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RESEARCH ARTICLE

Extragenic Suppression of Elongation Factor P Gene Mutant Phenotypes in *Erwinia amylovora*

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ABSTRACT Elongation factor P (EF-P) facilitates the translation of certain peptide motifs, including those with multiple proline residues. EF-P must be posttranslationally modified for full functionality; in enterobacteria, this is accomplished by two enzymes, namely, EpmA and EpmB, which catalyze the β-lysylation of EF-P at a conserved lysine position. Mutations to *efp* or its modifying enzymes produce pleiotropic phenotypes, including decreases in virulence, swimming motility, and extracellular polysaccharide production, as well as proteomic perturbations. Here, we generated targeted deletion mutants of the *efp*, *epmA*, and *epmB* genes in the Gram-negative bacterium *Erwinia amylovora*, which causes fire blight, an economically important disease of apples and pears. As expected, the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants were all defective in virulence on apples, and all three mutants were complemented in *trans* with plasmids bearing wild-type copies of the corresponding genes. By analyzing spontaneous suppressor mutants, we found that mutations in the *hrpA3* gene partially or completely suppressed the colony size, extracellular polysaccharide production, and virulence phenotypes in apple fruits and apple tree shoots but not the swimming motility phenotypes of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants. The deletion of *hrpA3* alone did not produce any alterations in any characteristics measured, indicating that the HrpA3 protein is not essential for any of the processes examined. The *hrpA3* gene encodes a putative DEAH-box ATP-dependent RNA helicase. These results suggest that the loss of the HrpA3 protein at least partially compensates for the lack of the EF-P protein or β-lysylated EF-P.

IMPORTANCE Fire blight disease has relatively few management options, with antibiotic application at bloom time being chief among them. As modification to elongation factor P (EF-P) is vital to virulence in several species, both EF-P and its modifying enzymes make attractive targets for novel antibiotics. However, it will be useful to understand how bacteria might overcome the hindrance of EF-P function so that we may be better prepared to anticipate bacterial adaptation to such antibiotics. The present study indicates that the mutation of *hrpA3* could provide a partial offset for the loss of EF-P activity. In addition, little is known about EF-P functional interactions or the HrpA3 predicted RNA helicase, and our genetic approach allowed us to discern a novel gene associated with EF-P function.

KEYWORDS DEAH-box RNA helicase, elongation factor P, *Erwinia amylovora*, apple, fire blight, ribosome

The translation of mRNA into protein by ribosomes is an essential function of all cells. The ribosome may pause during this translation as a function of steric hindrance of amino acids or uncharged tRNA, which reduces the rate of protein synthesis (1). These pauses may either require intervention for cell function or serve as a regulatory step in protein synthesis, such as pauses regulating the transcription of downstream genes (2).

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Elongation factor P (EF-P), a protein discovered in *Escherichia coli*, is associated with the 70S ribosome and stimulates peptide bond formation (3, 4). EF-P is a ubiquitous prokaryotic homologue of eukaryotic eIF5A, a protein that promotes translation of proline-rich motifs (5). The association of EF-P with the ribosome is transient and dynamic (6). EF-P can diminish ribosomal stalling at proline-rich regions of polypeptides by stimulating peptide bond formation in the ribosome (7–9). In addition, other motifs, both with and without proline, have been shown to require EF-P for efficient translation (10–12). EF-P-mediated alleviation of translational stalling is thought to play a role in protein abundance only when translation initiation is not the rate-limiting step in the synthesis of a protein (13).

EF-P must be posttranslationally modified for full functionality. In *E. coli* and *Salmonella* spp., EF-P is β-lysylated at a conserved lysine position by two proteins, namely, EpmA (YjeA and PoxA) and EpmB (YjeK) (14). EpmB is a lysine-2,3-aminomutase that converts L-lysine into (*R*)-β-lysine, which is ligated to the conserved Lys34 residue of EF-P by EpmA. While EF-P β-lysylation was originally described in enterobacteria, it is now clear that several other posttranslational modification pathways exist in more divergent bacteria. A lysine residue is posttranslationally modified in *Bacillus subtilis* by a range of enzymes (15). In *Pseudomonas aeruginosa*, EF-P is instead rhamnosylated at a conserved arginine residue by EarP (16). Unlike most species in which mutations to modifying enzymes produce a similar phenotype to *efp* mutants, *B. subtilis*-modifying enzyme mutants show distinct phenotypes, including altered proline translation rate and sensitivity to certain chemicals (15).

EF-P is required for full virulence in several species, including *Salmonella enterica*, *Shigella flexneri*, and *Agrobacterium tumefaciens* (17–19). The mutants also exhibit a wide range of other phenotypes, including increased chemical sensitivity, decreased swimming motility, and decreased extracellular polysaccharide production (17, 20, 21).

Fire blight is a serious bacterial disease of rosaceous plants, including apple and pear, which is caused by the enterobacterium *Erwinia amylovora*. Bacteria typically enter the plant through flowers (22) and are able to spread systemically within the plant, causing severe tissue necrosis, yield losses, and potentially tree mortality. Antibiotics, such as streptomycin, are heavily used for fire blight disease management, which not only increases environmental levels of antibiotics but also produces high pressure for antibiotic resistance (23). Like many other enterobacteria, *E. amylovora* requires both a type III secretion system and the production of extracellular polysaccharides for pathogenicity (24, 25).

