔfDR mutant is different relative to fsΔfRD and ΔfRA

Last, we introduced *fasR* constructs developed in this work into the rsΔfRD previously developed (Temmerman *et al.,* 2000). Relative to mock-inoculated seedlings, the length of roots of plants inoculated with wildtype strain D188 were significantly reduced in length (Figure 3.6A and B). As expected, rsΔfRD failed to inhibit root elongation to the same level as wildtype D188 and the length of roots

of treated seedlings was not significantly different than that of mock-inoculated seedlings (Figure 3.6A and B). Galls formed on mature plants that had been inoculated with strain D188. In contrast, no galls formed on mature plants inoculated with rsΔfRD (Figure 3.6C). These findings were consistent with previously published conclusions suggesting that rsΔfRD is not pathogenic (Temmerman *et al.* 2000).

We transformed each of the constructs of *fasR* into rsΔfRD and assayed the strains for pathogenicity on *N. benthamiana* (Table 3.1). DNA construct fRD1 complemented mutant rsΔfRD, as root growth was inhibited and plants formed galls (Figure 3.6). We also cloned the same fragment (Xf) as used by Temmerman *et al*., (2000), and showed the DNA fragment was sufficient to complement rsΔfRD on the basis of a root inhibition assay (Table 3.1; Figure 3.9). However, no other DNA construct could reliably complement rsΔfRD (Figure 3.6, Figure 3.7, and Figure 3.9). This was unexpected, as fRA and fRD1s2, which has a nonsense substitution 3’ to ATG1 could complement both the fsΔfRD and ΔfRA mutants (Figure 3.3, Figure 3.5). Mutant rsΔfRD + fRD1s2 was assayed repeatedly for pathogenicity on *N. benthamiana* and the ability to inhibit root growth was more variable than that of other strains a (Figure 3.6A and B). However, while still variable, rsΔfRD + fRD1s2 was able to cause galls to form on mature plants (Figure 3.6C).

Considering the differences between DNA constructs and their inconsistencies in complementing strain rsΔfRD, we next asked if the 45 bp region upstream of ATG2 in $fasR_{D188}$ is necessary to express a functional *fasR.* We

constructed a chimeric molecule that includes the sequence from the first to the second ATG of D188 *fasR* fused to the sequence starting at the second codon of A21d2 *fasR.* This chimera sequence (fRc) was cloned and verified by PCR and Sanger sequencing. The construct was transformed into each of the three loss-of-function mutants of D188 and A21d2 and assessed for pathogenicity. In mutants fsΔfRD and ΔfRA, fRc conferred upon the strains, the ability to inhibit root growth of seedlings (Figure 3.7A and B). Additionally, mature plants infected with either strains were able to cause galls to form (Figure 3.7C). In contrast, the mutant rsΔfRD carrying fRc was unable to inhibit root growth of *N. benthamiana* seedlings (Figure 3.7A and B). But, the mutant rsΔfRD carrying fRc was able to form galls on mature plants infected (Figure 3.67).

Finally, all strains developed in this study were verified via PCR. Complemented strains were screened for other virulence loci, such as *fasD* or *fasDF*, to confirm their background (Figure 3.8A). Complemented strains were also screened for the construct they were transformed (Figure 3.8B). Oligonucleotides used to for screening can be found in Table 3.1.

DISCUSSION

Plant-symbiotic *Rhodococcus* is unique in that the transition of a beneficial symbiont to a pathogen can occur seamlessly via horizontal acquisition of a cluster of three virulence loci vectored on a conjugative plasmid (Savory *et al.,* 2017). Once acquired, the phytopathogenic lineages can cause growth deformations to the plant. The mechanism of virulence has been explained by the cytokinin mixture model, which predicts the *fas* genes are necessary for phytopathogenic

Rhodococcus to synthesize and secrete cytokinins into the plant tissue and upset homeostatic levels, leading to disease (Stes *et al.,* 2013). The cytokinin mixture model also proposes that *fasR* regulates the expression of the *fas* operon. However, there is a preponderance of data that are inconsistent with the cytokinin mixture model and there is little support for the hypothesis that *fasR* is necessary for the regulation of *fas* (Galis *et al.,* 2005; Creason *et al.,* 2014; Dhandapani *et al.,* 2017; Savory *et al.,* 2017). Moreover, our lab has additional unpublished data that do not fit the cytokinin mixture model (Savory, Fuller, Stevens, and Chang, unpublished).

We suggest that data are more consistent with predictions of the virulence gene co-option model. This model suggests that horizontally acquired virulence genes repurpose the chromosome for pathogenicity (Letek *et al*., 2010). FasR is a predicted AraC-type transcriptional regulator and is potentially key to the co-option model in phytopathogenic *Rhodococcus*. Here, we describe the development of a set of strains and constructs and characterization of 30 combinations to understand the functionality of *fasR* in the virulence of *Rhodococcus* (Fig. 3.9).

We developed mutants of *fasR* in two phytopathogenic *Rhodococcus* strains, D188 and A21d2. We made a new mutant in D188 because the previously described *fasR* mutant spanned two genes and is likely polar (Temmerman *et al.,* 2000). We made a *fasR* mutant of A21d2 because the necessity of *fasR* in this strain had not yet been tested (Fig. 3.2B). Deletion mutants of *fasR* in both strains are nonpathogenic when tested on plants indicating that *fasR* is necessary for pathogenicity of *Rhodococcus* (Fig. 3.3). Additionally, constitutive expression of

fasR in a plasmid cured strain of *Rhodococcus* was unable to restore pathogenicity, indicating that *fasR* is not sufficient for pathogenicity (Fig. 3.3). This result was expected since genes within the *fas* locus is also necessary for pathogenicity (Pertry *et al.,* 2010; Creason *et al.,* 2014; Savory, unpublished).

Previous comparisons of the *fasR* alleles from strains D188 and A21d2 showed that the two are polymorphic, with a higher number of differences in a region predicted to encode the ligand binding domain (Creason *et al.,* 2014). To test whether the two alleles are functionally homologous, the alleles of *fasR* were swapped between the *fasR* mutants of D188 and A21d2 (Fig. 3.3). Mutants carrying the *fasR* allele from the other strain were pathogenic, suggesting they are homologous in function. This observation would suggest that if FasR can be explained by the light switch model, then the protein variants from D188 and A21d2 are activated by a ligand that is produced by both strains. In addition, results suggest that FasR of D188 and A21d2 bind and regulate the same set of genes, indicating that its target sequence is conserved between the two genotypes of *Rhodococcus*.

There are discrepancies in the annotation of *fasR* of D188*.* The first annotation of D188 *fasR* predicted the coding sequence is 834 bp in length (Temmerman *et al.,* 2000). Subsequent annotation efforts of whole genome sequences of D188 and other strains of phytopathogenic *Rhodococcus* predicted *fasR* is 1047 bp in length while in A21d2, *fasR* was predicted to be 993 bp in length (Creason *et al.,* 2014). In most strains, the *fasR* gene has three in-frame ATG

codons whereas in A21d2 *fasR*, there are two in-frame ATG codons that correspond to the latter two of *fasR* from D188 (Figure 3.4).

As a first step towards identifying the start codon, different sized coding sequences of *fasR*, each starting with a different ATG were tested (Figure 3.5). We found that only the *fasR* construct which had all three ATG codons could restore pathogenicity of the *fasR* mutants. A nonsense substitution between the first and second ATG in D188 *fasR* was introduced to confirm the full length *fasR* allele is correct. In contrast to what was expected, the nonsense mutant restored pathogenicity. This would suggest the second or third ATG is the beginning of the coding sequence of *fasR*. A nonsense mutation between the second and third ATG would need to be tested to determine which of these is the start codon of D188 *fasR*.