Recently, we described an *E. amylovora epmB* (*yjeK*) mutant that displays environmentally dependent changes to its proteome, as well as a decrease in virulence and the production of the extracellular polysaccharide amylovoran (21). Amylovoran is an essential virulence factor for *E. amylovora*, and mutations in the amylovoran biosynthesis operon (*ams*) result in a total loss of pathogenicity (25), and reduced amylovoran production in the *epmB* mutant likely contributes to its reduced ability to cause fire blight. In the present study, we identified extragenic suppressor mutants of an *efp* deletion mutant that were able to restore multiple phenotypes to wild type. These suppressors all contained mutations in *hrpA3* (DEAH family RNA helicase-like protein A [26]), which encodes a predicted ATP-dependent RNA helicase. These data help elucidate the precise role of EF-P in protein translation.

RESULTS AND DISCUSSION

Δ*efp*, Δ*epmA*, and Δ*epmB* mutants display similar virulence defects. Previously, we analyzed the virulence defect of an *E. amylovora epmB* (*yjeK*) Tn*5* transposon mutant (21). The *epmB* Tn*5* mutant was defective in virulence in apple fruits and tree shoots. In addition, the *epmB* Tn*5* mutant had defects in extracellular polysaccharide production and swimming motility, increased chemical sensitivity, and major alterations to the proteome (21). In several systems, the phenotypes of *efp*, *epmA*, and *epmB* mutants have been shown to be nearly identical, which is consistent with their roles related to EF-P production and function (20).

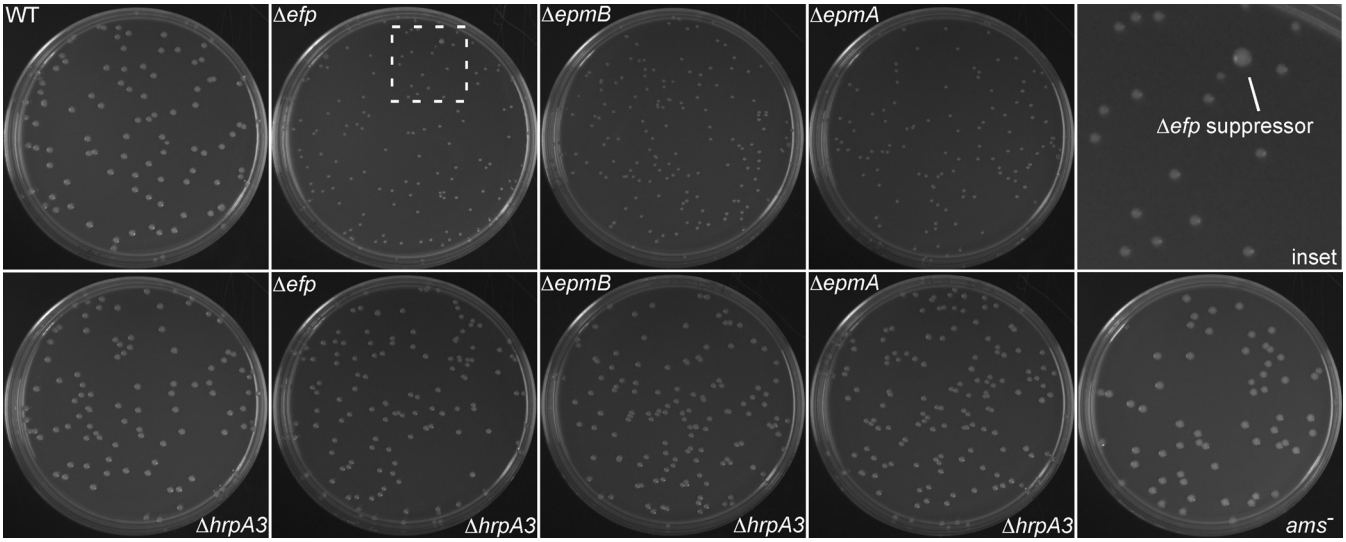

FIG 1 Deletion of *hrpA3* restores colony size to that of the wild type in Δ*efp*, Δ*epmA*, and Δ*epmB* genetic backgrounds. LB plates are shown after 72 hours of growth at 28°C. Genotypes are indicated on the figure. "Inset" represents a magnification of the boxed area in the Δ*efp* panel and contains an example spontaneous suppressor colony. Plates are 100- by 15-mm petri dishes.

Here, we created individual, targeted deletion mutants of the *efp*, *epmA*, and *epmB* genes in *E. amylovora* (Δ*efp*, Δ*epmA*, and Δ*epmB*) (see Table S1 in the supplemental material). These mutants were inoculated onto apple fruitlets, and all produced severely attenuated disease development (see Fig. S1 in the supplemental material). The virulence defect phenotypes of all three mutants were complemented in *trans* with plasmid-borne copies of the respective genes (Fig. S1). These results indicate that *efp*, *epmA*, and *epmB* all have similar mutant phenotypes in *E. amylovora*, and genetic complementation confirms the specific role of each gene in the phenotype.

Spontaneous suppressors of the Δ*efp*, Δ*epmA*, and Δ*epmB* small-colony phenotype. While growing the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants on rich-medium LB agar plates, it was noted that all of them produced small colonies compared to the wild type (Fig. 1 and 2). When plated for single colonies, Δ*efp*, Δ*epmA*, and Δ*epmB* strains periodically displayed occasional larger colonies with sizes similar to wild-type *E. amylovora* colonies (Fig. 1, inset). When these wild-type-sized colony strains were isolated, they consistently produced only large colonies, suggesting that the phenotype was stable. PCR tests verified the Δ*efp*, Δ*epmA*, and Δ*epmB* genotypes of the wild-type-sized colony strains, indicating that these represented potential suppressor mutants of the small-colony phenotype of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants. Six independent wild-type-sized colony isolates from independent cultures of the Δ*efp* mutant (Δ*efp*^{L1} to Δ*efp*^{L6}) and one wild-type-sized colony isolate in the Δ*epmB* genetic background (Δ*epmB*^{L1}) were obtained in pure culture (Table S1). All suppressors were isolated from unique parent strains to ensure that suppressors were not simply clones.

All sequenced suppressors had mutations in *hrpA3*. The complete genome sequences of seven wild-type-sized colony isolates (Δ*efp*^{L1} to Δ*efp*^{L6} and Δ*epmB*^{L1}) were determined, assembled, and compared with the sequence of the parental wild-type HKN06P1 *E. amylovora* lab strain. Only one gene contained sequence polymorphisms in all seven of the suppressors. This gene, *hrpA3*, encodes a putative RNA helicase protein (coding DNA sequence [CDS] GenBank accession no. CBA20795 in *E. amylovora* strain CFBP1430).

Suppressors Δ*efp*^{L1} to Δ*efp*^{L6} all contained frameshift mutations predicted to disrupt the C-terminal half of the HrpA3 protein, while suppressor strain Δ*epmB*^{L1} contained a large deletion of the 5′ region of the *hrpA3* gene (Fig. 3; see File S1 in the supplemental material). Despite being isolated from independent parental strains, suppressors Δ*efp*^{L2}, Δ*efp*^{L3}, and Δ*efp*^{L5} all contained the same *hrpA3* mutant allele, suggesting that they

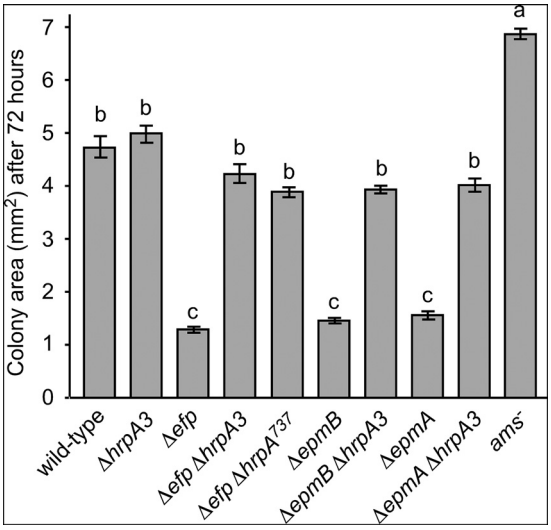

FIG 2 Quantification of colony sizes shown in Fig. 1. Colony sizes for the indicated strains were measured after 72 hours of growth on LB plates at 28°C. Colony size was quantified using NIH ImageJ to measure the area of each colony. Bars represent the average areas of 10 colonies for each genotype from a single representative experiment, and error bars indicate standard error (SE). Strains sharing a letter have no statistically significant difference based on a Tukey test. The entire experiment was performed at least four times with similar results each time.

were actually independent mutation events resulting in the same *hrpA3* allele. These results imply that the mutation of *hrpA3* can suppress the small-colony phenotype of *efp* and *epmB* mutants and that the loss of a functional HrpA3 protein can compensate in some way for the loss of a functional EF-P protein, at least in terms of colony size. All of the mutations resulted in major predicted alterations to the HrpA3 protein structure, and no amino acid substitution mutations were obtained. This finding suggests that a major disruption of the HrpA3 protein structure is required in order to suppress the effects of the loss of EF-P.

hrpA3 codes for a predicted DEAH-box RNA helicase. While these proteins are referred to as "helicases," their double-stranded unwinding activity is unclear (27). Characterized mostly in eukaryotes, DEXD/H box RNA helicases are thought to be involved in multiple cellular processes, including translational initiation and termination, ribosome biogenesis, and RNA degradation (28). In *Borrelia burgdorferi*, *hrpA3* is necessary for infectivity in mice (29), while in *E. coli* it is necessary for the processing of the *daa* fimbrial operon (30).