There are several explanations as to why the nonsense mutant could complement a loss-of-function mutant of *fasR* but DNA constructs starting with the second or third ATG are insufficient. It is possible that a ribosome binding site (RBS) sequence is necessary for translation and DNA constructs starting precisely at an ATG are not translated. However, the data argue against this, as the *tdTomato* gene, cloned from ATG to stop codon, is expressed in *Rhodococcus*, thus indicating an RBS sequence is present in the L5 promoter sequence (Savory, unpublished). The second and third possibilities are read-through of the nonsense mutant or use of an alternative codon to initiate translation. To eliminate these, additional experiments would need to be done to determine if the nonsense mutant is transcribed and translated. A fourth possibility is the region upstream of the

second or third ATG of *fasR* encompasses another coding sequence that is necessary for phytopathogenicity of *Rhodococcus*. However, searches for additional coding sequences failed to identify candidates.

The data are also consistent with the possibility for a 5' untranslated region (UTR) as being necessary for the expression of *fasR*. RNA-based mechanisms of regulation found in 5' UTRs are ubiquitous in bacteria. A variety of RNA-mediated regulators are found in Gram-positive bacteria and are frequently associated with virulence and bacterial fitness (Miller *et al.,* 2014). The 5' UTR may include *cis*-activating or *trans*-activating regulator RNAs, though given our use of the constitutive L5 promoter, less likely. Alternatively, the 5' UTR may mediate RNA stability, and protect the transcript against degradation (Jester *et al.,* 2011). Another example of RNA-mediated regulation in bacteria is a riboswitch. These form a variety of secondary structures within the 5' UTR and upon interactions with a ligand molecule, can change structure and regulate translation (Miller *et al.,* 2014).

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. Strains used in this study are listed in Table 3.1. *Rhodococcus* strains were maintained on solid LB medium at 28˚C or grown overnight in liquid LB medium at 28˚C with shaking. *Rhodococcus* strains were grown under selection with kanamycin (50 μg/ml) when necessary. *Escherichia coli* was maintained on solid LB medium at 37˚C or grown overnight

in liquid LB medium at 37˚C with shaking. *E. coli* mutants were grown on media supplemented with gentamycin (25 µg/ml) when necessary.

Cloning DNA constructs. PCR and Gateway cloning were used to make constructs for the purpose of generating mutants of *Rhodococcus* (Invitrogen, Carlsbad CA USA). The oligonucleotides D188_fasR_F and D188_fasR_R were used to PCR amplify the *fasR* coding region from genomic DNA extracted from strain D188. Use of the oligonucleotide, D188_fasR_shift_F and D188_fasR_shift_R, introduced a thymine residue at position 303 of *fasR*. Oligonucleotides A21d2_fasR_KO_F and A21d2_fasR_KO_R were used to amplify fragments that flank *fasR* of A21d2. These fragments were mixed together and subsequently amplified using oligonucleotides, B2_Fuse strands _F and B2_Fuse strands _R, to join the two fragments. The final amplicons were cloned into pDONR207 and transformed into *E.* coli DH5α or NEB10β cells and grown on LB with gentamycin (25 µg/ml) (Sigma-Aldrich, Germany) at 37˚C. DNA inserts were transferred to a Gateway-compatible pSelAct destination vector (Geize *et al.,* 2008).

Variants of *fasR* were amplified and cloned downstream of the L5 promoter in vectors pJDC60 or pJDC165. The coding sequences of *fasR* of D188 (fRD1) and A21d2 were amplified using primers, which added sites compatible to ends generated upon digestion with *Eco*RI and *Bam*HI and are described in Table 3.2. The vector pJDC60 was digested with restriction enzymes *Eco*RI and *Bam*HI (New England Biolabs, Ipswich MA USA) and ligated to the amplified products. PCR and restriction enzyme digestion were used to verify clones. Gibson assembly cloning

(New England Biolabs, Ipswich MA USA) was used to clone the shorter variants of *fasR* (fRD2 and fRD3), *fasR* chimera (fRc), and *Xho*I_*Xho*I *fasR* (Xf) into pJDC165 vector (Gibson *et al.,* 2009); the sequences of the oligonucleotides that were used as described in Table 3.2.

PCR and the oligonucleotides listed in table 3.2 were used to introduce a nonsense substitution into fRD1 at position 13. The product was treated with *Dpn*I (New England Biolabs, Ipswich MA USA) and transformed into *E. coli* NEB10β cells (New England Biolabs, Ipswich MA USA).

Preparation of genomic and plasmid DNA. The Wizard Genomic DNA Purification Kit was used, following the instructions of the manufacturer, to extract bacterial genomic DNA (Promega Corporation, Madison, WI, USA). The Qiagen QIAprep Miniprep Kit was used, following the instructions of the manufacturer (Qiagen Company, Hilden, Germany) to extract plasmid DNA. A nanodrop spectrometer was used to quantify DNA concentration.

Bacterial transformation. Overnight cultures, distilled water, and 10% glycerol were chilled on ice for at least 10 minutes. Bacterial cultures (3 mL) were spun down, washed with 1 mL water and 1 mL 10% glycerol. Cells were resuspended in 50 µl of 10% glycerol and a minimum of 50 ng of plasmid DNA was added. Cells were added to 1 mm gap cuvettes and electroporated (Bio-Rad Micropulser; company information) at 2.2 kV. Cells were resuspended in 350 µl of LB media and incubated overnight in 28 ˚C shaker. Transformed bacteria (200 µl) were plated on selective media (LB agar with 50 µg/ml of kanamycin) the next day. For mutant variants, merodiploids were selected for on LB plates with apramycin (50

µg/ml) (Sigma-Aldrich, Germany) at 28˚C and recombinants were subsequently selected for by growing cells on mineral acetate medium with 5-fluorocytosine (100 µg/ml) (Sigma-Aldrich, Germany) (Geize *et al.,* 2008). Mutants fsΔfRD and ΔfRA were verified using PCR and confirmed via Sanger sequencing.

PCR assays and sequencing. The sequences of primers used are listed in Table 3.2 The reaction mixture for PCRs are as follows: 1x Standard Thermopol buffer (New England Biolabs, Ipswich MA USA), 200 µM dNTPs, 0.2 µM of each primer, 20 to 100 ng genomic DNA template, 0.625 units *Taq* DNA polymerase (New England Biolabs, Ipswich MA USA), and ddH2O for a final volume of 20 µl (New England Biolabs, Ipswich MA USA). PCR conditions were 95˚C for 10 minutes; 30 cycles of 95˚C for 30 seconds, 60˚C for 30 seconds, 72˚C for 1 minutes and 30 seconds; 72˚C for 10 minutes. Extension times were adjusted to approximate 1 minute per kilobase of DNA amplified.

For Sanger sequencing, PCR products were treated with 2.5 units of Exonuclease I (New England Biolabs, Ipswich MA USA) and 0.25 U of Shrimp Alkaline Phosphatase (New England Biolabs, Ipswich MA USA) and incubated at 37˚C for 40 minutes and 80˚C for 20 minutes. Products were sequenced on an ABI3730 capillary sequencing machine at the Oregon State University Center for Genome Research and Biocomputing (CGRB).

Root inhibition seedling assay. *Nicotiana benthamiana* seeds were sterilized in a 1.5 mL tube containing 800 µl water, 200 µl bleach, and a drop of polyoxyethylene sorbitan monolaurate (Tween 20) and shaken for 20 to 30 minutes. Seeds were washed twice with distilled water and resuspended in 1 mL

distilled water. Sterilized seeds were plated on MS agar medium (half-strength MS, 0.5 M MES (Creason *et al.,* 2014). Bacteria were grown overnight in LB media in 28 ˚C shaker. Cells were spun down, washed and resuspended at an OD_{600} = 0.5 in a final volume of 500 µl of water. Three days after germination, 5 µl of a suspension of bacteria were drop-inoculated onto the plants. $MgCl_2$ or water were used for mock inoculations. Images of inoculated plants were taken 7 days post inoculation (dpi). Roots were measured via ImageJ (Schneider *et al.,* 2012). Standard error of the mean (SEM) was calculated. A one-way ANOVA was performed, followed by Tukey's Multiple Comparatives Test, to determine statistical significance (McHugh, 2011). At least three replicates were performed with 40 individuals per experiment.

Gall assay. Plants were grown under a 10:14 hour light:dark cycle. Bacteria were prepared as previously described and 10 µl of OD_{600} = 0.5 of bacteria were dropped onto meristems of plants that had been pinched with forceps. Plants were inspected up to 28 dpi and images were taken 28 dpi.