Deletion of *hrpA3* suppresses the small-colony phenotype of Δ*efp*, Δ*epmA*, and Δ*epmB* mutants. To confirm that mutations to *hrpA3* were responsible for the wild-type-sized colony phenotype of the Δ*efp* suppressor strains, the *hrpA3* gene was deleted in wild-type, Δ*efp*, Δ*epmA*, and Δ*epmB* genetic backgrounds. In addition, a partial genomic deletion of *hrpA3* (Δ*hrpA3*^{737}) was constructed in the Δ*efp* genetic

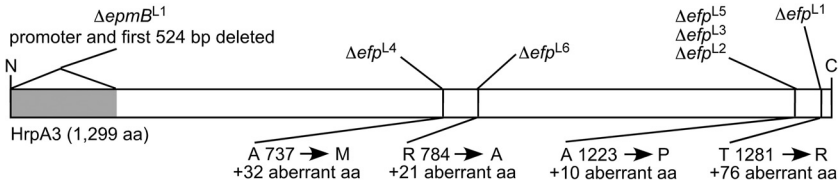

FIG 3 Polymorphisms in the *hrpA3* gene detected in seven independent suppressors of the Δ*efp* and Δ*epmB* small-colony phenotype. Δ*efp*^{L1} to Δ*efp*^{L6} are from six independent Δ*efp* suppressors; Δ*epmB*^{L1} is from a Δ*empB* suppressor. For the frameshift mutations, alterations to the predicted protein product of the *hrpA3* gene are indicated as the position of the first altered amino acid, along with the length of additional, aberrant amino acids produced by the frameshift. N and C indicate the amino and carboxy termini, respectively, of the HrpA3 protein.

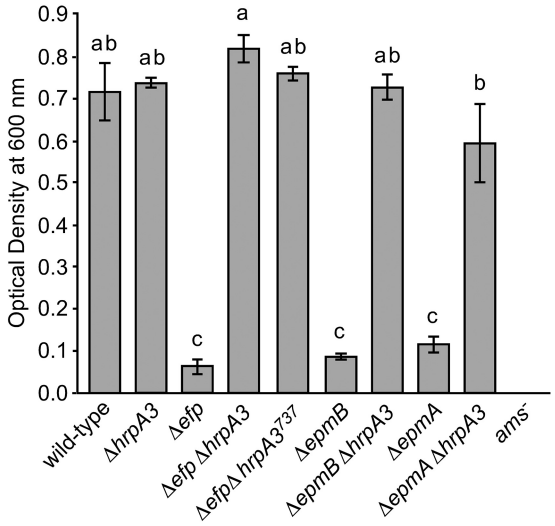

FIG 4 Deletion of *hrpA3* restores the amylovoran extracellular polysaccharide production level to that of the wild type in Δ*efp*, Δ*epmA*, and Δ*epmB* genetic backgrounds. Amylovoran production in the indicated strains was assayed via a cetylpyridinium chloride precipitation assay, where the precipitate OD at 600 nm is measured using a spectrophotometer. The experiment was repeated three times, with similar results each time. Error bars represent SE. Strains sharing the same letter have no statistically significant difference based on a Tukey test.

background. This deletion terminated the predicted protein product at amino acid position 737, which corresponded to the earliest change seen among the frameshift mutations (Δ*efp*^{737}) (Fig. 3). The Δ*efp* Δ*hrpA3*, Δ*efp* Δ*hrpA3*^{737}, Δ*epmA* Δ*hrpA3*, and Δ*epmB* Δ*hrpA3* double mutants all had colony sizes similar to those of the wild type on LB agar plates (Fig. 1 and 2). The Δ*hrpA3* single mutant had colonies similar in size to those of the wild type (Fig. 1 and 2). In addition, a mutant with a Tn*5* insertion in the promoter region of the *ams* operon (*ams*$^{-}$) (21) was analyzed for colony size, and the colonies were larger than the wild type (Fig. 1 and 2).

These results confirm that a mutation of *hrpA3* can suppress the small-colony phenotype of Δ*efp*, Δ*epmA*, and Δ*epmB* mutants, as implicated by the suppressor genome sequencing results. In addition, a partial deletion of *hrpA3* (Δ*hrpA3*^{737}) was sufficient to suppress the small-colony phenotype of the Δ*efp* mutant. However, disruption of *hrpA3* alone did not affect colony size. The normal colony size of the Δ*hrpA3* single mutant indicated that the *hrpA3* gene is dispensable for growth on LB agar.

Deletion of *hrpA3* suppresses the amylovoran production defect of Δ*efp*, Δ*epmA*, and Δ*epmB* mutants. Previously, we reported that a Tn*5* mutation in *epmB* (*yjeK*) resulted in greatly diminished amylovoran production (21). Consistent with the previous findings, the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants produced a minimal amount of the extracellular polysaccharide amylovoran (Fig. 4). In contrast, the Δ*efp* Δ*hrpA3*, Δ*efp* Δ*hrpA3*^{737}, Δ*epmA* Δ*hrpA3*, and Δ*epmB* Δ*hrpA3* double mutants had amylovoran production levels similar to those of the wild type (Fig. 4). As expected, the *ams*$^{-}$ mutant did not produce detectable amylovoran; however, the Δ*hrpA3* single mutant had amylovoran production levels similar to those of the wild type. These results indicate that the mutation of *hrpA3* can suppress the amylovoran production defect of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants. However, the mutation of *hrpA3* alone does not affect amylovoran production, indicating that *hrpA3* is dispensable for amylovoran production by *E. amylovora*.

Deletion of *hrpA3* does not suppress swimming motility defects of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants. Previously, we reported that a Tn*5* mutation in *epmB* (*yjeK*) resulted in greatly diminished swimming motility in low-density agar medium, and this correlated with reduced abundance of particular motility-associated proteins

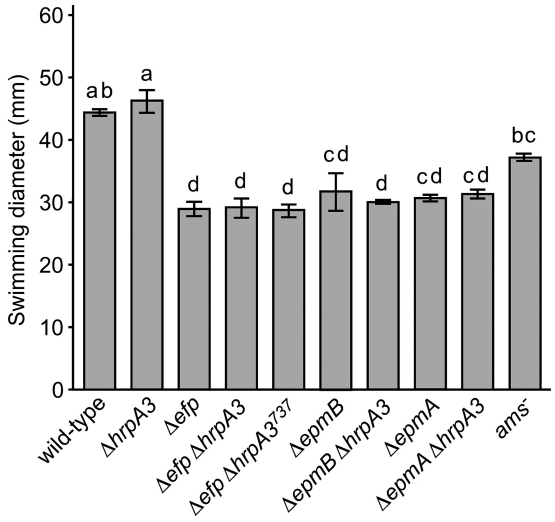

FIG 5 Deletion of *hrpA3* does not increase swimming motility in Δ*efp*, Δ*epmA*, and Δ*epmB* genetic backgrounds. Bars represent average diameters of visible bacteria for the indicated strains on M9 minimal medium plates after 48 hours at 28°C. The experiment was repeated four times with similar results each time. Error bars represent SE. Strains sharing the same letter have no statistically significant difference based on a Tukey test.

(21). Similarly, the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants all had reduced swimming motility (Fig. 5). In addition, the swimming motility of the Δ*efp* Δ*hrpA3*, Δ*efp* Δ*hrpA3*^{737}, Δ*epmA* Δ*hrpA3*, and Δ*epmB* Δ*hrpA3* double mutants was similar to the cognate Δ*efp*, Δ*epmA*, and Δ*epmB* single mutants, respectively (Fig. 5). The Δ*hrpA3* mutant had a similar swimming motility as that of the wild type. These results indicate that disruption of *hrpA3* cannot suppress the swimming motility defect of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants and that *hrpA3* by itself is not required for *E. amylovora* swimming motility. This finding indicates that not all phenotypes of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants are suppressed by the mutation of *hrpA3*, suggesting that the loss of the HrpA3 protein cannot compensate for all the defects caused by a lack of the EF-P protein.

Deletion of *hrpA3* partially suppresses virulence defects of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants. Previously, we reported that a Tn*5* mutation in *epmB* (*yjeK*) resulted in greatly diminished virulence and growth on apple fruitlets and virulence in apple tree shoots (21). Consistent with this, the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants were all minimally virulent on apple fruit, producing little of the necrosis and bacterial ooze indicative of fire blight disease development, while the wild-type strain produced severe necrosis and ooze on all fruits inoculated (Fig. 6A). In contrast, the Δ*efp* Δ*hrpA3*, Δ*efp* Δ*hrpA3*^{737}, Δ*epmA* Δ*hrpA3*, and Δ*epmB* Δ*hrpA3* double mutants produced symptoms on apple fruitlets similar to those produced by the wild type, and the Δ*hrpA3* single mutant also produced similar disease symptoms on apple fruitlets as those of the wild type (Fig. 6A). As expected, the *ams*$^{-}$ mutant produced no symptoms on the fruit, which is consistent with amylovoran being essential for *E. amylovora* virulence (25). These results indicate that deletion of *hrpA3* partially suppresses the virulence defect of the Δ*efp*, Δ*epmA*, and Δ*epmB* mutants.

E. amylovora growth in apple fruit tissues typically correlates with severity of disease development (31). Growth of the wild-type, Δ*efp*, Δ*efp* Δ*hrpA3*, Δ*hrpA3*, and *ams*$^{-}$ *E. amylovora* strains in the apple fruitlets was quantified 2 days after inoculation, when the majority of population increase is typically observed (Fig. 6B). Populations of the Δ*efp* Δ*hrpA3* double mutant were numerically higher in inoculated fruitlet tissue than populations of the Δ*efp* mutant, and Δ*efp* Δ*hrpA3* populations were not statistically separable from either the wild-type or the Δ*efp* mutant populations. This indicates that deletion of *hrpA3* partially corrected the growth defect of the Δ*efp* mutant in immature apple fruit tissues. The growth of the Δ*efp* mutant was similar to that of the *ams*$^{-}$