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Table 3.1. Strains and mutants of *Rhodococcus* and constructs used in this study.

Code	Full Name	Pathogenic	Linear Plasmid	Virulence Loci	Reference	Type
	D188	Yes	Yes	*att*+, *fas*+, *fasR*+	Desomer et al., 1988	Wildtype Strains
	A21d2	Yes	No	*att*+, *fasDF*	Creason et al., 2014	
ΔpFD	D188ΔpFID188	No	No/cured	None	Savory et al., 2017	Non-Functional Mutants
Δatt	D188Δ*att*	Reduced	Yes	*fas*+, *fasR*+	Maes et al., 2001	
ΔfRA	A21d2Δ*fasR*	No	Yes	*att*+, *fas*+	This work	
fsΔfRD	Frameshift D188Δ$fasR_{shift}$	No	Yes	*att*+, *fas*+	This work	
rsΔfRD	Restriction sites D188Δ$fasR_{912}$	No	Yes	*att*+, *fas*+	Temmerman et al., 2000	

fRA	$fasR_{A21d2}$	Constructs
fRD1	$fasR_{D188\_ATG1}$	
fRD2	$fasR_{D188\_ATG2}$	
fRD3	$fasR_{D188\_ATG3}$	
fRD1s2	$fasR_{D188\_ATG1\_stop\_ATG2}$	
fRc	$fasR_{D188\_ATG1\_ATG2}$ + $fasR_{A21d2}$	
Xf	XhoI_XhoI_*fasR* (from Temmerman *et al.,* 2000)	

Table 3.2. Oligonucleotides used in this study.

Gene	Purpose	Oligo Name	Sequence (5'->3')[1]
A21d2 *fasR*	Knockout Development	A21d2_fasR_KO_F	GGACGCGAGCACAAATTACG
		A21d2_fasR_KO_R	GGAATAGGGTGACCAGCGAG
		A21d2_fasR_5' flank_F	CAAAAAAGCAGGCTCCAAAGATCACTCTGATGTCCG
		A21d2_fasR_5' flank_R	ATTATCTAGCTTTCGCCCCCCGGCACGAAT
		A21d2_fasR_3' flank_F	GGGGGCGAAAGCTAGATAATTTCGGTCGTA
		A21d2_fasR_3' flank_R	GAAAGCTGGGTGACCAGGTTGGGTATGGCTCC
		B1_Fuse strands_F	GGGGACAAGTTTGTACAAAAAAGCAGGCT
		B2_Fuse strands _R	AGATTGGGGACCACTTTGTACAAGAAAGCTGGGT
	Gene Specific	A21d2_fasR _F	CAAAAAAGCAGGCTCCATGACTGCAGCCGATAGAGAA
		A21d2_fasR_R	GAAAGCTGGGTGCGCGTGAAACCTTCTGAA
	Cloning	pJDC165_keep_L5_R	GAATTCCCTCCTATTGGATCGGA
		pJDC165_remove_GFP_F	TAAccaatATCTAGATGCAT
A21d2 *fasDF*	Gene Specific	A21d2_fasDF_F	TTCGATAGAGAAAGCGGTTGA
		A21d2_fasDF_R	TGATCACTGTTCTCGCGGTT
D188 *fasD*	Gene Specific	D188_fasD_F	CAAAAAAGCAGGCTCCATGAAGGAATCAACCATGGCA
		D188_fasD_R	GAAAGCTGGGTGTCTGGCGGTCACACCTGGGGC
D188 $fasR_{1047}$	Knockout Development	D188_fasR_shift_F	gctctagaactagtggatccCGTCACCTTGAGCAAGACCT
		D188_fasR_shift_R	atcggcttcGAGaATCAACCTGTCGACGAGT
	Gene Specific	D188_fasR_F	CAAAAAAGCAGGCTCCATGAGCAAATTAAAGACC
		D188_fasR_R	GAAAGCTGGGTGGCTGGGTTCCGCGTAAAACCT
	Cloning	fasR_1047_pJDC165_F	gatccaataggagggaattcATGAGCAAATTAAAGACCGAACACGATTCGCGCCG
D188 $fasR_{1002}$	Cloning	fasR_1002_pJDC165_F	gatccaataggagggaattcATGGCTGCAACCGATAGAGAACCTTCAAAAAGGTTC
D188 $fasR_{834}$	Cloning	fasR_834_pJDC165_F	gatccaataggagggaattcATGACACGACTCACTGCGCGAATAGTCGATGGGGA
		fasR_pJDC165_R	atgcatctagatattggttaTTAGCTGGGTTCCGCGTAAAACCTTCTGAATTCCG
D188 *fasR* 1s2	Site-Directed Mutagenesis	D188_fasR_1047_stop_1002_F	ATGAGCAAATTATAGACCGAACAT

		D188_fasR_1047_stop_1002_R	GCGAATCATGTTCGGTCTATAAT
A21d2 *fasR*	Cloning	fasR_A21d2_Top_long	gatccATGACTGCAGCCGATAGAGAACC
		fasR_A21d2_Bottom_short	TACGCGTGAAACCTTCTGAATTC
		fasR_A21d2_Top_short	cATGACTGCAGCCGATAGAGAACC
		fasR_A21d2_Bottom_long	gaaTTACGCGTGAAACCTTCTGAATTC
D188 *fasR* 1047	Cloning	fasR_D188_Top_long	gatccATGAGCAAATTAAAGACCGAACACG
		fasR_D188_Top_short	cATGAGCAAATTAAAGACCGAACACG
		fasR_D188_Bottom_long	gaaTTAGCTGGGTTCCGCGTAAAACC
		fasR_D188_Bottom_short	TAGCTGGGTTCCGCGTAAAACC
D188 *fasR* chimera	Cloning	pJDC165_D188_1047_1002_A21d2_fasR_F	gatccaataggagggaattcATGAGCAAATTAAAGACCGAACACGATTCGCGCCGGGGGGGCGAAAACTGCAGCCGATAGAGAAC
	Cloning; PCR	pJDC165_A21d2_fasR_R	cgcgaatgcatctagatattggttaTTACGCGTGAAACCTTCTG
	PCR	D188_1047_1002_A21d2_fasR_Step_1_F	CAAATTAAAGACCGAACACGATTCGCGCCGGGGGGCGAAAACTGCAGCCGATAGAGAAC
D188 XhoI_XhoI *fasR*	Cloning; PCR	X_X_D188_fasR_F	cagtcgatcgtacgctagttTCGAGGAAGTGTGCACGGATC
		X_X_D188_fasR_R	atgcatctagatattggttaGCAAGGTCAAGAATTTTGTCT
N/A	Plasmid Backbone Screen	pJDC_vec_L5_F	GCGTGCGTTCGACCGAATCATCGA
		pJDC_vec_R	TTCCGCTGAATATCGTGGAGC

[1]Lowercase nucleotides signifies DNA sequence that are homolog to vector (for Gibson Assembly).

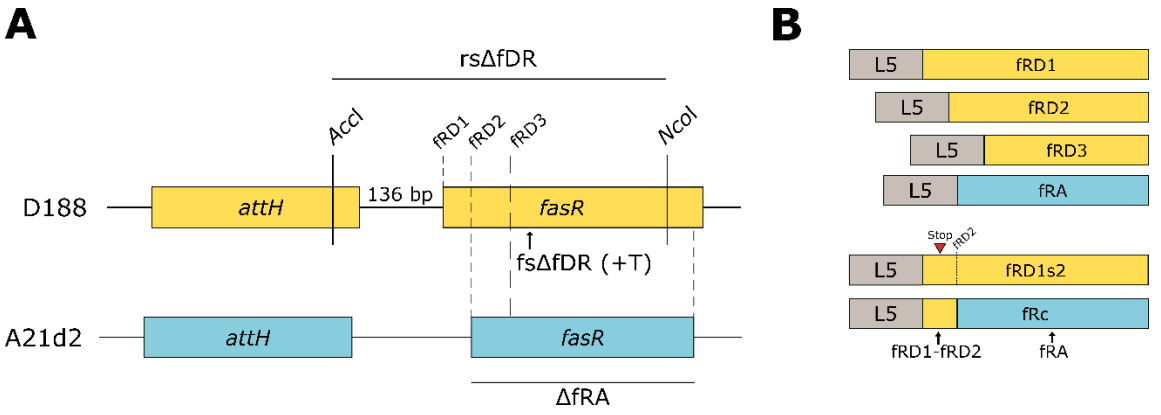

Figure 3.1: Schematic of *fasR* in strains D188 and A21d2, *fasR* mutants, and *fasR* constructs. Key features of the *fasR* coding sequence of D188 (top) are compared to those of *fasR* of A21d2 (bottom). The *attH* coding sequence is adjacent to *fasR.* The *fasR* allele of D188 has three ATG codons predicted to be in frame and are denoted fRD1 through fRD3. Also depicted are two mutants of *fasR* in D188. In the first, the *Acc*I and *Nco*I restriction sites are depicted and demark the region (912 bp) that was deleted (rsΔfRD) (Temmerman *et al*., 2000). In a second mutant, a frameshift was produced by inserting a thymine nucleotide at position 303 (fsΔfRD). The *fasR* allele of A21d2 has two ATG codons predicted to be in frame. There is one non-polar deletion mutant of *fasR* in strain A21d2 (ΔfRA). Boxes = coding sequences; lines = non-coding regions. **(B)** DNA constructs were made with different sequences of the *fasR* coding sequence fused downstream of the constitutive L5 promotor. The constructs fRD1-3 = fragments of $fasR_{D188}$, starting from the first, second, and third ATG, respectively; fRA = $fasR_{A21d2}$ starting from its first ATG; fRD1s2 is $fasR_{D188}$ fRD1 with a nonsense mutant at position 13; and fRc is a chimeric molecule consisting of the sequence from ATG1-ATG2 of $fasR_{D188}$ fused to fRA.

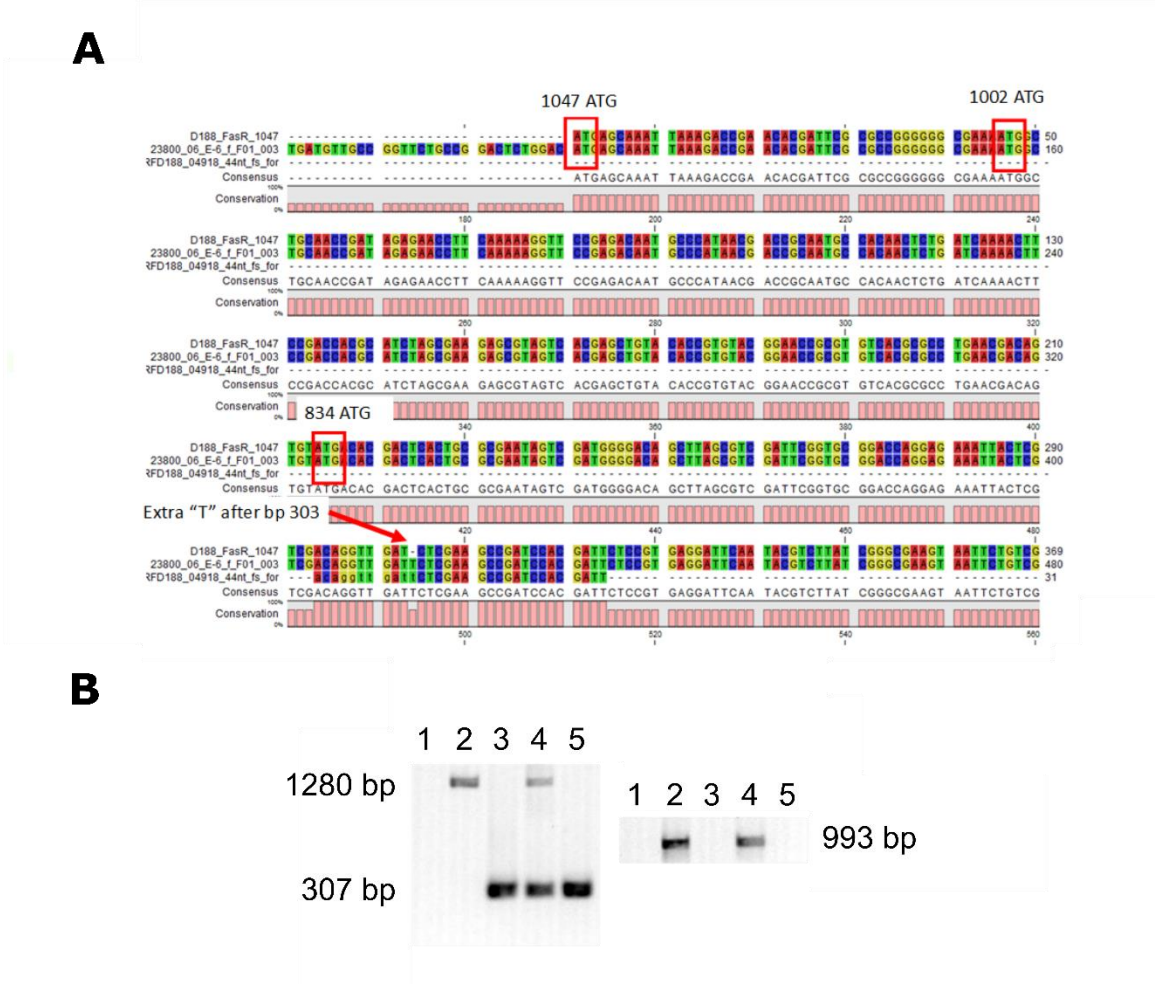

Figure 3.2. Nonfunctional *fasR* mutants developed in strain D188 and A21d2. (A) Verification of allelic exchange of *fasR* in mutant fsΔfRD. Nucleotide alignment of the D188 *fasR* coding sequence, the oligonucleotide use to introduce a thymine nucleotide, and the fsΔfRD mutant sequence. CLC Sequence Viewer (Qiagen Company, Hilden, Germany) was used to generate the alignments. **(B)** Resolution of PCR products on a 1%, 1XTAE agarose gel. The image on the left shows PCR products following amplification using primers which anneal regions that flank the *fasR* locus of A21d2. The image on the right shows PCR products following amplification using primers that anneal to the region of the A21d2 *fasR* coding sequence. Lanes are products from reactions which included the following as templates: 1: water; 2: DNA from A21d2; 3: DNA from *E. coli* carrying pSelAct_A21d2*fasR*_KO; 4: DNA from the merodiploid; 5: DNA from ΔfRA. The estimated sizes of the PCR products are depicted (basepairs).