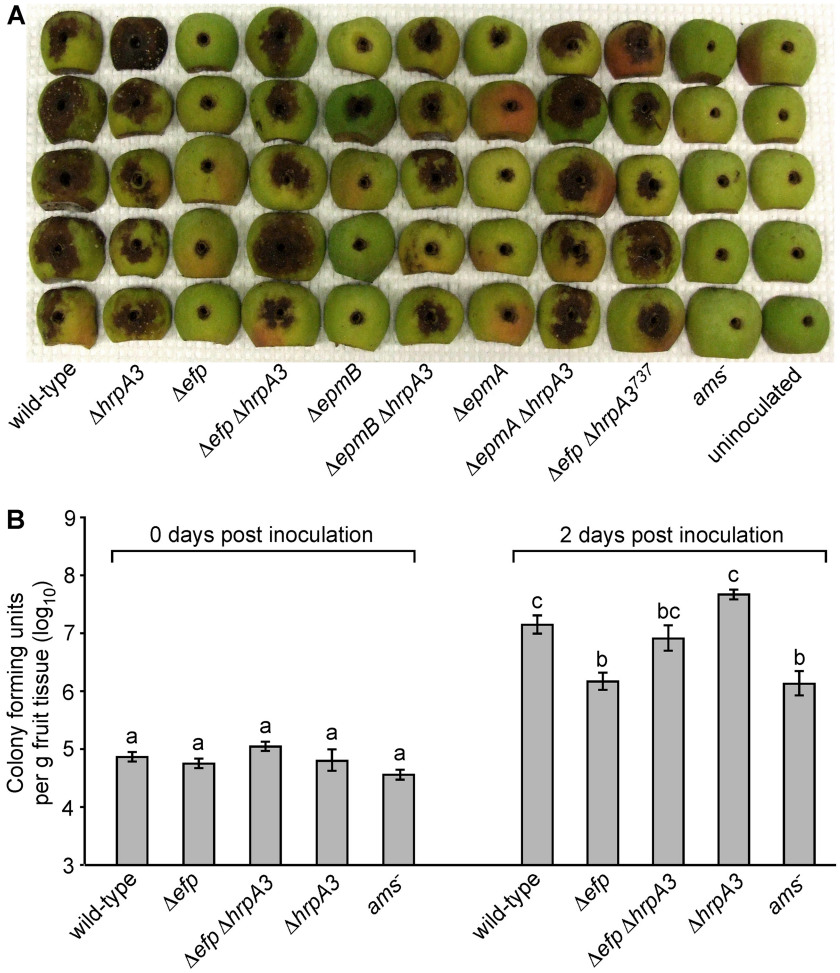

FIG 6 Deletion of *hrpA3* partially restores virulence in the Δ*efp*, Δ*epmA*, and Δ*epmB* genetic backgrounds. (A) Symptom development in apple fruitlets at 7 days after inoculation with 2×10^6 cells of the indicated *E. amylovora* strains. (B) Growth of the indicated bacterial strains in apple fruitlets inoculated as in panel A, over the first 2 days of infection, expressed as CFU per gram of apple tissue. The experiment was repeated three times, with similar results each time. Within a given time point, bars sharing a letter have no statistically significant difference according to a Tukey test, and error bars represent SE.

mutant, indicating that the Δ*efp* mutant has a severe defect in growth similar to that of the avirulent *ams*$^-$ mutant.

Shoot blight is one of the most common and devastating manifestations of fire blight disease. While there is a good correlation between virulence in apple fruitlets and shoot blight virulence in whole trees (31), the shoot tissue host environment poses different challenges to *E. amylovora*. Therefore, the virulence of four representative spontaneous suppressor strains (Δ*efp*^{L1}, Δ*efp*^{L3}, Δ*efp*^{L4}, and Δ*efp*^{L6}) was tested on second-leaf 'Gala' apple tree shoots by shoot tip wound inoculation. The selected suppressor strains represented the four different *hrpA3* truncation alleles (Fig. 3). Each mutant was inoculated onto actively growing shoot tips, and the length of symptomatic branches as a percentage of total branch length was measured at 1, 2, and 3 weeks postinoculation.

While not as virulent as the wild type, Δ*efp*^{L1}, Δ*efp*^{L3}, Δ*efp*^{L4}, and Δ*efp*^{L6} produced numerically higher disease severity values than the Δ*efp* mutant at all time points (Fig. 7). At 1 week after inoculation, disease severity produced by Δ*efp*^{L4} and Δ*efp*^{L6} had a statistically significant difference from the Δ*efp* mutant. After 2 weeks, the disease severity produced by Δ*efp*^{L3} and Δ*efp*^{L6} was not statistically separable from the disease severity caused by the wild type, and Δ*efp*^{L3}, Δ*efp*^{L4}, and Δ*efp*^{L6} produced significantly