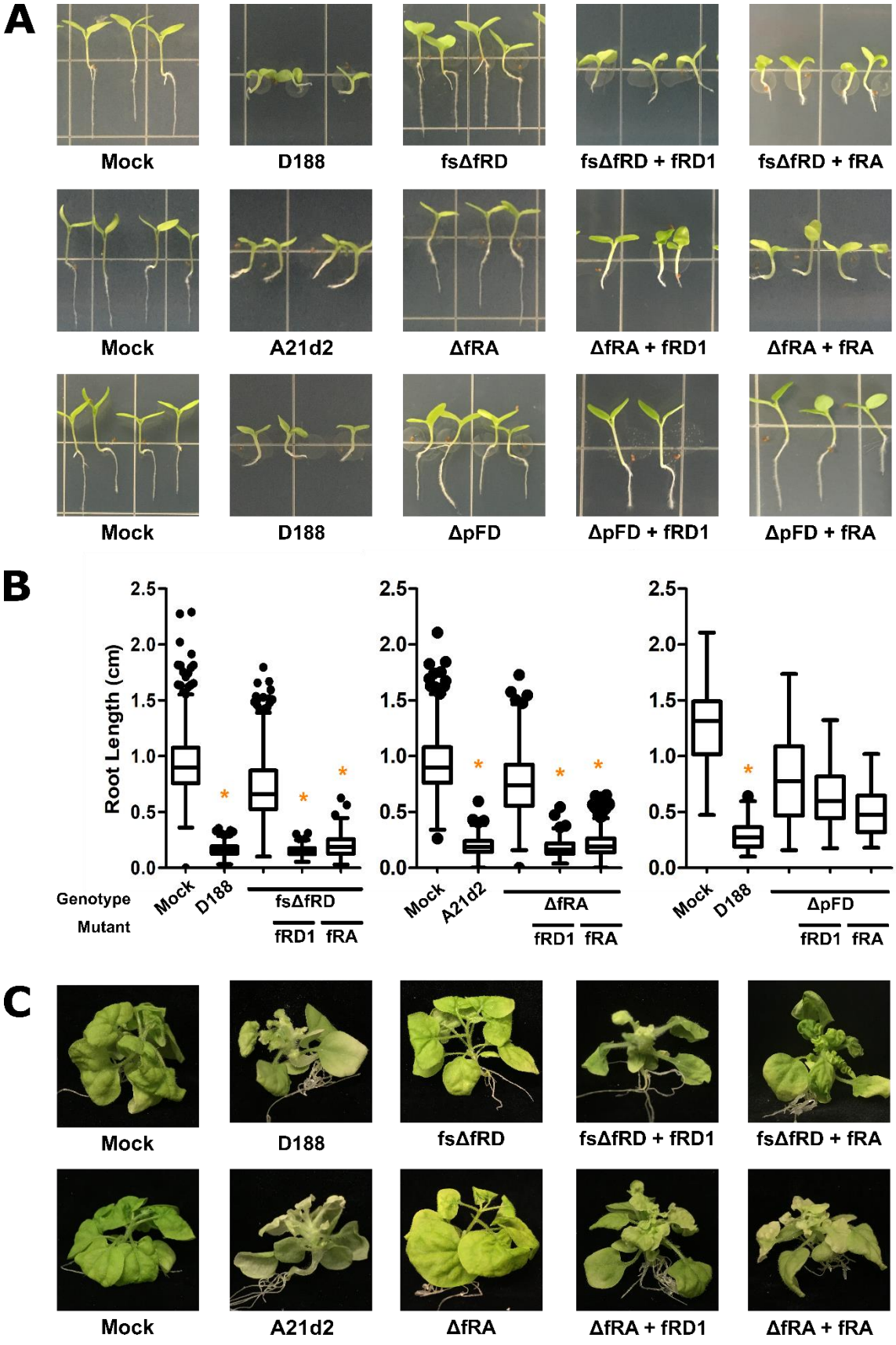
A
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRA
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRA
Mock
D188
ΔpFD
ΔpFD + fRD1
ΔpFD + fRA
B
Root Length (cm)
2.5
2.0
1.5
1.0
0.5
0.0
Genotype
Mutant
Mock
D188
fsΔfRD
fRD1
fRA
Mock
A21d2
ΔfRA
fRD1
fRA
Mock
D188
ΔpFD
fRD1
fRA
C
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRA
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRA

Figure 3.3. The *fasR* gene is necessary and its alleles are homologous in function but neither are sufficient for phytopathogenicity of *Rhodococcus.* **(A)** Inhibition of root elongation of *N. benthamiana*. Strains of *Rhodococcus* or water (mock) were inoculated onto 3-day old *N. benthamiana* seedlings. Photos of representative plants were taken at 7 dpi. At least three replicates were performed with 40 individuals per experiment. **(B)** Quantification of root lengths of *Rhodococcus*-inoculated seedlings. The roots of inoculated plants were photographed and measured 7 dpi. Error bars indicate standard error of the mean (SEM); * represents a significant difference (p-value < 0.001) relative to water (mock)-treated seedlings. **(C)** Gall formation on *N. benthamiana.* Strains of *Rhodococcus* or water (mock) were inoculated onto 4-week old *N. benthamiana* plants. Photos were taken at 18 dpi. At least three replicates were performed with 12 individuals per experiment.

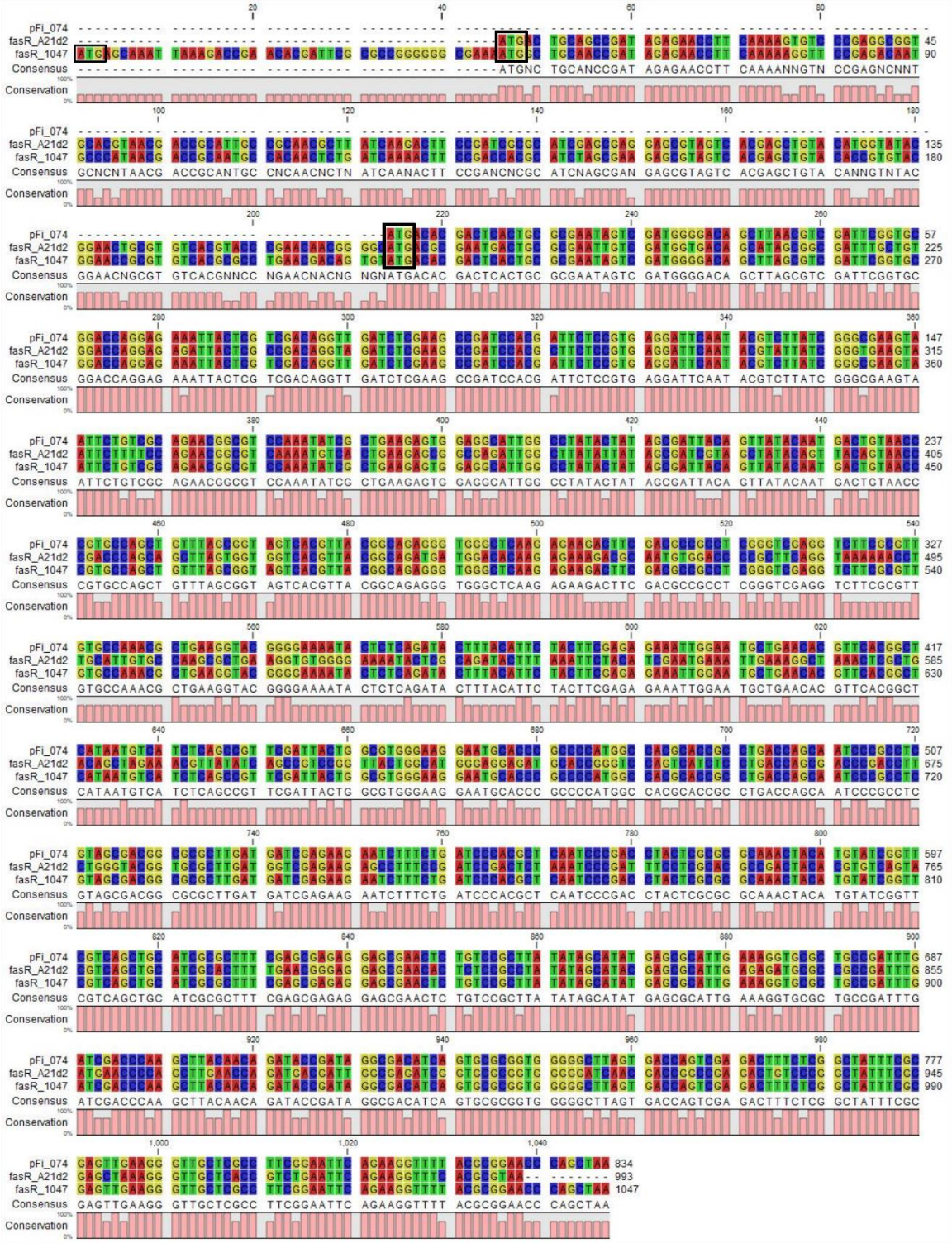
pFi_074
fasR_A21d2
fasR_1047
Consensus
Conservation

Figure 3.4. Nucleotide alignment shows the *fasR* alleles have multiple predicted in-frame ATG codons. Three sequences were aligned: pFi_074 is the originally annotated *fasR* of D188, *fasR*_A21d2 is the fasR from strain A21d2, and *fasR*_1047 is the annotation of the longer *fasR* of D188. Each predicted ATG codons is highlighted in a black square.