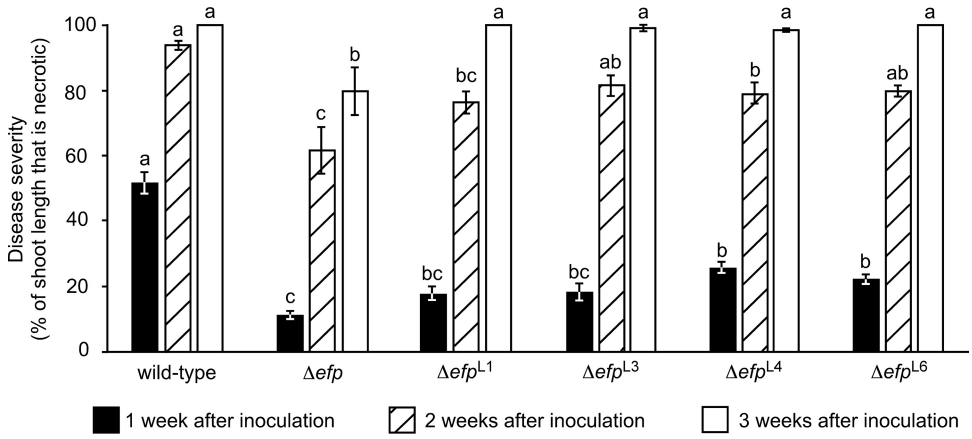

FIG 7 Δ*efp* suppressor mutants have increased virulence in apple tree shoots compared with that of the Δ*efp* parent strain. Apple trees were inoculated through shoot tip wounds with the indicated *E. amylovora* strains, and the extent of resulting necrosis in the inoculated shoots was measured at 1-week intervals. Bars represent the extent of necrosis in 25 shoots per strain; error bars indicate SE. Within a given time point, bars sharing a letter have no statistically significant difference according to a Tukey test. This experiment was performed twice with similar results each time; results of one representative experiment are shown.

more severe symptoms than the Δ*efp* mutant. After 3 weeks, the disease severity caused by all four suppressor strains had a statistically significant difference from the Δ*efp* mutant, and most tree shoots inoculated with the suppressor strains and the wild type were completely necrotic. Together, the results indicate that the loss of HrpA3 results in significantly increased virulence of the Δ*efp* mutant.

CONCLUSIONS

Suppressor mutants have long been used to uncover novel partners in biological pathways, in this case allowing us to identify an otherwise unknown gene related to EF-P function, *hrpA3*. The present study has revealed that mutations disrupting the predicted RNA helicase HrpA3 can partially compensate for the loss of functional EF-P. Since *hrpA3* deletions and truncations display nearly identical phenotypes in Δ*efp*, Δ*epmA*, and Δ*epmB* mutants, it follows that the loss of HrpA3 appears to compensate specifically for the loss of a functional EF-P and that this compensation does not involve a restoration of function to the EF-P protein.

The restoration of amylovoran production in the Δ*efp* Δ*hrpA3*, Δ*efp* Δ*hrpA3*^{737}, Δ*epmA* Δ*hrpA3*, and Δ*epmB* Δ*hrpA3* double mutants to wild-type levels could at least partly account for their increased virulence compared to that of the Δ*efp*, Δ*epmA* and Δ*epmB* single mutants. However, increased amylovoran production is unlikely to account for the restoration of colony size to that of the wild type in the Δ*efp* Δ*hrpA3*, Δ*efp* Δ*hrpA3*^{737}, Δ*epmA* Δ*hrpA3*, and Δ*epmB* Δ*hrpA3* double mutants, as the *ams*$^{-}$ mutant displayed colonies that were larger than the wild type. This effect is possibly due to a decrease in the colony dome shape sometimes seen in extracellular polysaccharide mutants (32, 33). Thus, at least two unique measurable phenotypes (colony size and amylovoran production) are restored by the mutation of *hrpA3* in Δ*efp*, Δ*epmA*, and Δ*epmB* strains.

Deleting *hrpA3* in a wild-type *E. amylovora* background produced no measurable phenotypic change in any assay performed in this study, indicating that *hrpA3* is not an essential gene in *E. amylovora*. Several other putative ATP-dependent RNA helicase genes are found in the *E. amylovora* genome, including *hrpB*, *deaD*, and *dbpA*. It is possible that aspects of HrpA3 function are duplicated by one or more of these other putative RNA helicases; however, our results indicate that HrpA3 has some unique functions related specifically to EF-P.

It is possible that HrpA3 directly or indirectly interacts with or structurally changes the ribosome complex in a way that partially alleviates ribosomal stalling, either

universally or under certain circumstances. In an interactome study in *E. coli*, HrpA3 was found to interact with both 50S and 30S subunits of the ribosome, as well as elongation factor thermounstable (EF-Tu), uncharacterized tRNA/rRNA methyltransferase YfiF, and several other proteins (34). It is not immediately clear why disruption of a DEAH-box RNA helicase would ameliorate the effects of the loss of EF-P. However, DEAH family RNA helicases have a number of functions related to mRNA processing and translation that could be related to this effect, including ribosomal biogenesis, translation initiation, and the bacterial degradosome (35).

Given the role of HrpA3 orthologues in the degradation of particular RNAs (29, 30, 36), it is possible that deletion of *hrpA3* in *E. amylovora efp* mutants simply alters the abundance of certain transcripts in a manner which happens to suppress specific Δ*efp* phenotypes. For example, mutation of the DEAH-box RNA helicase gene *hrpB* of *Xanthomonas citri* pv. *citri* reduced biofilm formation, increased sliding motility, and reduced symptom development in sweet orange (37). Furthermore, mutation of DEAH-box RNA helicase Z5898 of *E. coli* O157:H7 resulted in the absence of flagella (38).

Mechanistically, Δ*hrpA3* suppression of Δ*efp* phenotypes could occur through altered processing of translational stall events. mRNAs in stalled ribosomes may be cleaved and both the mRNA and defective protein targeted for degradation (39, 40). HrpA3 has been found to be associated with the degradosome of *E. coli* (41). It is possible that HrpA3 helps enable degradation of certain mRNAs in stalled ribosomes. Thus, the Δ*efp* Δ*hrpA3* mutant may have more time than the Δ*efp* mutant to resolve ribosomal stalls (42) without aborting translation, leading to a partial alleviation of Δ*efp* mutant phenotypes in the Δ*efp* Δ*hrpA3* mutant. Long transcripts, such as the 16-kb *ams* transcript (43), might actually be translated more efficiently in the Δ*efp* Δ*hrpA3* mutant than in the Δ*efp* mutant, resulting in an energetic benefit to the cell.