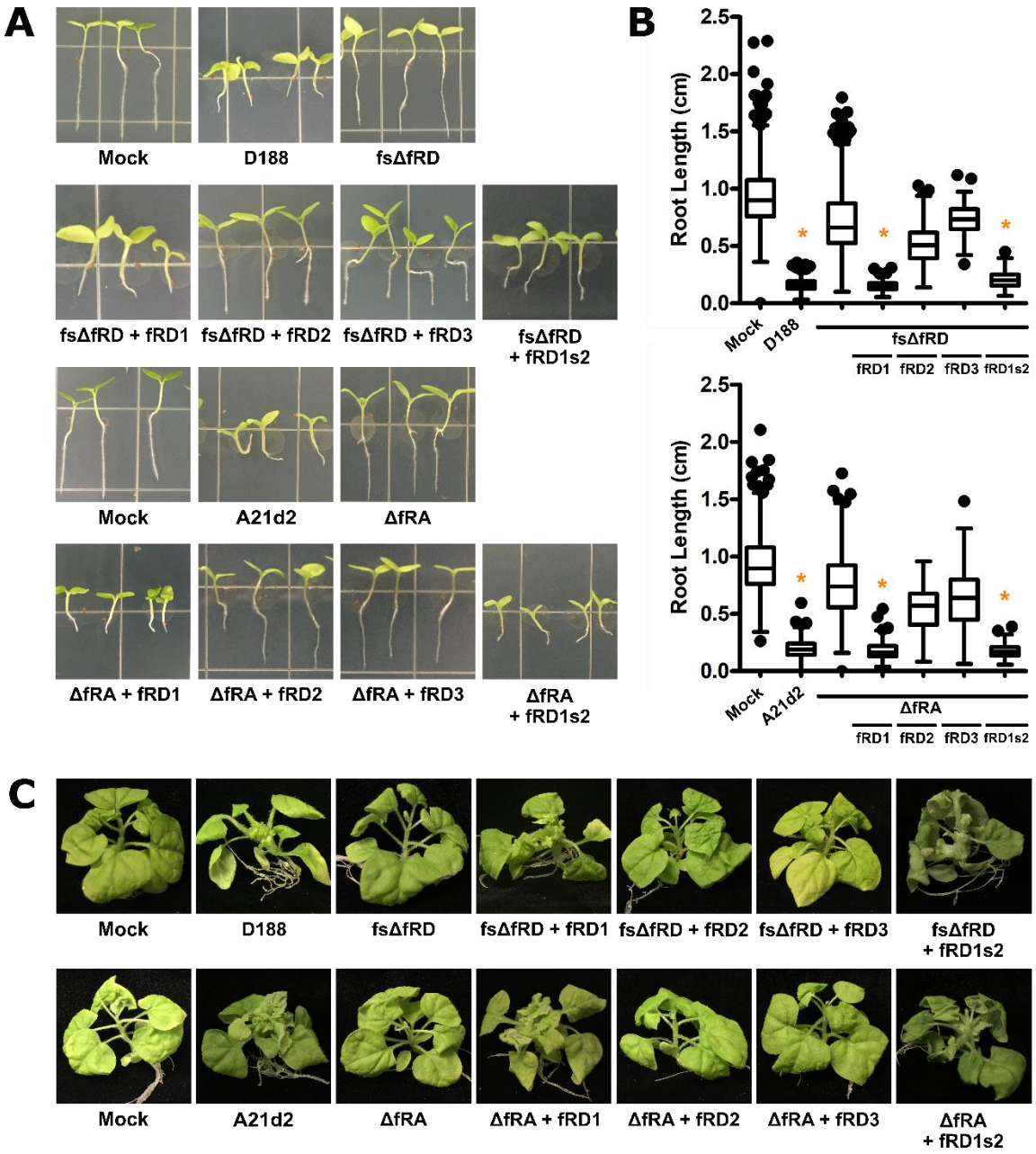
A
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRD2
fsΔfRD + fRD3
fsΔfRD + fRD1s2
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRD2
ΔfRA + fRD3
ΔfRA + fRD1s2
B
Root Length (cm)
2.5
2.0
1.5
1.0
0.5
0.0
Mock
D188
fsΔfRD
fRD1
fRD2
fRD3
fRD1s2
Root Length (cm)
Mock
A21d2
ΔfRA
fRD1
fRD2
fRD3
fRD1s2
C
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRD2
fsΔfRD + fRD3
fsΔfRD + fRD1s2
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRD2
ΔfRA + fRD3
ΔfRA + fRD1s2

Figure 3.5. Translation of FasR of D188 is inferred to start at ATG2 or ATG3. **(A)** Inhibition of root elongation of *N. benthamiana*. Strains of *Rhodococcus* or water (mock) were inoculated onto 3-day old *N. benthamiana* seedlings. Photos of representative plants were taken at 7 dpi. At least three replicates were performed with 40 individuals per experiment. **(B)** Quantification of root lengths of *Rhodococcus*-inoculated seedlings. The roots of inoculated plants were photographed and measured 7 dpi. Error bars indicate standard error of the mean (SEM); * represents a significant difference (p-value < 0.001) relative to water (mock)-treated seedlings. **(C)** Gall formation on *N. benthamiana.* Strains of *Rhodococcus* or water (mock) were inoculated onto 4-week old *N. benthamiana* plants. Photos were taken at 18 dpi. At least three replicates were performed with 12 individuals per experiment.

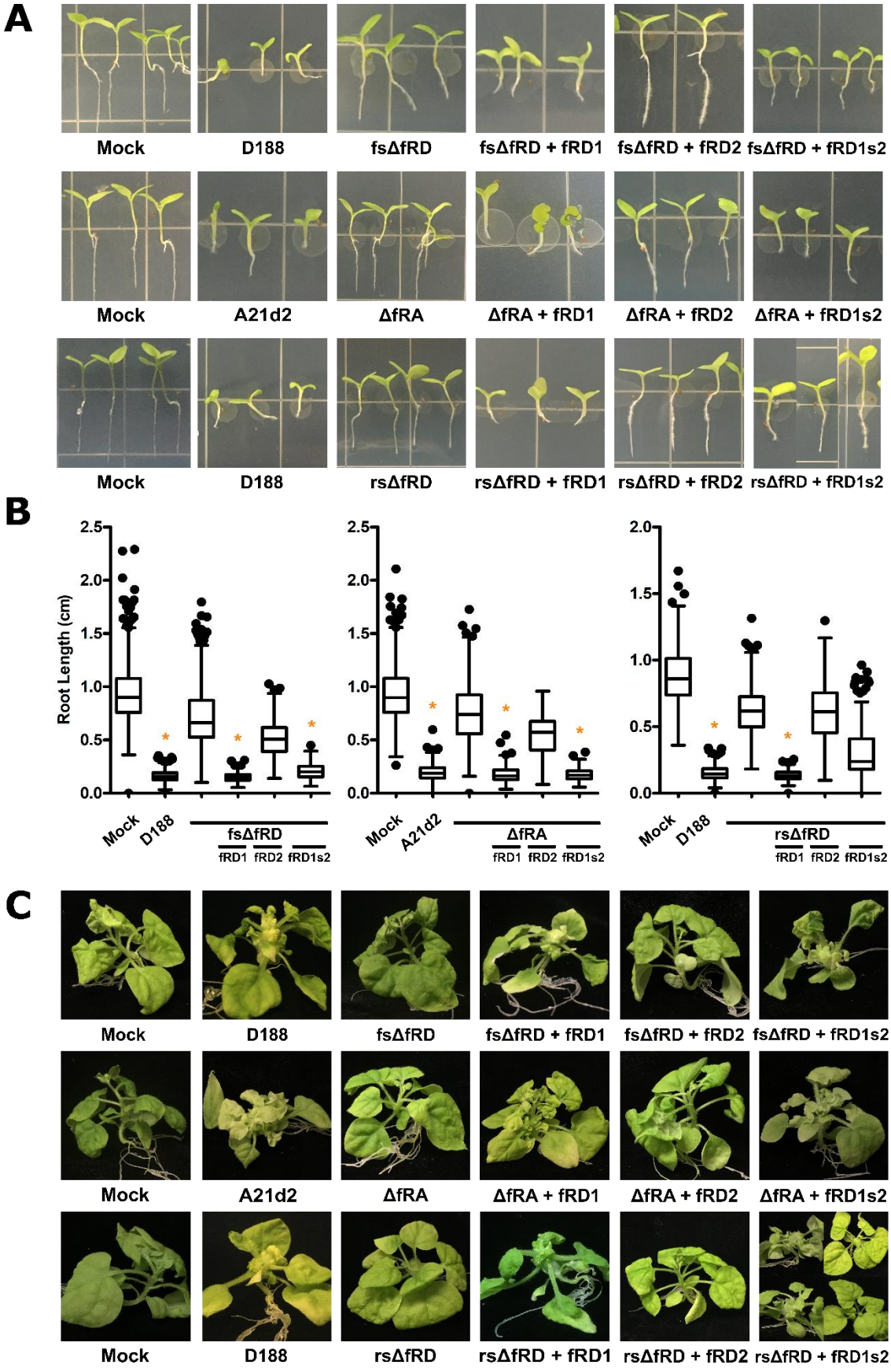
A
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRD2
fsΔfRD + fRD1s2
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRD2
ΔfRA + fRD1s2
Mock
D188
rsΔfRD
rsΔfRD + fRD1
rsΔfRD + fRD2
rsΔfRD + fRD1s2
B
Root Length (cm)
2.5
2.0
1.5
1.0
0.5
0.0
Mock
D188
fsΔfRD
fRD1
fRD2
fRD1s2
Mock
A21d2
ΔfRA
fRD1
fRD2
fRD1s2
2.0
1.5
1.0
0.5
0.0
Mock
D188
rsΔfRD
fRD1
fRD2
fRD1s2
C
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRD2
fsΔfRD + fRD1s2
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRD2
ΔfRA + fRD1s2
Mock
D188
rsΔfRD
rsΔfRD + fRD1
rsΔfRD + fRD2
rsΔfRD + fRD1s2