MATERIALS AND METHODS

Growth media and conditions. A nalidixic acid-resistant derivative of *E. amylovora* strain HKN06P1 (31) (referred to as wild type) was used throughout. *E. amylovora* strains were routinely grown at 28°C in lysogeny broth (LB) or M9 minimal medium (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1.0 g NH_4Cl, and 0.24 g $MgSO_4$ [all per liter], supplemented with 0.4% sorbitol and 0.2 g/liter of nicotinic acid). *E. coli* strains were grown in LB at 37°C. When appropriate, antibiotics were used at the following concentrations: nalidixic acid, 20 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; carbenicillin, 100 μg ml^{-1}; and chloramphenicol, 25 μg ml^{-1}. Bacterial suspensions of approximately 10^8 CFU/ml were prepared by resuspending the indicated strains in 10 mM $MgCl_2$ at a specific optical density (OD) at 600 nm. At this wavelength, an OD of 0.1 for wild-type and *hrpA3* deletion strains or 0.09 for Δ*efp*, Δ*epmB*, and Δ*epmA* strains corresponded to approximately 10^8 CFU/ml.

General DNA manipulations. Standard molecular biology techniques were used throughout (44). All PCRs were performed using Q5 polymerase (New England Biolabs) and products were initially cloned using the TOPO-TA kit (Invitrogen). Plasmids and oligonucleotides used in this study are listed in Tables S2 and S3 in the supplemental material, respectively.

Creation of deletion and complementation strains. Deletions of indicated genes were generated using overlap extension PCR to generate segments of DNA homologous of DNA upstream and downstream of the target gene, without the open reading frame present. The resulting products were cloned into the suicide plasmid pDS132 (45). These plasmids were transferred into the nalidixic acid-resistant wild-type strain through biparental mating with *E. coli* SM10λpir. Initial recombination events were selected by plating onto chloramphenicol and nalidixic acid, and secondary recombination events were selected by plating onto 5% sucrose. Successful deletions were selected for the loss of the gene by PCR amplification of the cloned area. Complementation plasmids were produced as described previously (21) and are listed in Table S2.

Isolation of spontaneous suppressor strains. Suppressor strains were selected based on colony size. To ensure that these suppressor strains were not simply clones, multiple independent *efp* deletions were created and used as separate parent strains. The background genotypes of all suppressor mutations were confirmed by PCR.

Quantification of colony size. Images of 100-mm-diameter plates were taken 72 hours after plating approximately 50 CFU of each strain. Ten colonies from each strain were selected at random and their areas were quantified using ImageJ (46). This experiment was repeated at least four times.

Genome sequencing and SNP mapping. Full genomes of the wild-type HKN06P1, Δ*efp*, six independently derived Δ*efp* suppressor lines, and one Δ*epmB* suppressor line were sequenced on an Illumina MiSeq instrument using 250 × 250 paired-end sequencing at the Penn State Genomics Core Facility at University Park. The DNA libraries were constructed at the Penn State Genomics Core Facilities using the TruSeq DNA PCR-free kit. Results were analyzed using Geneious 11.0.2 (https://www.geneious.com). The genome of Δ*efp* was aligned to the wild type, and suppressor genomes were subsequently aligned to Δ*efp* using the Geneious mapper. Variations were identified both inside and outside coding

sequences, with a minimum variant frequency of 0.25 and a maximum variant *P* value of 10^{-6} and a minimum strand bias *P* value of 10^{-5} when exceeding 65% bias. Only putative single nucleotide polymorphisms (SNPs), insertions, and deletions with coverage of over 50×, variant frequency over 95%, and a variant *P* value of less than 0.001 were considered for further analysis.

Amylovoran production and motility assays. Amylovoran was quantified using cetylpyridinium chloride as described in reference 32. Motility assays were conducted as described previously (21) on M9 minimal medium plates with 0.3% agar. Motility was quantified as the visible diameter of bacterial spread after 48 hours using ImageJ (21). All experiments were performed at least three separate times.

Disease assays. Growth in immature 'Gala' apple fruitlets was quantified as described in (31). Briefly, bacterial suspensions of 2×10^6 CFU/ml in $MgCl_2$ were added into apples, and CFU/g was determined by serial dilution and plating of macerated apples on the indicated days. This experiment was performed at least 3 times. To determine strain virulence on apple trees, greenhouse-grown second-leaf 'Gala' apple trees on Malling.9 rootstocks were inoculated as previously described (31). The first emerging leaves were cut with scissors dipped in bacterial suspensions of 1×10^8 CFU/ml in $MgCl_2$. Twenty shoots were inoculated per strain, on approximately five separate trees. The length of the shoot showing visible symptoms was recorded every week and was expressed as a percentage of the total length of the shoot. The entire experiment was performed twice.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00722-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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