Figure 3.6. Strain rsΔfRD + fRD1s2 is more variable in phytopathogenicity. **(A)** Inhibition of root elongation of *N. benthamiana*. Strains of *Rhodococcus* or water (mock) were inoculated onto 3-day old *N. benthamiana* seedlings. Photos of representative plants were taken at 7 dpi. For rsΔfRD + fRD1s2, photographs of three different plants are presented to show the higher variation in results. At least three replicates were performed with 40 individuals per experiment. **(B)** Quantification of root lengths of *Rhodococcus*-inoculated seedlings. The roots of inoculated plants were photographed and measured 7 dpi. Error bars indicate standard error of the mean (SEM); * represents a significant difference (p-value < 0.001) relative to water (mock)-treated seedlings. **(C)** Gall formation on *N. benthamiana.* Strains of *Rhodococcus* or water (mock) were inoculated onto 4-week old *N. benthamiana* plants. Photos were taken at 18 dpi. At least three replicates were performed with 12 individuals per experiment.

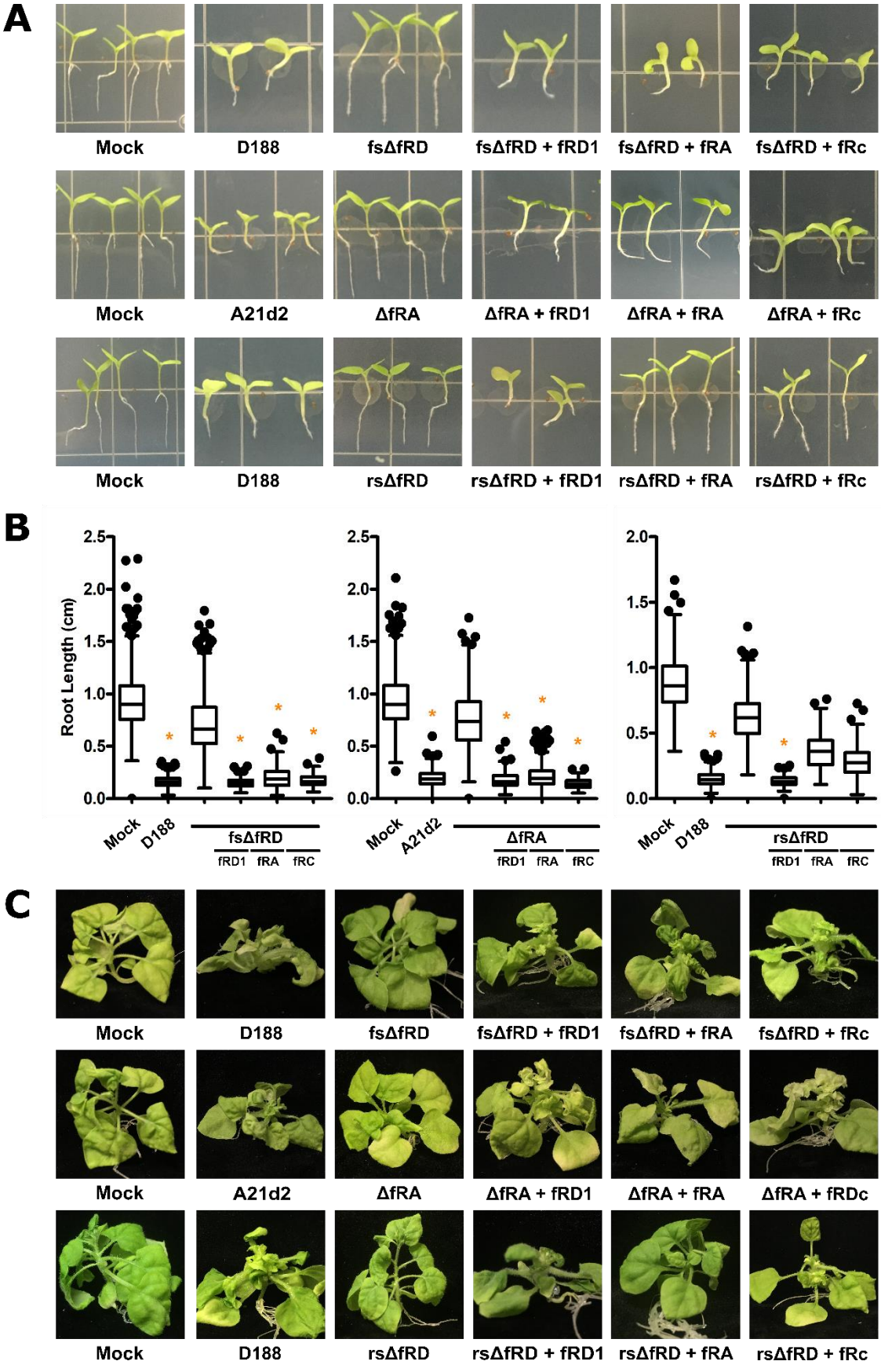
A
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRA
fsΔfRD + fRc
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRA
ΔfRA + fRc
Mock
D188
rsΔfRD
rsΔfRD + fRD1
rsΔfRD + fRA
rsΔfRD + fRc
B
Root Length (cm)
2.5
2.0
1.5
1.0
0.5
0.0
Mock
D188
fsΔfRD
fRD1
fRA
fRC
Mock
A21d2
ΔfRA
fRD1
fRA
fRC
2.0
1.5
1.0
0.5
0.0
Mock
D188
rsΔfRD
fRD1
fRA
fRC
C
Mock
D188
fsΔfRD
fsΔfRD + fRD1
fsΔfRD + fRA
fsΔfRD + fRc
Mock
A21d2
ΔfRA
ΔfRA + fRD1
ΔfRA + fRA
ΔfRA + fRDc
Mock
D188
rsΔfRD
rsΔfRD + fRD1
rsΔfRD + fRA
rsΔfRD + fRc

Figure 3.7. A chimera between *fasR* alleles does not complement rsΔfRD. (A) Inhibition of root elongation of *N. benthamiana*. Strains of *Rhodococcus* or water (mock) were inoculated onto 3-day old *N. benthamiana* seedlings. Photos of representative plants were taken at 7 dpi. At least three replicates were performed with 40 individuals per experiment. **(B)** Quantification of root lengths of *Rhodococcus*-inoculated seedlings. The roots of inoculated plants were photographed and measured 7 dpi. Error bars indicate standard error of the mean (SEM); * represents a significant difference (p-value < 0.001) relative to water (mock)-treated seedlings. **(C)** Gall formation on *N. benthamiana.* Strains of *Rhodococcus* or water (mock) were inoculated onto 4-week old *N. benthamiana* plants. Photos were taken at 18 dpi. At least three replicates were performed with 12 individuals per experiment.

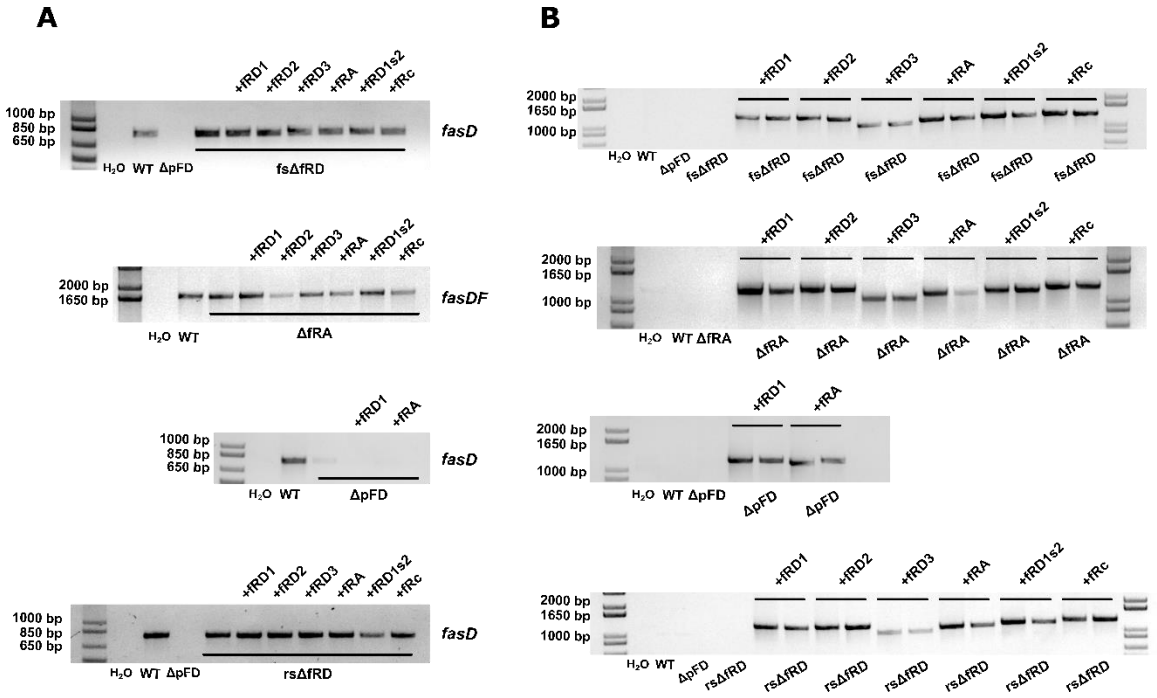

Figure 3.8. PCR-based verification of genetic background and presence of constructs in modified strains. (A) Inverse image of a 1%, 1XTAE agarose gel. The presence of the virulence plasmid (D188) was determined on the basis of amplifying positively for *fasD.* Strain A21d2 was verified on the basis of amplifying positively for *fasDF*. Sizes of marker bands (bp) are listed to the left of the images. **(B)** Inverse image of a 1%, 1XTAE agarose gel. The presence of *fasR* constructs was determined on the basis of amplifying positively for *fasR*. Sizes of marker bands (bp) are listed to the left of the images.

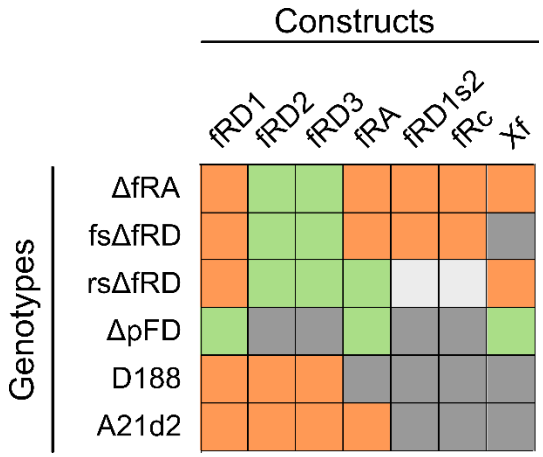

Figure 3.9: Summary of pathogenicity phenotype of 30 genotype x construct combinations. Different colors are used to summarize the pathogenicity phenotype of the mutant x construct combination: orange = pathogenic; green = nonpathogenic; light grey = intermediate phenotype, and dark grey = not tested. Pathogenicity is based on the ability to inhibit root growth of *N. benthamiana* and cause galls.

Conclusions and Future Directions

Danielle M. Stevens

Rhodococcus causes disease to a large number of plant species and can be responsible for significant economic damage in US agriculture. Three loci are known to be necessary to influence or be necessary for the virulence of this Gram-positive bacterial pathogen (Francis *et al.,* 2012). Here, we focused on *fasR*, which is hypothesized to be member of the AraC-type transcription factors. Our overarching hypothesis is that *fasR* is necessary for virulence and functions to co-opt genes of the chromosome, and their misregulation leads to pathogenicity.

As a first step towards addressing this hypothesis, we developed new mutant strains that are predicted to be compromised only in the *fasR* coding sequence. We demonstrated the *fasR* virulence gene is necessary but not sufficient for *Rhodococcus* to cause disease to plants (Figure 3.3). This was necessary because previous conclusions on the necessity of *fasR* in virulence were drawn from a deletion mutant that affected the adjacent *attH* gene and is predicted to be polar (Temmerman *et al.,* 2000).

Second, we developed and tested variants of *fasR* to determine which of the three in-frame ATG sequences is likely the start codon. Our findings showed that only the variant that included all three ATG codons was sufficient to complement loss-of-function mutants (Figure 3.5). However, the observation that an introduced non-sense mutation immediately downstream of the first ATG was functional was unexpected and suggests translation may initiate at either position 1002 or 834 (Figure 3.4, Figure 3.5).

There are several explanations for the observed findings. First, there is potential for a 5’ untranslated region (UTR) to be necessary for regulating the

expression of *fasR*. Alternatively, it is possible that there is read through of the introduced non-sense mutation or use of an alternative start codon.

To distinguish between the most plausible possibilities, qRT-PCR and western blot analysis of tagged proteins could be used to measure transcription and translation of the variants of *fasR*. If a 5' UTR is necessary to provide stability to the mRNA, we would expect to detect no transcription and no translation of *fasR*. If readthrough is occurring in the mutant allele, then we could expect to detect both transcription and translation of *fasR*. If results were consistent with readthrough, we would need to introduce multiple nonsense substitutions and test whether a polymutant can complement the knockout mutants. This would allow us to more confidently determine which ATG is the start codon and whether a 5' UTR is influencing the expression of *fasR*.

Based on the co-option model, we hypothesize that FasR, a predicted transcriptional regulator, has a key role in misregulating the expression of chromosome-encoded genes for pathogenicity. Having developed non-polar mutants of *fasR* and taken important steps towards defining the sequences sufficient for expression of *fasR*, we can couple our genetically-modified strains with whole transcriptome analyses such as RNA-Seq or ChIP-Seq to identify and characterize the FasR-regulon and test the co-option model.

Based on the data obtained in this work, we can conclude that *fasR* is a necessary virulence locus that is essential for determining the mechanism of virulence in phytopathogenic *Rhodococcus.* Further work on this gene as well as other indicated virulence genes within the *fas* locus is necessary to refine the

model of phytopathogenicity in *Rhodococcus.* In doing so, we can better understand how only a few mobile genes carried on a conjugative plasmid can drive the evolution of pathogens of plants.

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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*. J. Bacteriol. 182: 5832-5840.